EFFECT OF HIGH FIBER AND HIGH STARCH ALTERNATIVE FEEDSTUFFS ON THE GROWTH PERFORMANCE AND GUT HEALTH OF BROILER CHICKENS

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Effects of high fiber and high starch alternative feedstuffs on the growth performance and gut health parameters of broiler chickens

Abstract

Corn, wheat and soybean meal (SBM) have been used as primary feedstuffs in animal feeding programs. However, it is necessary to explore and evaluate alternative feedstuffs to deal with variable price and supply of primary feedstuffs in current market. Two independent studies were conducted using Macadamia nut cake (MNC) and Cassava Root Chips (CRC) as high fiber and high starch feedstuff, respectively that could partially replace corn, wheat and SBM in broiler chicken diets. In each study, 180 d-old chicks were randomly and equally assigned to one of the treatments with corn-SBM based control diet for 42 d. In the MNC study 0, 5, 10, 15 and 20% MNC included whereas, in the CRC study 0, 12.5, 25, 37.5 and 50% of CRC was included in the total diet. Weight of birds and feed were recorded to calculate body weight (BW), average daily feed intake (ADFI), average daily gain (ADG) and feed conversion ratio (FCR) as growth performance (GP) parameter and gut microbiota (MNC study), ileal morphology (CRC study) and volatile fatty acid (VFA, both studies) as gut health indicators.

In MNC study, ADFI and FCR increased significantly at 20% MNC inclusion in starter and 15% in finisher and overall period compared to control group. Out of 89 sequences analyzed for 3 treatments, 0% MNC fed birds had mainly Ruminococcus (29%), Faecalibacterium (19%) and Bacteroides (16%); 10% MNC has Bacteroides (50%), Clostridium (20%), and Ruminococcus (10%); and 20% MNC consists Bacteroides (36%), Ruminococcus (29%), Clostridium (14%) and Faecalibacterium (11%) as the predominant bacteria. There was no compromise on growth performance of chicken up to 15% MNC inclusion due to high feed intake, growth of selective bacteria and their metabolites.

In CRC study, inclusion of 37.5 and 50% of CRC in the starter phase showed negative effects as BW ($P < 0.01$) and ADG ($P < 0.05$) decreased whereas, FCR increased ($P < 0.05$). There was no significant difference in finisher except lower FCR in 50% inclusion level than 37.5%, suggesting that CRC can be included up to 50% in finisher diets. No statistical differences ($P > 0.05$) in villus height, crypt depth, villus height to crypt depth ratio and villus surface area across treatments was found. These results suggest that inclusion of CRC up to 25% in starter and 50% in finisher broiler diets have comparable growth performance than corn-SBM based diets and can be advantageous from economic perspective as CRC are cheaper than corn. Further inclusion level can be optimized after economic analysis and supplementation with exogenous enzymes to enhance nutrient utilization.

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1. Introduction

The word “poultry” comes from the French word poule, itself derived from the Latin word Pullus, which means small animal. Term “poultry” is used for any kind of domesticated bird, especially raised for utility purpose. In the past, this word was used to refer wildfowl and waterfowl. Now, the definition is broad and includes domestic fowls such as chickens, turkeys, geese and ducks, raised for meat or eggs. Sometimes Japanese quail, squabs and common pheasant are also included in the list. Worldwide, more chickens are kept than any other type of poultry, with over 50 billion birds being raised per year just for meat and egg purpose. Global production of chicken meat started since 1960s and has been one of the fastest growing and accepted in developing and developed countries. Traditionally, they were reared in small flocks and in foraging in the day time and housed at night. However, rising world populations and urbanization have led to production in large scale and in more intensive specialist units. Despite of many advantages, positive market for poultry meat, the world poultry sector faces challenges such as feed ingredient price, availability, product quality, animal welfare, and environmental issues associated with commercial poultry production systems (Shane, 2004). Profitability of the production depends very much on the price of feed as it contributes about 60% of the total cost.

Energy and protein are the most expensive component of poultry feeds, thus the increase in the cost of energy/protein yielding feedstuffs will increase the cost of poultry production. Corn, wheat and soybean meal (SBM) are the most widely used feedstuffs in poultry diets. The price of corn has increased with some fluctuation over last decade; thereby making poultry feed cost uncertain. Increase in price of corn was because it is used for ethanol production (renewable fuel), thereby increasing the demand for corn by the ethanol industry (Tyner and Taheripour,

2007), and reduced production of corn due to the 2012 U.S. drought. The price of SBM, the most widely used protein source in poultry diets, has soared also due to increased acreage used to grow corn at the expenses of soybean acreage (Schmit et al., 2009) and reduced production of soybean due to the 2012 U.S. drought. Prices of U.S. corn and SBM more than doubled over the last 7 years (Patience, 2013) though currently its low. Moreover, market availability of these ingredients is variable, and the costs of these ingredients are predictable to increase continuously due to competition among food for humans, feed for animals and fuel for vehicles and their inadequate production (USDA, 2012). Therefore, nutritionists and producers are continuously looking for alternatives to expensive energy and protein sources to combat high feed costs. Reasons to include alternative feedstuffs in monogastric diets might be a desire also to modulate intestinal health (Pieper et al., 2008; Jha et al., 2010), improve carcass quality, (Jha et al., 2012a) and nutrient management (Jha and Leterme, 2012). Potential alternative feedstuffs are available and can fulfill the nutritional requirements of livestock (FAO, 1997) and reduce feed costs (Woyengo et al., 2014). Several alternative feedstuffs, including macadamia nut cake (Tiwari and Jha, 2016b), cassava (Morgan and Choct, 2016), roots, bananas, plantains, organic waste (FAO, 1997), tropical fruits, leaves, tubers, and agro-industrial coproducts (Leterme et al., 2006; Tiwari and Jha, 2016a), taro (Buntha et al., 2008), novel grains and pulses (Beltranena and Zijlstra, 2007), distiller's dried grains with solubles (Avelar et al., 2010), oilseed cakes (Seneviratne et al., 2010), wheat millrun (Nortey et al., 2008; Jha et al., 2012) and copra meal, palm kernel expellers, or palm kernel meal (Jaworski et al., 2014) have been evaluated and used successfully in monogastric diets. However, agro-climatic conditions and farming system largely differ from location to location which influence the nutritional profile of feedstuffs and their

utilization in animals. Therefore, evaluating available local novel feedstuffs is necessary to develop animal feeding program using such feedstuffs.

1.1 History of chicken meat consumption

Chicken as a meat has been depicted in Babylonian carvings from around 600 BC. Chicken was one of the most common meats available in the Middle Ages. It was widely believed to be easily digested and considered to be one of the most natural foods. It was eaten over most of the Eastern hemisphere and number of different kinds of chicken such as capons, pullets and hens were eaten. It was one of the basic ingredients in the so called white dish, a stew usually consisting of chicken and fried onions cooked in milk and seasoned with spices and sugar.

Recently, the poultry industry is one of the fastest developing meat producing industries. Feed efficiency and high performance of the birds are the major goals in poultry production. Quality of diet, along with environmental condition and health issues, need to be considered to achieve this goal. Feed cost contributes to around two-thirds of the total cost of poultry production. Usually corn and soybean meal are the major ingredients of chicken feed, which do not have any alternatives that completely replace them, although wheat is included in prominent levels in some parts of the world. Corn, wheat and SBM are the most widely used feedstuff in chicken diets. Prices of these energy/ protein yielding feedstuffs have been quite variable over past years.

1.2 Status of poultry production in Hawaii

In 1945, many thousands of chicks and poults were imported in Hawaii first time by plane from Pacific Coast states to see the effect of how this new method of shipping chicks works. During the world war time citizens of Hawaii were urged to produce as much vegetable

and animal as possible. In response, many undertook back yards gardening and others combined poultry raising with vegetable. Later on, some of them continued growing chicken because of their interest while others moved on to some other job once their herd was affected by disease outbreak.

In 1999, poultry broiler production in Hawaii declined by 30 percent within year 1989-1999, a reduction and trend largely due to high production costs associated with land, housing, and imported feeds. In addition, problem is former farmers retired and new generations changed their profession. However, demand for poultry broiler products was in continuously increasing, due to marketing efforts by the national poultry industry. To meet the market demand, in-shipment of broilers to Hawaii increased by 27 percent between 1987 and 1991 and an average of 3.5 percent on each of the past ten years. Pasture-based poultry production were also considered as more land became available due to decline in the plantation industries (sugarcane and pineapple) in Hawaii. This got also attraction as it does not require costly equipment or structures. In this system, up to 30 percent of the broiler diet is provided by pasture so decrease the feed cost making broiler production more sustainable. Along with that, birds get natural balance diets from forage, grain, insects and worms.

With the objective to establish a task force to develop long term solutions to effectively protect the livestock industry in Hawaii, a meeting of the poultry industry was called in 2007 with the motive to identify issues, problems and opportunities. Committee was formed to summarize ideas and develop plan, also strength and weakness were reviewed (Zaleski, 2007).

1.2.1 Pros of chicken production in Hawaii

1. Local producers can provide fresh meat and eggs.

2. Product diversification of egg, and meat attracts consumer of diverse culture to consume local chicken products.
3. Most of the local farms are family farms with better understanding of management and produce more environment friendly organic and antibiotic-free chickens.
4. Compost and manure provide excellent sources of nutrients to the local land.

1.2.2 Cons of chicken production in Hawaii

1. Cost of labor is expensive.
2. Small number of producer and have very low or no successors interested in their parent's occupation which makes this field less sustainable.
3. Innovative waste management and environment pollution control is needed.
4. The cost of production and marketing is very high, mainly regarding feed import costs, transportation and shipping costs, tax, fuel and regulatory costs
5. Very limited land because of re-zoning, loss of agriculture land, and urban encroachment
6. Lack of state resources to support the industry
7. Very limited expert or veterinarian specialized in poultry industry in Hawaii
8. Small farmers have limited economic scale
9. Food security depends on having local supplies rather than depending on mainland supplies. 10. The industry depends on a consistent supply of replacement chicks

1.2.3 Statistics of Poultry in Hawaii

Table 1: Egg and chicken production in Hawaii (USDA, 2012)

Year	Data Item	Quantity
2010	Egg production, number	69,500,000

2005	Egg production, number	114,500,000
2000	Egg production, number	142,900,000
2010	Chicken, number	117,000
2009	Chicken, number	58,000
2008	Chicken, number	78,000

1.3 Feedstuffs for chickens in Hawaii

From the beginning of poultry farming in Hawaii, commercial feed manufacturers have played a major role in the development of local poultry industry. Hawaii is primarily dependent upon mainland for ready mix formulated feed or import feed ingredients and mix here by some local manufacturers. Import is mainly because of limited land with high price, makes growing of grains unprofitable. Again, shipping cost make it illogical bringing individual bags of organic feed from the mainland. The only way it makes sense economically when whole shipping container is with feed, which are one or more tons. This is not practical until there are lots of people or very large farm-buying organic feed on a regular basis. Though it is imported as organic but transporting thousands of miles in fossil fuel-burning vehicles should pay environmental cost. In this case, it is likely that local is more environmentally sound than imported organic. Several products and by-products from agricultural and industries are available in Hawaii in copious quantities and could together provide the basis for producing more affordable feeds.

1.3.1 Conventional feedstuffs

Corn, wheat, and SBM are the most widely used feedstuffs in chicken diets. The price of corn has soared because corn is used for ethanol production (renewable fuel), human consumption, and reduced production of corn due to the 2012 U.S. drought. The price of SBM has also soared due to increased acreage used to grow corn at the expenses of soybean acreage (Avalos, 2014). No any grain based farming for feed purpose is done in Hawaii. Therefore, it is necessary to look for alternatives energy and protein sources to combat high feed costs which should be feasible to produce locally in Hawaii.

1.3.2 Unconventional/ alternative feedstuffs

Although no grain is cultivated in the state of Hawaii because of land cost, soil, water availability, fungus infection, climate, and infrastructure also does not scale down efficiently, still several products and byproducts from agricultural resources and industries are available in Hawaiian Islands in large quantities and could together provide the basis for producing more affordable feeds. To meet Hawaii's 2030 Clean Energy Initiative (HCEI) goals, about 20 million gallons of biodiesel is proposed to be produced in Hawaii (HCEI, 2011). These biodiesels will primarily come from oilseeds; macadamia nut and sunflower will serve as primary crop to meet the HCEI goal.

Alternatives that are available locally are Grass (pasturing), sorghum, amaranth, legumes like pigeon peas, chayote (leaves and tubers), tubers (taro, potatoes), azolla, coconuts, papaya, nuts, cassava, restaurant wastes, and agriculture by-products.

Grass

Chickens in free range consume as much as 30% of their calories from grass. In Hawaii, its beneficial as we have abundant grass in most parts and has health benefit to both chicken and

meat consumer. They get grass hoppers and other insects once in free range. Pasture improves flavor, texture and appearance of meat and eggs.

Sorghum

This is one of the most potential chicken feed crops for Hawaii as it can be grown in wider range of climates and this is fifth most important cereal crop in the world.

Pigeon peas

This is high in protein (15-20%) of mix. Pigeon peas used to be grown for feed in Hawaii on 8,000 acres to feed animals. The harvested pigeon pea can be left on the stem and fed intact to the hens (Fukumoto, 2009).

Chayote

Chayote grow wildly in wet parts of Hawaii with no care or maintenance required. All parts of this plant are edible to chickens. Fruit of this plant mostly contains water, fiber, a little starch, vitamin K and vitamin C.

Worms

Worms could probably be raised as a source of protein for chickens. Red worm can be one of them. It can be directly incorporated in chicken diet and the manure from chicken can be fed back to worm, to make a natural cycle. Worm proteins are rich and complete so no need to balance as carefully as vegetables and other grains.

Larvae

Larvae of flies that look like worm, also known as grubs can be raised. The nutrition of the larvae is complete and they eat wide variety of things. One of the most popular fly is Black

Soldier Fly, *Hermetia illucens.* This fly is omnipotent and present in Hawaii as well. Only problem is harvesting efficiently rather than growing.

Roots and tubers (sweet potato, taro etc.)

These are the traditional carbohydrate sources for Hawaii also known to grow abundantly and sustainably. There are some negative aspects of growing these root vegetables such as these root starches grow very slowly and gives output in long period of time. Need intensive labor to dig, complexity in chopping and cooking them.

Breadfruit:

Similar to tubers, at low elevation and sufficient rainfall, breadfruit can produce huge amount of nutritious starches that is consumable by chicken. Again, labor is the main constraints in harvesting and preparing the fruits for feed.

Papaya, other fruits and vegetables

They are good sources of vitamins and seeds high in protein. There is the overhead of chopping and cooking them for the chickens.

Restaurant wastes

Restaurant wastes are easily available and are potential feedstuff as there are still many nutritional factors from macro-nutrient to minerals and sodium that chicken can consume and get enough feed and perform well.

Macadamia nut cake

Hawaii is one of the largest producers of macadamia nut globally. Macadamia nut cake (MNC) is a byproduct of macadamia nut oil extraction, which is available at large scale in

Hawaii, and is expected to increase its production by over 60,000 tons per year by 2020 (*Pacific Biodiesel, Hawaii, Personal communication*). There is not much information available on nutritional value of these novel feedstuffs and their effects on growth performance and gut health of poultry, limiting the use of these feedstuffs in animal feeding program. Thus, most of the byproducts, including MNC are sent to landfill, additional burden to environment. Disposal of these byproducts results in economic loss and potential environmental impacts in Hawaii each year (DOA Hawaii, 2007).

Although, alternative feedstuffs are variable in their nutrient profile they may reduce the feed cost, modulate intestinal health, and improve carcass quality of chicken. Skenjana et al. (2006) reported relatively high *in vitro* digestibility and *in situ* degradability of MNC in sheep. Similarly, Tiwari and Jha (2016b) evaluated MNC as a potential feed ingredient for swine diets and found that MNC is rich in energy with relatively high in vitro digestibility. Recently, Berrocoso et al. (2017) determined the basic nutrient profile and apparent metabolizable energy content of MNC for broiler chicken to be 2897 kcal/kg, which is comparable to the AME of conventional feedstuffs used in chicken diets.

Macadamia nut cake is commonly available in Hawaii (USA), Australia, New Zealand, Kenya, Brazil, Israel. The MNC is not well utilized in animal feeding programs due to limited information on its nutritional value although it has potential to be utilized as a cost-effective feed ingredient in animal diets. MNC as other coproducts, are high in fiber, fat residue, gross energy and fair to high amount of protein and is expected to affect the growth performance, gut microbiota, metabolites produced from those microbiota and bone mineralization (Acheampong et al., 2008; Skenjana et al., 2006; Tiwari and Jha, 2016b).

Therefore, the effects of utilizing novel feedstuffs such as MNC on nutrient utilization, growth performance, gut health of broiler chickens, and their potential economic and environmental impact in poultry industry need to be studied.

Cassava

Corn is the major feed ingredient providing energy to the broiler chickens. Its diverse use in the food, feed and biofuel production has caused fluctuation in cost and availability making it imperative to look for alternative feed ingredient to replace corn to sustain supply of energy in the broiler production. Cassava (*Manihot esculenta* Crantz) is one of the alternative to corn being rich in starch content. It is available globally with 70% of the production coming from Nigeria, Brazil, Thailand, Indonesia, and the Democratic Republic of the Congo (FAO, 2014). Cassava if used in animal feed helps to mitigate feed scarcity and decrease the environmental problem as millions of tons of waste can be converted into valuable animal feed. Cassava root chips are carbohydrate source having poor quality of protein with some limiting amino acids. It contains cyanogenic glucosides, linamarin and lotaustralin, which on hydrolysis yield hydrocyanic acid (HCN). HCN in cassava can be considerably reduced to the acceptable limit by boiling, drying, grating, soaking, fermentation, or combination of these processes. Cassava having high moisture content is usually dried in the sun. According to Tewe et al. (1980) sun drying is more effective than oven-drying to reduce the HCN level of cassava chips. Sun drying is also more cost-effective method and energy efficient method as compared to oven-drying.

Lots of animal scientists advocate for the use of cassava in animal feeding program, at the same time nutritional characteristics requires careful attention in balancing feed formulation as methionine and lysine are the limiting factor in cassava feed (Oke, 1978). Cassava can make significant impact if it can be incorporated in commercial poultry feed to certain extent. Most of

the previous research works done using cassava in chicken feed have found inclusion of around 10% to obtain satisfactory result without any deleterious effect. Some of the studies have also shown positive response with inclusion up to 50%.

1.4 Growth performance of broiler chickens

Growth performance is the visible changes in size or maturation over a period that can be accounted and compared between treatments. For this purpose, all birds are weighed individually on the first day of hatch and at the end of each week. Also, weight of feed is considered along with consumption, leftover and waste of feed.

Body Weight

Each bird is weighed on d 1, 7,14, 21, 28, 35, and 42, which provide information that dietary treatment did not limit growth. Then, the weight of each pen birds is averaged and at the end each treatment groups are averaged and data is used to compare the body weight of different treatments statistically.

Body Weight (BW)= Final weight- initial weight

Average daily feed intake

As we are weighing feed for all the chickens in the pen on weekly basis. So, when the final feed remaining in the feeder and feed loss in the pen is reduced from the initial feed in feeder, it gives the amount of feed intake per week and dividing it by 7 to get the average daily feed intake of all the birds from a pen. For stats, we take the average of all the feed consumed by birds in same treatment.

Average daily feed intake (ADFI)= Feed offered- Feed remaining/ Days

Average daily gain

Average daily gain is the ratio of weight gain to number of days. Where, weight gain is obtained when initial weight is subtracted from finisher weight. Here we weigh the birds weekly so average daily gain will be weight after 7 days minus weight of bird today divided by 7 days.

Average daily gain (ADG)= Final weight- Initial weight/ Days

Feed conversion ratio

Feed conversion ratio or FCR is the proportion of feed that is consumed and converted to meat. So, its formula is the feed intake divided by average daily gain. FCR is also reciprocal to the feed efficiency and the lower value is desirable to have high meat converting efficiency from that feed.

Feed conversion ratio (FCR)= Feed intake/ Weight gain

1.5 Gut health of broiler chickens

The notion of "gut health" is difficult to define and, at present, it is an ill-defined notion (Montagne et al., 2003). According to Conway (1994) there are three major components of "gut health", namely the diet, the mucosa and the commensal flora. The maintenance of gut health is complex and relies on a delicate balance between these 3 components the diet, the commensal microflora and the mucosa, including the digestive epithelium and the mucus overlying the epithelium (Lindberg, 2014). Beyond maintenance, improving "gut health" is another important aspect to enhance growth performance of host by the physiological role of gut health components in nutrient utilization and improving overall health (Bach Knudsen et al., 2012). One approach for improving "gut health" is the dietary manipulation, especially inclusion of fibers and to modulate fermentation characteristics in the hindgut (Pieper et al., 2008; Jha et al., 2010, Jha et

al. 2012b; Jha and Leterme, 2012) or use of exogenous enzymes to enhance the utilization of fibers in the gut (Jha et al., 2015).

At hatching, the digestive tract is sterile and settlement of microflora depends on the egg microbial condition and contamination from hen at hatching, also the species of bacteria entering the GI is determined during hatching depending upon their ability to colonize and their interaction in the GI (Apajalahti et al., 2004; Kelly and King, 2001). Microbial diversity keeps changing throughout maturation and is influenced by many factors including chicken strain, sex and the rearing environment with individuals housing their own digestive bacterial community (Zhu et al., 2002). As the host grows, the microbiota becomes more diverse and tends to be relatively stable in later age. Increased breeding density and thermal stress increases harmful bacteria over beneficial ones (Suzuki et al., 1989). Using environmental factors to modulate the intestinal microbiota is quite irregular and variable to control; instead gut microbiota is directly related with the dietary ingredients or additives as they are potential substrate for bacterial growth. The presence of water soluble non-starch polysaccharides (WS-NSP) leads to modification of gut flora. Mathlouthi et al. (2002) found an increase in lactobacilli and coliforms along with other facultative bacteria population when the bird diet is switched from maize-based to wheat and barley-based. In case of WS-NSP rich diets, the increase in digestive content viscosity and the increase in transit time is noticed along with a higher production of VFA which regulate ileal motility in a beneficial way (Cherbut, 2003). Change in gut microbiota with antibiotic supplements in a day-old bird shows negative effect on immune development (Schokker et al., 2017).

GIT is the transition between external environment and the internal body where feed plays crucial role. The intestinal tract of chicken is home to a complex and dynamic microbial

community, most of which are bacteria (Zhu et al., 2002). Culture-independent molecular techniques have been used recently to characterize microbial diversity and opened the possibility to study the effect of environmental factors on this microbiota. The most important environmental factor is the diet, and initial studies have revealed fascinating results on the interaction of diet with microbiota such as microbial communities shift (Apajalahti et al., 2002), energy source for bacteria and selective growth of target bacteria (Apajalahti and Bedford, 2000). Gut microbiota interact within themselves, with their host and with the diet of the host, where commensal bacteria plays a pivotal role in host health and metabolism and pathogenic bacteria cause direct or indirect harmful effects. Thus, feed should be selected to favor gut condition and maintain balance between the environment, host and microbiota. The total number of bacteria in the digestive tract is higher than the number of eukaryotic cells of the host body. There are mainly three types of bacteria in the host: dominant bacteria ($>10^6$ CFU/g), subdominant bacteria (10^6 to 10^3 CFU/g) and residual bacteria ($<10^3$ CFU/g). The poultry digestive tract consists of a substantial proportion of Gram positive mainly facultative anaerobes from crop to lower ileum, whereas the ceca is composed of *lactobacilli, enterococci, coliforms*, and yeasts (Savage 1977; Ewing and Cole 1994; Mackie et al., 1999; Gaskins 2001). In the gizzard and proventriculus, low pH causes a decrease in the bacterial population. In the duodenum enzymes, high oxygen pressure, and bile salts are responsible for a reduction in floral concentration whereas in other segments of the small intestine and large intestine the environment is exact opposite to favor growth of bacteria. According to Oviedo-Rondon et al. (2006) intestinal and cecal symbiotic bacteria functions for specific metabolic, trophic and protective role as the first line of defense against pathogenic bacteria is the beneficial gut microbiota of the birds. Both cecal and intestinal bacterial communities undergo changes and

were found to diversify with age (Knarreborg et al., 2002; Lu et al., 2003). Apajalahti et al. (2004) states that the ceca have a favorable environment for bacterial growth so they have as high as 10^9 to 10^{11} bacteria per gram of content respectively. The authors found 640 distinct species and 140 bacterial genera in the gastrointestinal (GI) of chicken, where about 90% of the species are yet to be described.

Dietary fiber (DF) plays important role in the diet of monogastric animals so it should be included at least minimum level to maintain or enhance normal physiological function of the gastrointestinal tract (GIT, Wenk, 2001; Mateos et al., 2012). Dietary fiber interacts both with the mucosa and the microbiota and consequently plays a vital role in the control of gut health (Bach Knudsen et al., 2012). The inclusion of fiber in the diet has been shown to enhance intestinal function and modify the composition and quantity of the microbiota population in the GIT of pigs and poultry, both in vivo (Shakouri et al., 2006, Pieper et al., 2008) and in vitro (Dunkley et al., 2007). The effects of DF on gut health were more noticeable in weanling pigs (Mateos et al., 2006; van der Meulen et al., 2010), but they might also affect broilers and all types of poultry species in general (Kalmendal et al., 2011). In a recent study, Jiménez-Moreno et al. (2011) studied the effects of including 5% oat hull or sugar beet pulp in the diets of broilers on *Lactobacillus* counts in the crop and ceca and on *Clostridium perfringens* and *Enterobacteriaceae* counts in the ceca. The authors reported that *Lactobacillus* counts in the crop increased with the inclusion of sugar beet pulp, but not with oat hull. However, no effects of DF on *Lactobacillus* counts were detected in the ceca. On the other hand, the counts of *C. perfringens* and *Enterobacteriaceae* in the ceca decreased significantly with oat hull inclusion but were not affected by sugar beet pulp. Therefore, DF might reduce the growth of pathogenic microorganisms and the incidence of digestive disturbances, such as wet litter in chickens and

incidence of diarrhea in pigs, by different mechanisms, depending on diet composition, including the nature of the fiber fraction. Also, solubility, viscosity, and fermentation capability are 3 key physicochemical properties of fiber sources that affect microflora diversity of the GIT.

It can be noted that the host has multiple ways to control microbial growth and proliferation but the interaction among microbiota and between the microbiota and host mucosa is important to maintain gut balance. Intervention of dietary factors should consider all these interactions and mechanisms and how they relate to each other.

1.5.1 Gastrointestinal microbiota in chicken

The GIT of chicken consists of the esophagus, crop, proventriculus, gizzard, duodenum, jejunum, ileum, cecum, colon, and cloaca. Chicken GIT is much shorter than other mammals in respect to their body length. Thus, microbiota growing in such a tract with relatively low transit time requires distinctive adaptations to adhere to the mucosal wall and grow rapidly. The ceca have lower passage rate and are favorable to diverse groups of bacteria, which affect host nutrition and health.

Cecal microbiota analysis started in the 1970s (Barnes, 1979) and after that modern techniques such as culture-independent molecular techniques have been in use to characterize individual microbiota genera and their diversity in the GI. Traditional anaerobic culture techniques were used to assess and identify bacteria. Some of the problems with these culture-dependent methods are: they only culture selected bacteria out of the diverse digestive microbiota; they lack phylogenetically-based classification scheme; they are unable to detect those present in very low abundance; and bacterial species live in a community and are dependent on one another as well as to the host environment, therefore, isolating and growing in

culture might not be the same as in the host (Apajalahti et al., 2004). Culture- independent techniques is carried out to overcome this difficulty in selective culture, and to identify individual bacteria, the modern approach of examining the microbial DNA extracted from the sample (Zhu et al., 2002; Lan et al., 2002). Molecular techniques are in harmony with the culture method in contest of quantity and diversification of complex microbiota during different phase of life. The modern molecular techniques are able to reveal that 90% of the bacteria that were previously unknown species in the chicken GI tract of chicken (Lan et al., 2002). Among the molecular techniques, such as whole genome shotgun sequencing provides more in-depth understanding with valid differentiation between treatments microbiota profile (Schwiertz et al., 2010). Techniques as the terminal restriction fragment length polymorphism (TRFLP) is used to compare and contrast microflora in the duodenum, jejunum, ileum, and ceca (Gong et al., 2002). Similarly, next generation sequencing has made it possible to determine microbiota dynamics with maximum coverage and accuracy (Shaufi et al., 2015). Also, the %G+C profiling technique is based on %G+C content of chromosomal DNA and is independent of DNA sequences based techniques (Apajalahti et al., 2002).

In a phylogenetic diversity census study of bacteria in the GI of chicken 915 species-equivalents operational taxonomic units (defined at 0.03 phylogenetic distances) were found where chicken sequences represent 117 established bacterial genera (Wei et al., 2013). The GI tract harbors more than 100 billion bacteria. It consists of multiple times more bacteria than number of cell in host body, including thousands of species dominated by anaerobic bacteria. Out of these bacteria, beneficial bacteria make up 85% and pathogenic bacteria make up 15% of a balance microbial community. Some of the commonly found microbes are: *Lactobacillus sp.,*

Bacteroides sp., Eubacterium sp., Clostridium sp., Escherichia coli., Streptococcus sp., Prevotella sp., Fusobacterium sp., Selenomonas sp., Megasphaera sp., and *Bifidobacterium sp.*

1.5.2 Gut histology

Gut histology gives idea about the immunological condition of the gut and the level of nutrient absorbed depending upon the nature of feed. The size and intensity of villus of small intestine is directly related to the absorption capacity of the birds. Previous works confirmed that crypt depth and villi length are related to the rate of sensory activity and increase/decrease in cell turn over (Langhout et al., 1999; Hedemann et al., 2003). In a research, feeding ethanol-treated castor oil seed (ECAM) 100 g/kg and the control diet showed improved absorption and hydrolysis potential of nutrient leading to high duodenal villus length and crypt depth. Whereas, reduction in crypt depth with higher that 100 g/kg of ECAM could be due to lower secretory activity of mucus (Langhout et al., 1999). Engberg et al. (2004) reported that reduced intestinal crypt depth could be linked with reduced intestinal microbial population.

1.5.3 Volatile Fatty Acid production

Dietary fiber present in MNC and resistant starch present in CRC are the main substrate for bacterial fermentation, particularly in the large intestine of non-ruminant animals. The main products of fermentation are volatile fatty acids (VFA), predominantly acetate, propionate and butyrate. Acetate is formed by hydrolysis of acetyl CoA once pyruvate is broken down into acetyl CoA, hydrogen and carbon-dioxide (Pryde et al., 2002). Acetate diffuses through the large intestine and reaches muscle tissue, where it is substrate for fatty acid production and cholesterol synthesis. Propionate is formed via electron transfer chain through PEP (Miller and Wolin, 1996). Propionate gets absorbed into the blood stream and reaches liver and produces glucose via

gluconeogenesis. Butyrate, is preferred energy source for colonic epithelial cells, regulates cell proliferation, maintain mucosal integrity and reduces cellular apoptosis. When lactate utilizing bacteria produces acetyl CoA, butyrate is formed after condensation and reduction of acetyl CoA and butyryl CoA, respectively (Pryde et al., 2002). One of the ways to promote butyrate producing microbes is by inclusion of dietary fermentable carbohydrates (Sakata and Inagaki, 2001). According to Von Engelhardt et al. (1989) report around 95 and 99% of total VFA produced is absorbed before reaching the rectum in a number of non-ruminant species. Therefore, VFA contribute to the energy requirement to the gut and to the whole body of non-ruminant animals. This higher production of VFAs not only satisfy the energy demand of the host, but also participate in the gut immunity and recycling of inorganic ions by enhancing ion transport processes within the ceca (Rice and Skadhauge, 1982).

1.6 Cecal microbiota profile

Like all the vertebrates, chickens have wide range of highly populated niches of microbiota within the guts with major role in host nutritional physiology and health benefits (Waite and Taylor, 2015). Studies using molecular techniques have elucidated that culture-based analysis may underestimate the bacterial diversity of cecal microbiota. So, the use of 16S rRNA next-generation sequencing platform for the identification and analysis of microbial communities has been widely used in microbial ecology and is replacing the conventional cultural methods. In a research by Hird et al. (2014) gut microbiota profile is correlated with diet, age and location. Author also suggested that gut microbiota is the product of environment rather than bird trait per se. Genetic fingerprinting techniques provide rapid and relatively easy alternative to the analysis of microbial communities. Denaturing gradient gel electrophoresis (DGGE) is a fingerprinting technique by which PCR-amplified DNA fragments are separated according to their sequence

information. The basis of this technique is that DNA fragments of the same size but with different base pair sequences can be separated. This separation by DGGE relies on the electrophoretic mobility of partially denatured DNA molecules in a polyacrylamide gel, which is encumbered in comparison to the completely helical form of the molecule. The revolutionary 16S rRNA gene sequencing has been used for studies of microbiota role in birds, complemented with investigation in microbiota diversity and profile (Waite and Taylor, 2015). Although role of some specific bacteria can be inferred but due to significant evolutionary specialization of gut-associated bacteria, much of the role of these microbiota are still unclear (Foley et al., 2013). This thesis presents a fingerprint analysis of microbiota sampled from cecal excreta using DGGE and sequencing techniques. Any marked difference between the microbial composition of high fiber MNC included diet and the corn-SBM based control diet would further advance our understanding of how specific bacteria are associated with, and respond to different nature of feed and gut environment.

1.7 Objectives for this study

The general goal of this study was to use by-products from agro-industry as alternative feedstuffs in broiler chicken diet. To accomplish the goal, two independent studies using alternative feedstuffs were conducted. The objective of the study-1 was to determine the effect of 0 (corn-SBM based control diet), 5 (5% MNC + 95% control diet), 10 (10% MNC + 90% control diet), 15 (15% MNC + 85% control diet) and 20 (20% MNC + 80% control diet) % MNC inclusion on growth performance and gut health parameters such as cecal microbiota profile and volatile fatty acid production. The objective of study two was to determine the effect of CRC inclusion, 0 (corn-SBM based control diet), 12.5 (12.5% CRC + 87.5% control diet), 25 (25% CRC + 75% control diet), 37.5 (37.5% CRC + 62.5% control diet), and 50 (50% CRC + 50%

control diet) on the growth performance, gut histology of broiler chickens and volatile fatty acid production.

Chapter 2: Materials and Methods

Study 1: Effect of different levels of MNC inclusion on the growth performance and gut health parameters of broiler chickens

2.1 Study setup and experimental design

All the animal experimentation was carried at the Small Animal Facility (SAF) of University of Hawaii at Manoa. The study was conducted in accordance with the guideline and ethics committee approved by the Institutional Animal Care and Use Committee (IACUC, Protocol #13-1639).

A total of 180, day-old mixed-sex broiler chicks (Cobb 500) obtained from a local hatchery (Asagi Hatchery, Honolulu, HI) were used in the growth performance study. Birds were raised in group floor pen with-standard commercial broiler rearing environment (temperature, humidity, light and built up litters). On day one, all 180, day-old chicks were weighed individually, wing tagged and placed randomly in one of 30 pens (six birds/ pen), making 6 replicates of each treatment. Birds in each pen were fed with one of 5 diets where diet was treatment and pen was experimental unit. All the birds had ad libitum access to feed and water.

2.2 Diets

Preparation of MNC

The MNC sample was sourced from a local macadamia nut processing plant (Oils of Aloha, Kunia, HI). MNC was ground to pass through a 3/16 mesh sieve using a Wiley mill (Thomas Model 2 Wiley Mill, Thomas scientific, Swedesboro, NJ). Prior to diet formulation, proximate nutrient analysis of MNC were determined. Nutrient profile and Metabolizable energy value was used from a previous study conducted in our lab (Berrocoso et al., 2017).

Experiment

The diets used in this study were corn-soybean meal based and were fed in mash form. The feeds were formulated for two phases- grower (0-21d) and finisher (22-42 d), to meet or exceed the nutrients requirements of broilers (NRC, 1994). The diets were formulated to have 5 different level of MNC: 0% as control, and 5, 10, 15 and 20% as other treatments. These treatments were allotted to chickens in completely randomized design. The diets formulation is presented in Table 2.

Table 2: Ingredient used and nutrient composition of different treatment diets

	Starter					Finisher				
Ingredients, %	MNC 0	MNC 5	MNC 10	MNC 15	MNC 20	MNC 0	MNC 5	MNC 10	MNC 15	MNC 20
Corn	55.38	52.34	48.36	44.87	41.38	60.39	56.68	53.25	50.25	47.14
SBM	37.00	35.00	34.00	33.00	31.50	31.25	30.00	28.50	27.50	25.50
MNC	0.00	5.00	10.00	15.00	20.00	0.00	5.00	10.00	15.00	20.00
Soybean oil	2.50	2.50	2.50	2.00	2.00	4.00	4.00	4.00	3.00	3.00
Limestone	1.40	1.40	1.40	1.40	1.40	1.30	1.30	1.30	1.30	1.30
Mono-cal Phos	1.50	1.50	1.50	1.50	1.50	1.20	1.20	1.20	1.20	1.20
Lysine	0.31	0.30	0.28	0.27	0.31	0.15	0.13	0.11	0.11	0.15
Methionine	0.30	0.30	0.30	0.30	0.30	0.20	0.20	0.20	0.20	0.20
Threonine	0.20	0.20	0.20	0.20	0.20	0.10	0.10	0.05	0.05	0.10
Tryptophan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Choline Cl	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nacl	0.40	0.45	0.45	0.45	0.40	0.40	0.38	0.38	0.38	0.40
Vitamin mix[1]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mineral mix[1]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Phytase	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Calculated content, %										
MEn, Kcal/kg	2918	2916	2906	2864	2858	3100	3093	3090	3016	3013
CP	21.38	21.11	21.21	21.36	21.32	18.71	18.72	18.59	18.77	18.58
Ca	0.93	0.93	0.93	0.93	0.93	0.82	0.82	0.82	0.82	0.82
Total P	0.71	0.71	0.71	0.71	0.71	0.62	0.62	0.62	0.63	0.63
nnP	0.44	0.44	0.43	0.42	0.42	0.37	0.37	0.36	0.35	0.35
Lysine	1.38	1.35	1.33	1.32	1.33	1.12	1.09	1.06	1.06	1.06
Methionine	0.62	0.62	0.61	0.61	0.61	0.50	0.50	0.49	0.49	0.48
Cysteine	0.41	0.41	0.40	0.40	0.40	0.39	0.39	0.38	0.38	0.38
Threonine	0.99	0.97	0.97	0.97	0.95	0.81	0.80	0.74	0.74	0.77
Tryptophan	0.31	0.30	0.29	0.29	0.28	0.27	0.26	0.26	0.25	0.24
Methionine +Cysteine	1.03	1.02	1.02	1.02	1.01	0.89	0.88	0.87	0.87	0.86
Arginine	1.52	1.52	1.55	1.57	1.58	1.35	1.37	1.38	1.41	1.41
Valine	1.16	1.13	1.12	1.11	1.09	1.05	1.03	1.01	1.01	0.97
Isoleucine	0.89	0.86	0.86	0.85	0.84	0.79	0.78	0.76	0.76	0.73
Leucine	1.81	1.75	1.72	1.70	1.66	1.66	1.63	1.59	1.57	1.51
NDF	8.91	10.21	11.54	12.92	14.24	8.72	10.05	11.38	12.79	14.09
CF	3.81	4.26	4.76	5.28	5.76	3.52	4.01	4.49	5.01	5.46
Na	0.17	0.19	0.19	0.19	0.17	0.17	0.17	0.17	0.17	0.17
Cl	0.28	0.31	0.31	0.31	0.27	0.28	0.27	0.26	0.26	0.27
Choline (mg/kg)	1354	1280	1228	1179	1117	1228	1171	1108	1063	989

[1]Providing the following (per kg of diet): vitamin A (trans-retinyl acetate), 10,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E (all-*rac*-tocopherol-acetate), 30 mg; vitamin B_1, 2 mg; vitamin B_2, 8 mg; vitamin B_6, 4 mg; vitamin B_{12} (cyanocobalamin), 0.025 mg; vitamin K_3 (bisulphatemenadione complex), 3mg; choline (choline chloride), 250 mg; nicotinic acid, 60 mg; pantothenic acid (D-calcium pantothenate), 15 mg; folic acid, 1.5 mg; betaíne anhydrous, 80 mg; D-biotin, 0.15 mg; zinc (ZnO), 80 mg; manganese (MnO), 70 mg iron ($FeCO_3$), 60 mg; copper ($CuSO_4 \cdot 5H_2O$), 8 mg; iodine (KI), 2 mg; selenium (Na_2SeO_3), 0.2 mg.

2.3 Proximate analysis/ Nutrient profile

Table 3: Proximate analysis of starter and finisher feed and MNC

	Proximate As is basis	Crude Protein W/W%	Crude Fat W/W%	ADF W/W%	NDF W/W%	Lignin W/W%
Starter	0MNC	20.90	2.30	3.92	6.48	0.70
	5MNC	21.13	3.08	6.18	9.18	2.08
	10MNC	20.51	4.12	6.82	9.31	2.53
	15MNC	21.51	4.14	9.66	13.70	4.37
	20MNC	20.10	4.62	9.82	14.18	3.17
Finisher	0MNC	18.07	4.68	3.85	7.22	0.78
	5MNC	19.03	5.33	4.98	8.53	1.67
	10MNC	18.26	5.47	6.62	11.37	2.40
	15MNC	18.25	4.45	9.37	13.49	3.59
	20MNC	18.75	4.97	9.40	16.21	3.70
	MNC	16.61	6.80	41.96	49.67	19.58

All feed samples including 5 starters and 5 finishers as well as MNC sample were analyzed for their nutrient profile- DM, GE, N (for CP), EE, ADF, NDF, Lignin, ash, amino acids, and starch using standard procedures of AOAC (AOAC, 2006).

Dry matter

Dry matter content of the MNC and all the starter and finisher treatment diet was determined at 135°C for 2 h in oven, (method 930.15), and ash (method 942.05).

Gross energy

Gross energy content of samples was determined in duplicates using an adiabatic oxygen bomb calorimeter (Parr Isoperibol Bomb Calorimeter 6200, Parr Instruments Co., Moline, IL) with benzoic acid as a calibration standard.

CP analysis

CP was calculated by determining nitrogen (N) by dry combustion using a LECO analyzer (LECO CN-2000, Leco Corp., St. 88 Joseph, MI; method 976.05, CP = N × 6.25). Blank ethylene diamine tetra acetic acid (EDTA) samples were run to correct for drift before starting the actual protein analysis.

Ether extract: Ether extract was determined by method 920.39; using Soxhlet apparatus and petroleum ether.

Acid detergent fiber: by method 973.18 and **Neutral detergent fiber**: by method 2002.04 were determined using Ankom^{200} Fiber Analyzer (Ankom Technology Macedon, NY).

Total starch content: method 996.11 was applied to determine total starch using a Megazyme test kit (Megazyme International, Ireland, UK).

Nitrogen-corrected apparent metabolizable energy (AMEn) value of macadamia nut cake

Two energy balance studies were carried out in our lab (Berrocoso et al., 2017), to determine the AME_n value of (MNC) for broiler chickens at different ages. In study 1, two dietary treatments were fed from 4 to 10 d of age. Dietary treatments consisted of a control diet with no MNC and a diet containing 6% added MNC (94% control diet). In experiment 2, four dietary treatments were provided from 17 to 23 d of age. Diets in second experiment were four treatment diets including control diet (0% MNC); 3% added MNC (97% control diet); 6% added MNC (94% control diet); and 9% added MNC (91% control diet) were fed 100, 97, 94 and 91 % of ad libitum respectively. So, the differences in AMEn consumption were only due to MNC source. A single source of MNC was used in both experiments. Feed intake, body weight, energy intake, energy excretion, N intake, N excretion, AME_n intake and AME_n were determined in both experiments. In experiment 1, the AME_n was estimated using the difference method by

subtracting AME_n of the basal diet from AME_n of the test diet. In experiment 2, AME_n intake was regressed against feed intake with the slope estimating AME_n of MNC. Regression equation used was Y = 2,908.2x – 122.73 ($P < 0.001$; SEM of the slope = 11.7; $r^2 = 0.93$). The AME_n of MNC was found to be 2889.58 kcal and 2908 kcal/kg in experiment 1 and 2, respectively with an average of 2899 kcal/kg on DM basis (Berrocoso et al., 2017). The results indicate that AME_n of MNC is comparable to conventional feedstuffs with similar nutrient profile, and can be incorporated in broiler diets.

2.4 Sample collection and processing

2.4.1 Growth Performance

Body weights of individual birds were taken on d 1, 7, 14, 21, 28, 35 and 42. Feed offered to each pen were recorded. Any leftover fed in the feeder (and on the plastic sheet below feeder) were weighed back and recorded weekly. The data generated were used to calculate average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR).

2.4.2 Cecal microbiota

At the end of the experiment, all the broiler chickens sacrificed by CO_2 asphyxiation. The birds had free access to feed and water, in order to avoid a possible effects of feed withdrawal before slaughter on the excreta sample, which may mask some of the effects of factors in question or confound the interpretation of the results. Randomly selected two birds from each pen (a total of 12 birds per treatment, with the body weights approximating to the mean value of the representative treatment) were sacrificed to obtain cecal excreta sample. Cecal excreta sample was stored at -80^{o}C until further processing.

DNA isolation procedure

The -80^{o}C frozen cecal excreta sample was thawed and DNA isolation process was done using Repeated Bead Beating Plus Column (RBB + C) method as previously described (Yu and Morrison, 2004). Briefly, DNA was isolated as described below:

A. Cell lysis

Cecal excreta 0.25 g was transferred into a 2 mL screw cap tube. 1 mL of lysis buffer was added along with 0.3 g of 0.1 mm zirconia beads and 0.1 g of 0.5 mm zirconia beads. It was then homogenized for 3 minutes at maximum speed on bead beater. The homogenized excreta sample were then incubated in water bath for 15 minutes at 70^{o}C and made sure to shake manually every 5 minutes. After incubation, it was centrifuged at 4^{o}C for 5 minutes at 16000x g. Supernatant was transferred to new 2 mL Eppendorf tube. 300 µL of fresh lysis buffer was added to the lysis tube and steps were repeated from homogenizing to centrifuge and later supernatant were pooled.

B. Precipitation of nucleic acids

For precipitation, 200 µL of 10 M ammonium acetate was added to each lysate tube, mixed well, and incubated on ice for 5 min. Then incubated product was centrifuged at 4°C for 10 min at 16,000× g. The supernatant was transferred to two 1.5 mL eppendorf tubes, and one volume of isopropanol was added mixed and incubated on ice for 30 min. It was further centrifuged at 4°C for 15 min at 16,000× g, the supernatant was removed by aspiration, and the nucleic acids pellet was washed with 70% ethanol, dried under vacuum for 3 min. Nucleic acid pellet was dissolved in 100 µL of TE (Tris-EDTA) buffer and the two aliquots were pooled.

C. Removal of RNA, protein, and purification

2 µL of DNase-free RNase (10 mg/mL) was added and incubated at 37°C for 15 min. Then 15 µL of proteinase K was added to 200 µL of Buffer AL (from the QIAamp DNA Stool Mini Kit), mixed well, and incubated at 70°C for 10 min. Further 200 µL of ethanol was added

and mixed well. Transfered to a QIAamp column and centrifuged at 16,000× g for 1 min. The flow through was discarded and 500 µL of Buffer AW1 (Qiagen) was added, later centrifuged for 1 min at room temperature. Again, the flow through was discarded and 500 µL of Buffer AW2 (Qiagen) was added to centrifuge for 1 min at room temperature. The column was dried by centrifugation at room temperature for 1 min. Further, 200 µL of Buffer AE (Qiagen) was added and incubated at room temperature for 2 min. It was then, centrifuged at room temperature for 1 min to elute the DNA. The DNA solution was aliquoted into four tubes. 2 µL of obtained DNA was run on a 0.8% gel to check the DNA quality. The DNA solutions was stored at -20°C and used as template DNA in PCR to amplify the 16S ribosomal DNA for DGGE analysis and construction of 16S rDNA clone libraries was performed.

DNA was quantified using 2 µL sample by UV 260/280 absorbance in a nanodrop spectrophotometer (BioSpec-nano Shimadzu, Queensland, Australia) to check for the quantity and quality of DNA. Fifteen samples of DNA for the 5 treatments were used for DGGE.

PCR amplification of 16S ribosomal DNA for DGGE

The variable V3 region of 16S rDNA was enzymatically amplified in the PCR with a primer set of conserve regions of the 16S rRNA genes. The nucleotide sequences of the primer set are as follows: 341f-GC (5'-CCTACGGGAGGCAGCAG-3'); and 534r (5'-ATTACCGCG GCTGCTGG-3'). Primer 341f has an additional 40-nucleotide GC-rich sequence (GC clamp) at its 5' end. PCR amplification was performed with a MJ Research thermal cycle machine (MJ Research, Inc., Watertown, Mass.). A 20 µl volume PCR reaction contained with 10 µl of Bioline Shortmix, 1 µl 10 µM of each primer, 1 µl of concentration 500 ng of genomic DNA and 7 µl DNA/RNA free water. The samples were first incubated for 4 min at 94°C to denature the template DNA. This hot start technique was performed to minimize non-specific annealing

primers to non-target DNA. The initial hot-start was then followed by 35 cycles of the following parameters: 94°C for 1 minute, 56°C for 30 s, primary extension at 72°C for 30 s and final extension was carried out at 72°C for 10 min.

The yield of PCR amplification of each sample was assessed by running 1 µl of the PCR product on a 2% agarose gel. Gels containing 10% (wt/vol) polyacrylamide (37.5:1 acrylamide: bisacrylamide) were cast using a D-code system (Bio-Rad Laboratories, Inc., Hercules, CA). The gels contained a linear gradient of the denaturants urea and formamide, increasing from 30% at the top of the gel to 70% at the bottom (with 100% denaturants corresponding to 7 M urea and 40% [vol/vol] deionized formamide). Gels were run at 50 V for 30 minutes and 150 V for 13 hours at a constant temperature of 65°C in 7 liters of 1x Tris-acetate-EDTA (TAE) buffer. Gels were stained for 1 h in 100 mL of 1x TAE containing 10 µl of 10,000 x SYBR green nucleic acid gel stain (Molecular Probes, PoortGebouw, The Netherlands) and photographed under a UV light transilluminator (AlphaImager, San Leandro, CA). After visual confirmation of the PCR products on a 2% agarose gel, DGGE was performed.

DNA fingerprint from Denaturing Gel Gradient Electrophoresis images

GelJ a free software was used as a technique for comparing DNA patterns that allows to analyze genomic relatedness among different samples, as this software has features such as accurate lane- and band-detection, several options for computing migration models, several band-and curve-based similarity methods, different techniques for generating dendrograms, comparison of banding patterns from different experiments, and database support.

Different steps that were followed to analyze DGGE image using Gelj software:

1. *Pre-processing*: Image was saved in jpeg format and simple operation such as cropping, rotating and filtering was done in this step.

2. *Lane detection*: This step allows automatic detection of lanes of gel-images, and the lanes can be manually added or deleted. This step also allows to adjust the brightness and contrast of individual lanes.

3. *Normalization:* In this step, the migrating bands are normalized on the basis of reference band with some additional features such as 1^{st}-3^{rd} degree curves, cubic splines, logarithmic, gaussian or rodbard.

4. *Band detection*: This software provides automatic band detection, height threshold, manual picking, and densitometric-curve display. It also helps synchronization of the histogram with the gel-image. It also allows lane-by-lane threshold functionality to detect bands automatically.

5. *Fingerprint comparison*: This stage allows configure using 4 parameters such as lanes to compare, similarity method, clustering method and output. This allows to find lanes that are similar to such a lane.

Cloning

Since microbiota plays important role in competitive exclusion of pathogenic bacteria, there is need to identify the bacteria present in the cecum of the chicken. For this purpose, we need to retrieve 16S rRNA gene sequences from DNA isolated from cecum. Cloning was done to determine the 16S rRNA gene sequences. For cloning, DNA that was diluted to 20 ng/ μL was pooled and made 12 samples for each of the treatments 0%MNC, 10%MNC and 20%MNC. Following are the steps of cloning:

PCR product production for cloning

It was important to properly design PCR primers to ensure proper product that we were looking for our study. Primer pair 63F-1387R were used to obtain 16S rRNA from V3 to V8 region. In a mixture containing *Taq* polymerase and a proofreading polymerase, *Taq* was used in excess of a 10:1 ratio to ensure the presence of 3´ A-overhangs on the PCR product.

The 50 μL PCR reaction was set up. We used the cycling parameters suitable for our primers and template. We made sure to include a 7–30 minutes extension at 72°C after the last cycle to ensure that all PCR products are full length and 3´ adenylated.

Amplification products were analyzed by electrophoresis in 0.8% (wt/vol) agarose gels and SYBR green staining, then were stored at −20°C until they were used for construction of the clone libraries.

Construction of 16S rDNA clone libraries and sequencing

For the amplification of PCR products, TOPO TA Cloning vector (Invitrogen) was used. This provides a highly efficient, 5-minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. For further sub-cloning, primers were not required.

Once the PCR product was produced, the TOPO cloning reaction (mix gently together the PCR Product and TOPO vector) was set up. Incubated for 5 minutes at room temperature. Then the reaction was placed on ice and proceed to transform the TOPO cloning reaction into One Shot Competent *E. Coli* Cells. Also, sodium chloride and magnesium chloride was added to a final concentration of 200 mM NaCl, 10 mM MgCl2 in the TOPO® Cloning reaction which caused to increase the number of colonies over time.

Cloning procedure

X-gal 40 µl was spread with glass spreader and plates were incubated at room temperature. Ligation was set up in PCR tubes with 1 µl PCR product, 1 µl salt solution, 3 µl sterile water, and 1 µl of PCR II TOPO vector (i.e. 6 µl total from kit). It was then gently mixed and centrifuged for 30 second. Incubated at room temperature for 30 minutes, and then kept in ice. Then incubated in ice for 20 minutes, mixed by flicking, and again incubated for 10 minutes. Heat shock in water bath was given for 30 second @ 42°C (without shaking). Immediately transferred to ice for 2 minutes. 250 µl SOC medium was added to tightly closed tube to shake at 200 rpm, 37°C horizontally for 1 hour. For control group, 50 µl TOPO and 250 µl SOC was mixed and 100 µl was transferred to LB broth and flick tube. Spread 50 µl to warm plates which gave around 100 colonies after overnight incubation.

Tubes were labeled with different treatments and 36 white or light blue but not the dark blue colonies were taken from each dietary treatment. Then kept on the cap holder onto tubes and the tubes were boiled in floating rack for 5 minutes, and kept on ice.

Post plating PCR screening was performed for all the treatments with 20 µl of PCR mix in each tube. Each PCR screening product tube contained 10 µl 2x shortmix, 1 µl F primer T7, 1 µl R primer M13, 6 µl nuclease free water, and 2 µl of lysate. Screening before sequencing was done in 1% Agarose gel with 4 µl of PCR screening product, 1 µl of 6x loading dye and 1 µl of 100x SYBR green in each well. The retrieved sequences were compared with the GenBank database (http://blast.ncbi.nlm. nih.gov/) using Basic Local Alignment Search Tool of the National Center for Biotechnology Information algorithm. The sequence with the highest similarity to a distinct species/genus was chosen as the closest relative of the cloned sequences (Gong et al., 2008).

Shannon-Wiener index (H′)

The Shannon-Wiener index of diversity (H') was calculated to determine the diversity of the microbial community from different dietary treatments, by the following formula: $H' = -\sum (Pi)\,(\ln Pi)$, where Pi is defined as the proportion of each band (or specie) for the total bands (or species) in the sample (Liu et al., 2012).

2.4.3 Volatile fatty acid

Volatile fatty acid (VFA) especially acetic acid, propionic acid, and butyric acid are much studied because of its positive role in maintaining gut health and other physiological benefits to the host. For this work, a rapid and reliable gas chromatographic method was used to determine different VFAs. For this propose 4 chickens per pen (24 birds/ treatment) cecal excreta samples were collected and kept frozen until further analysis. During analysis, the samples were thawed, weighed 200mg each sample and diluted with distilled water in a sterile Eppendorf tubes. Add 100 μL of 25% metaphosphoric acid solution and TMA solution 50 μL. Dilute the sample again so that the total volume in Eppendorf tube is 1400 μL. Cecal digesta samples were homogenized and centrifuged at 12,000× g for 30 minutes at 4^{o}C. 700 μL of supernatant was then transferred to GC vial and were analyzed using a gas chromatograph (Shimadzu GC-2010, Shimadzu Co., Kyoto, Japan) coupled with a 30 m × 0.53 mm internal diameter column (Teknokroma TRB-FFAP, Teknokroma, Barcelona, Spain) and flame ionization detector to determine VFA concentrations in cecal digesta (Rebole et al., 2010; Zhang et al., 2003). The injector-port and flame ionization detector temperatures were fixed at 230^{o}C and 250^{o}C, respectively. In the temperature program, the initial temperature was held at 120^{o}C for 4 min after injection and then increased at 4^{o}C/min to 160^{o}C, where it was held for 4 min. Helium was used as the carrier gas. The injection volume was set at 1 μL and analyses were performed, and the run time for each analysis was 17.5 min. An aqueous stock standard solution was prepared

with a concentration of 1 mm, 2 mm, 3 mm, 4 mm, 5 mm, 6 mm, 7 mm, and 8 mm with final volume 1400 μL. All the stock standard solutions were stored at −20°C until used. The molar concentration of each VFA was calculated using methyl valeric acid, and the molar percentage of an individual VFA was calculated by dividing the micro molar (μM) concentration of the individual VFA by the μM sum of all the VFA multiplied by 100. Individual VFA concentrations were calculated as a percentage of the total VFA content to determine if the increase in dietary fiber shifted the concentration of one VFA to another.

Study 2: Effect of different levels of cassava root chips inclusion on the growth performance and gut health parameters of broiler chickens

2.5 Study setup and experimental design

All the animal experimentation was carried at the Small Animal Facility (SAF) of University of Hawaii at Manoa. The study was conducted in accordance with the guideline and ethics committee approved by the Institutional Animal Care and Use Committee (IACUC, Protocol #13-1639).

A total of 180, day-old broiler chicks (Cobb 500) from a local hatchery (Asagi Hatchery, Honolulu, HI) were used in the growth performance study. Birds were raised in group floor pen and standard commercial broiler rearing environment (temperature, humidity, light and built up litters) was managed. On day one, all 180 day-old chicks were weighed individually, wing tagged and placed randomly in one of 30 pens (six birds/ pen), making 6 replicates of each treatment. Each pen birds were fed with one of 5 diets where diets were treatment and pen was experimental unit. All birds received starter and finisher diets in mash form from day 1 to 21 and 22 to 42 d, respectively. The birds had ad libitum access to feed and water.

2.6 Diets

Preparation of Cassava root chips

The CRC sample was sourced from Ulupono Initiative which is Hawaii-focused impact investing firm that uses for-profit and non-profit investments to improve the quality of life for island residents in three areas: locally produced food; renewable energy; and waste reduction. We received the cassava root which were already chipped or sliced and sun dried once cassava roots were collected from farm and proper washing was done but not peeled. CRC was than

ground to pass through a 3/16 mesh sieve. Prior to diet formulation, proximate and other analysis of CRC were determined. Nutrient profile and Metabolizable energy value was used from a previous study.

Experiment

The diets used in this study were corn-soybean meal based and were fed in mash form. The feeds were formulated for two phases- grower (0-21d) and finisher (22-42 d), to meet or exceed the nutrients requirements of broilers (NRC, 1994). The diets were formulated to have 5 different level of CRC: 0% as control, and 12.5, 25, 37.5 and 50% as other treatments. These treatments were allotted to chickens in completely randomized design. The diet formulas are presented in Table

Table 4: Ingredient used and nutrient composition of different treatment diets

	Starter					Finisher				
Ingredients, %	CSV0	CSV12.5	CSV25	CSV37.5	CSV50	CSV0	CSV12.5	CSV25	CSV37.5	CSV50
Corn	56.22	41.69	27.41	12.79	0.00	61.75	47.68	32.85	18.35	5.26
SBM	36.58	37.60	39.00	40.00	40.10	31.00	32.00	33.50	35.00	35.10
Cassava chips	0.00	12.50	25.00	37.50	50.00	0.00	12.50	25.00	37.50	50.00
Soybean oil	2.50	3.50	4.00	5.00	5.14	3.49	4.08	5.00	5.50	5.80
Limestone	1.40	1.30	1.30	1.30	1.30	1.00	1.00	1.00	1.00	1.00
Mono-cal Phos	1.20	1.30	1.20	1.20	1.20	1.00	1.00	1.00	1.00	1.00
Lysine	0.31	0.30	0.28	0.30	0.30	0.15	0.13	0.11	0.11	0.15
Methionine	0.30	0.30	0.30	0.40	0.45	0.20	0.20	0.20	0.20	0.30
Threonine	0.20	0.20	0.20	0.20	0.20	0.10	0.10	0.05	0.05	0.10
Tryptophan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Choline Cl	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nacl	0.28	0.30	0.30	0.30	0.30	0.30	0.30	0.28	0.28	0.28
Vitamin mix[1]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mineral mix[1]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

Phytase	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Total	100	100	100	100	100	100	100	100	100	100
Calculated Content, %										
MEn, Kcal/kg	2937	2942	2916	2917	2878	3092	3073	3071	3040	3006
CP	21.25	21.04	21.01	21.01	20.61	18.68	18.49	18.42	18.44	18.20
Ca	0.87	0.88	0.89	0.91	0.93	0.67	0.69	0.72	0.75	0.77
Total P	0.65	0.65	0.61	0.58	0.56	0.58	0.56	0.54	0.52	0.50
nnP	0.38	0.40	0.39	0.39	0.39	0.33	0.33	0.34	0.34	0.34
Lysine	1.38	1.37	1.36	1.38	1.36	1.11	1.10	1.10	1.11	1.12
Methionine	0.62	0.61	0.59	0.67	0.70	0.50	0.48	0.47	0.46	0.54
Cysteine	0.41	0.46	0.51	0.56	0.61	0.39	0.44	0.49	0.54	0.59
Threonine	0.99	1.00	1.02	1.03	1.03	0.81	0.82	0.79	0.81	0.86
Tryptophan	0.30	0.30	0.31	0.30	0.30	0.27	0.27	0.27	0.27	0.26
Methionine+Cysteine	1.03	0.98	0.94	0.99	0.98	0.89	0.84	0.80	0.75	0.80
Arginine	1.51	1.48	1.46	1.42	1.36	1.35	1.32	1.30	1.28	1.22
Valine	1.16	1.11	1.07	1.02	0.96	1.05	1.01	0.97	0.93	0.87
Isoleucine	0.88	0.86	0.84	0.82	0.79	0.79	0.77	0.75	0.74	0.70
Leucine	1.80	1.69	1.60	1.48	1.36	1.67	1.56	1.46	1.37	1.24
NDF	8.93	7.77	6.66	5.49	4.37	8.81	7.68	6.54	5.43	4.29
CF	3.80	4.01	4.26	4.47	4.66	3.53	3.75	3.99	4.24	4.42
Na	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.12	0.12	0.12
Cl	0.21	0.22	0.22	0.22	0.22	0.22	0.22	0.21	0.21	0.21
Choline (mg/kg)	1348	1285	1235	1172	1095	1229	1170	1119	1070	991

[1]Providing the following (per kg of diet): vitamin A (trans-retinyl acetate), 10,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E (all-*rac*-tocopherol-acetate), 30 mg; vitamin B_1, 2 mg; vitamin B_2, 8 mg; vitamin B_6, 4 mg; vitamin B_{12} (cyanocobalamin), 0.025 mg; vitamin K_3 (bisulphatemenadione complex), 3mg; choline (choline chloride), 250 mg; nicotinic acid, 60 mg; pantothenic acid (D-calcium pantothenate), 15 mg; folic acid, 1.5 mg; betaíne anhydrous, 80 mg; D-biotin, 0.15 mg; zinc (ZnO), 80 mg; manganese (MnO), 70 mg iron ($FeCO_3$), 60 mg; copper ($CuSO_4 \cdot 5H_2O$), 8 mg; iodine (KI), 2 mg; selenium (Na_2SeO_3), 0.2 mg.

2.7 Proximate analysis/ Nutrient profile

All 10 feed samples including starter, finisher and CRC sample were analyzed for their nutrient profile- DM, GE, N (for CP), EE, ADF, NDF, ash, amino acids, and starch using standard procedures of AOAC (AOAC, 2006) same as for the MNC study.

2.8 Sample collection and processing

2.8.1 Growth Performance

Body weight of individual bird was taken on d 1, 7, 14, 21, 28, 35 and 42. Feed offered to each pen was recorded. Any leftover fed in the feeder (and on the plastic sheet below feeder) was weighed back and recorded weekly. The data generated was used to calculate total body weight, average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR). At the end of the experiment, all the broiler chickens destined for slaughter (euthanized by carbon dioxide asphyxiation) had free access to feed and water, in order to avoid a possible effects of feed withdrawal for a period of time before slaughter on the size and weight of tissues of interest, which may mask some of the effects of factors in question or confound the interpretation of the results. Randomly selected two birds from each pen (a total of 12 birds per treatment, with the body weights approximating to the mean value of the representative treatment) were used to collect ileum tissue sample for the histological analysis. Remaining 4 birds from each pen were selected for the VFA analysis.

2.8.2 Ileum Histology

At the end of the feeding trial (d 42), 2 birds per pen selected randomly (12birds/ diet) were killed by CO2 asphyxiation. Intestinal ileum segment samples (approximately 2 cm in length) was excised and flushed with 0.9% saline to remove the fecal contents. Then, it was fixed

in 10% neutral-buffered formalin for histology. The segment of intestine collected for ileum was midway between Meckel's diverticulum and the ileo-cecal junction. Samples were dehydrated, cleared, and paraffin embedded. Segments from each treatment was sectioned at a 6-μm thickness, placed on glass slides, and processed in HE stain for examination by microscopy. The morphological indices evaluated were villus height from the tip of the villus to the crypt, crypt depth from the base of the villus to the submucosa, villus height to crypt depth ratio and the villus surface area.

2.8.3 Volatile fatty acid

Volatile fatty acid or short chain fatty acid especially acetic acid, propionic acid, and butyric acid are being considered and much studied recently because of its positive role in maintaining gut health and other physiological benefits to the host. For this work, a rapid and reliable gas chromatographic method is used to determine different VFAs. For this propose 4 chickens per pen (24 birds/ treatment) cecal excreta samples were collected and kept frozen until further analysis. During analysis, the samples were thawed, weighed 200mg each sample and diluted with distilled water in a sterile Eppendorf tubes. Add 100 μL of 25% metaphosphoric acid solution and TMA solution 50 μL. Dilute the sample again so that the total volume in Eppendorf tube is 1400 μL. Cecal digesta samples were homogenized and centrifuged at 12,000 x g for 30 minutes at 4°C. 700 μL of supernatant was then transferred to GC vial and were analyzed using a gas chromatograph (Shimadzu GC-2010, Shimadzu Co., Kyoto, Japan) coupled with a 30 m × 0.53 mm internal diameter column (Teknokroma TRB-FFAP, Teknokroma, Barcelona, Spain) and flame ionization detector to determine VFA concentrations in cecal digesta (Rebole et al., 2010; Zhang et al., 2003). The injector-port and flame ionization detector temperatures were fixed at 230°C and 250°C, respectively. In the temperature program, the

initial temperature was held at 120°C for 4 min after injection and then increased at 4°C /min to 160°C, where it was held for 4 min. Helium was used as the carrier gas. The injection volume was set at 1 μL and analyses were performed, and the run time for each analysis was 17.5 min. An aqueous stock standard solution was prepared with a concentration of 1 mm, 2 mm, 3 mm, 4 mm, 5 mm, 6 mm, 7 mm, and 8 mm with final volume 1400 μL. All the stock standard solutions were stored at −20°C until used. The molar concentration of each VFA was calculated using methyl valeric acid, and the molar percentage of an individual VFA was calculated by dividing the micro molar (μM) concentration of the individual VFA by the μM sum of all the VFA multiplied by 100. Individual VFA concentrations were calculated as a percentage of the total VFA content to determine if the increase in dietary fiber shifted the concentration of one VFA to another.

2.9 Statistical analysis

Data were analyzed by ANOVA using Mixed procedure of SAS (SAS 9.2, SAS Institute Inc., Cary, NC). Significant differences among treatments were assessed by Tukey's test. The effect of increasing treatment diet inclusion was partitioned into linear and quadratic components by using polynomial trend analysis. Statistical significance was considered at P less than 0.05.

Chapter 3: Results

Study 1: Effect of different levels of MNC inclusion on the growth performance and gut health parameters of broiler chickens

3.1 Growth Performance

The broiler chicken fed with different dietary inclusion of macadamia nut cake did not shows any significant differences ($P > 0.05$) for BW and ADG during any phase of life. ADFI and FCR linearly increased with increasing level of MNC. The reason for increased feed intake and FCR could be due to reduced gross energy in feed with higher inclusion during feed formulation, which causes chicken to consume more feed to get same amount of weight gain. FCR increased with the increase in feed intake. The insignificant difference in BW and ADG was probably due to higher feed consumption. During starter phase ADFI is insignificant up to inclusion of 15% MNC compared to control diet whereas up to 10% MNC for FCR. In the finisher and overall stage 10% inclusion level showed the insignificance in ADFI. FCR in overall period is highly increased which is not desirable but comparing with the control diet in starter and finisher FCR is statistically similar to 10 and 15% MNC inclusion respectively.

Table 5: Growth Performance of broiler chicken fed control and different level of Macadamia nut cake

Items	Control	MNC 5	MNC 10	MNC 15	MNC 20	SEM	*P* – value	
							Linear	Quadratic
BW, g								
Starter	757.00	822.67	785.00	783.33	788.17	23.292	0.7574	0.3350
Finisher	1759.33	1721.83	1762.33	1755.17	1704.17	40.221	0.5504	0.6241
Overall	2516.00	2544.50	2547.50	2538.33	2492.33	48.231	0.7287	0.3803

ADFI, g								
Starter	48.83b	52.50ab	51.33ab	52.50ab	53.33a	1.072	**0.0136**	0.4140
Finisher	126.17b	134.50ab	132.33ab	140.83a	144.50a	3.523	**0.0007**	0.9202
Overall	94.33b	101.17ab	100.67ab	106.33a	107.83a	2.221	**0.0001**	0.5929
ADG, g								
Starter	35.83	39.17	38.33	37.17	37.83	0.874	0.4762	0.0956
Finisher	83.67	82.00	83.00	83.50	81.17	1.956	0.5765	0.8042
Overall	60.00	60.83	60.67	60.33	59.33	1.163	0.6224	0.3867
FCR								
Starter	1.35b	1.34b	1.34b	1.41a	1.41a	0.022	**0.0137**	0.2041
Finisher	1.51b	1.64ab	1.60b	1.69ab	1.79a	0.045	**0.0002**	0.6569
Overall	1.58c	1.67abc	1.66bc	1.77ab	1.82a	0.036	**0.0001**	0.8458

3.2 Microbiota profile

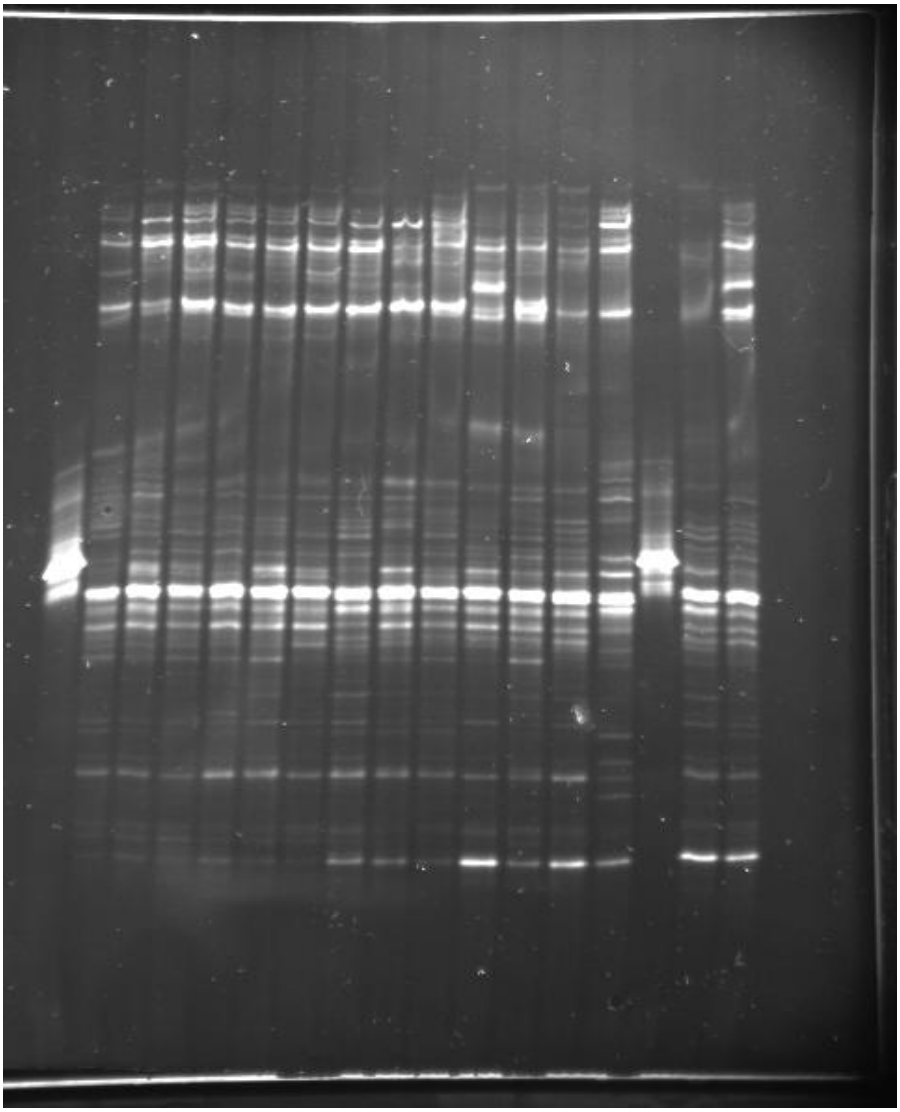

Figure 1: DGGE image of different treatments along with ladder bandsThis image shows Isolated DNA run through DGGE and different fingerprints were obtained. The denaturing gradient gel electrophoresis (DGGE) of chicken cecal bacterial populations at 42 days of age. Pattern of bands in the image is Ladder, T1R1, T1R2, T1R3, T2R1, T2R2, T2R3, T3R1, T3R2, T3R3, T4R1, T4R2, T4R3, T5R1, Ladder, T5R2, and T5R3 from left to right.

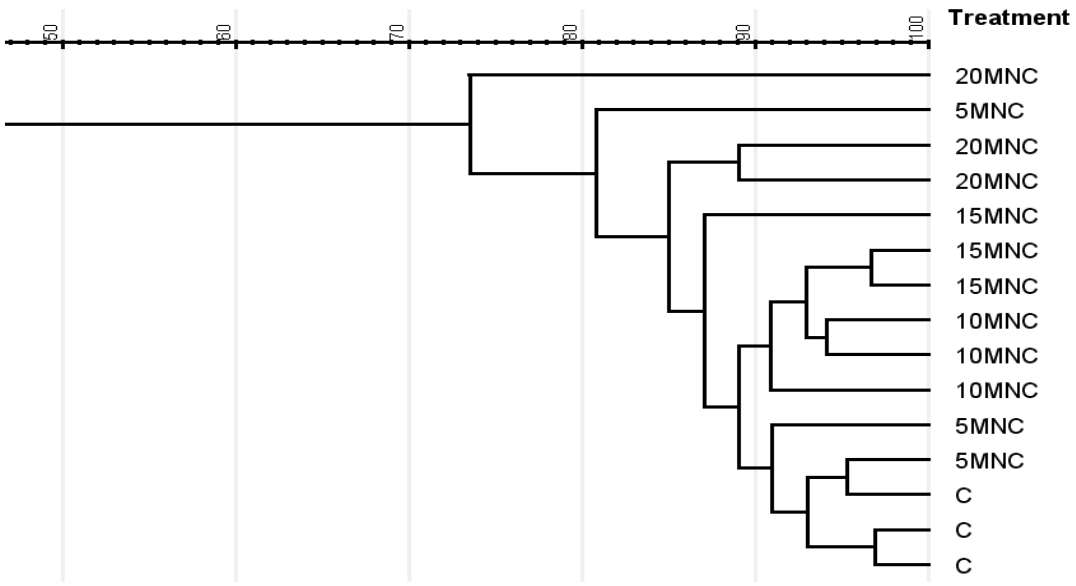

Figure 2: Dendrogram showing similarities between samples using Gelj software

This dendrogram constructed from the DGGE image shows that the bacteria in the control group and 5, 10, 15 % are around 90% and more similar whereas 20% has more of dissimilar bacteria.

Compare OTUs:

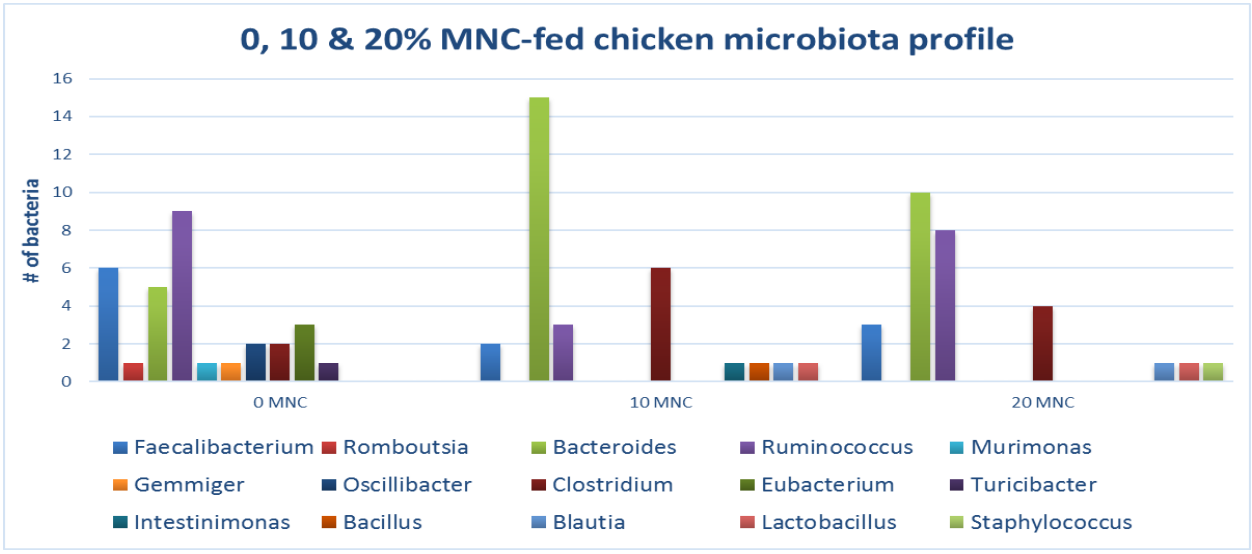

Figure 3: Comparing Operational Taxonomical Units (OTUs) across different treatments

Shannon Index

Table 6: Shannon Index across 0, 10 and 20%

Treatment	**H`**	**E_H**
0%	1.99	0.866
10%	1.53	0.737
20%	1.60	0.822

According to Shannon Index, among 3 treatment analyzed treatment 0% MNC or control group fed birds has more diverse (H`) and more even (E_H) bacterial composition compared to other MNC fed treatment groups birds.

Phylogenetic tree

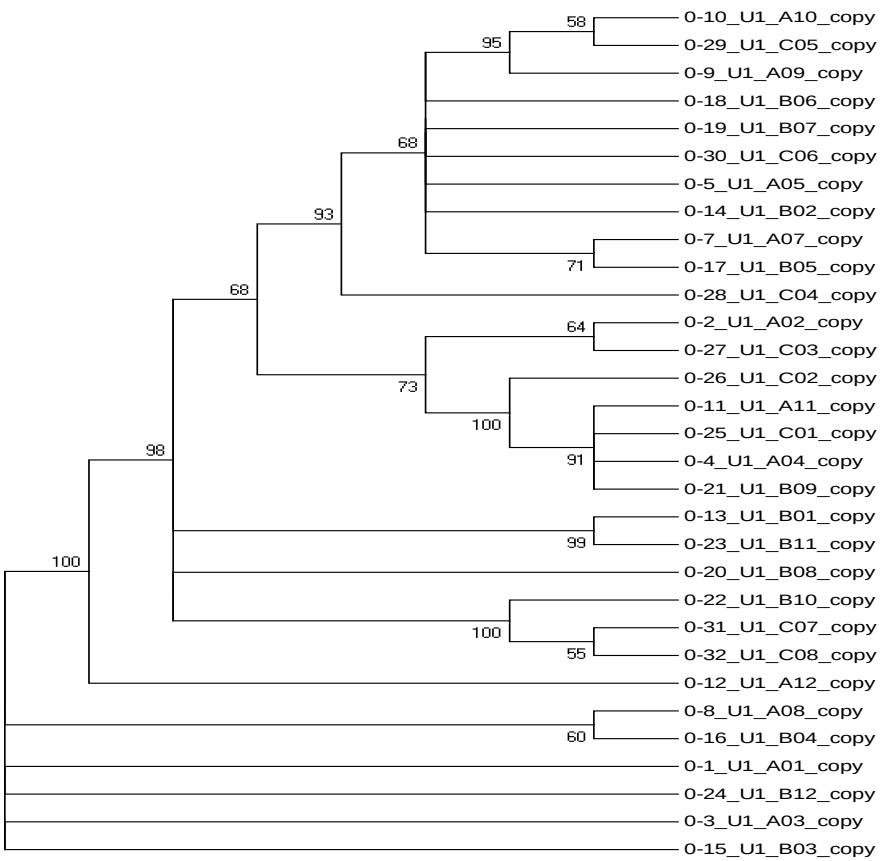

Figure 4: Molecular Phylogenetic analysis for 0% MNC fed birds microbiota profile by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were

eliminated. There was a total of 261 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

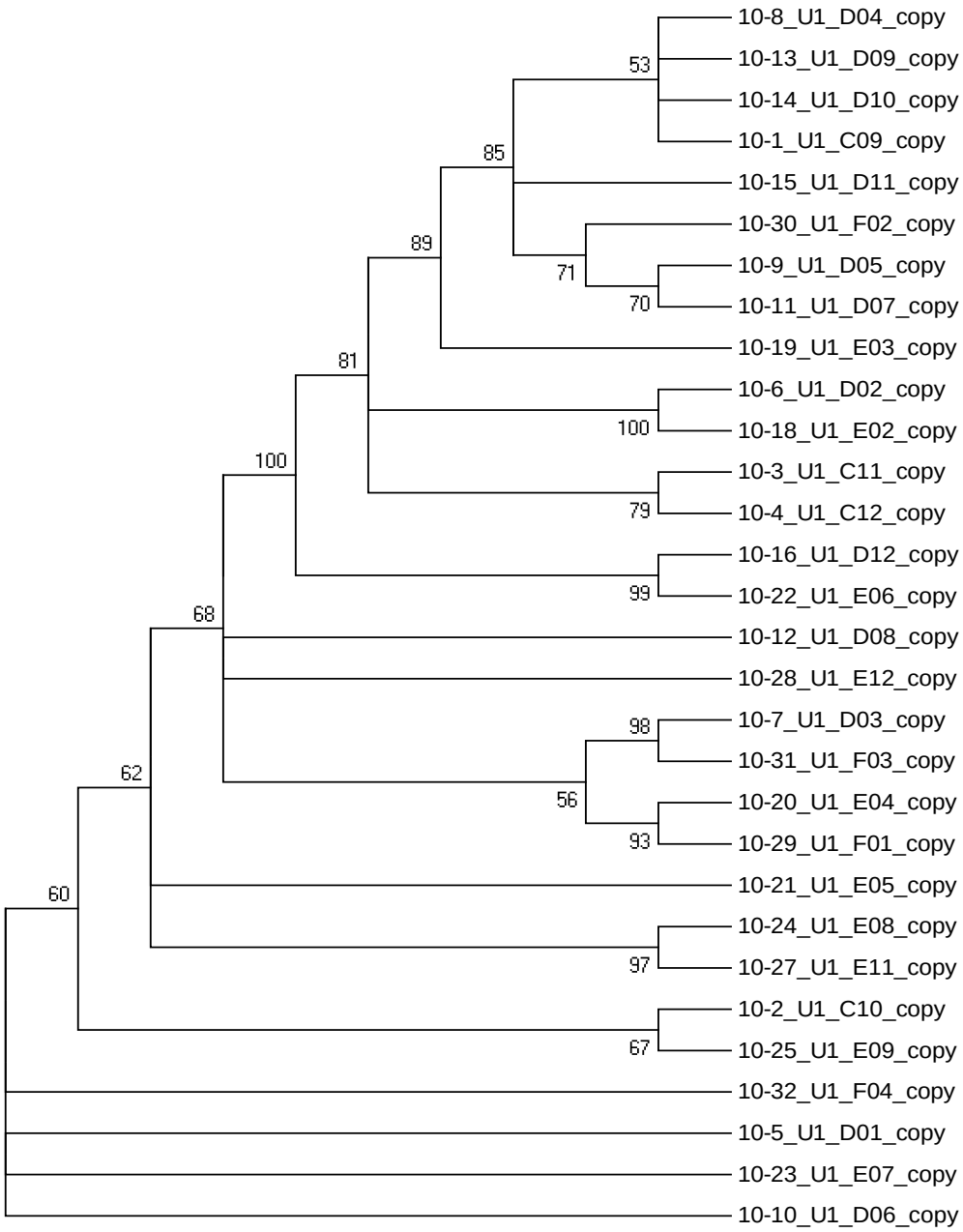

Figure 5: Molecular Phylogenetic analysis for 10%MNC fed birds microbiota profile by Maximum Likelihood method

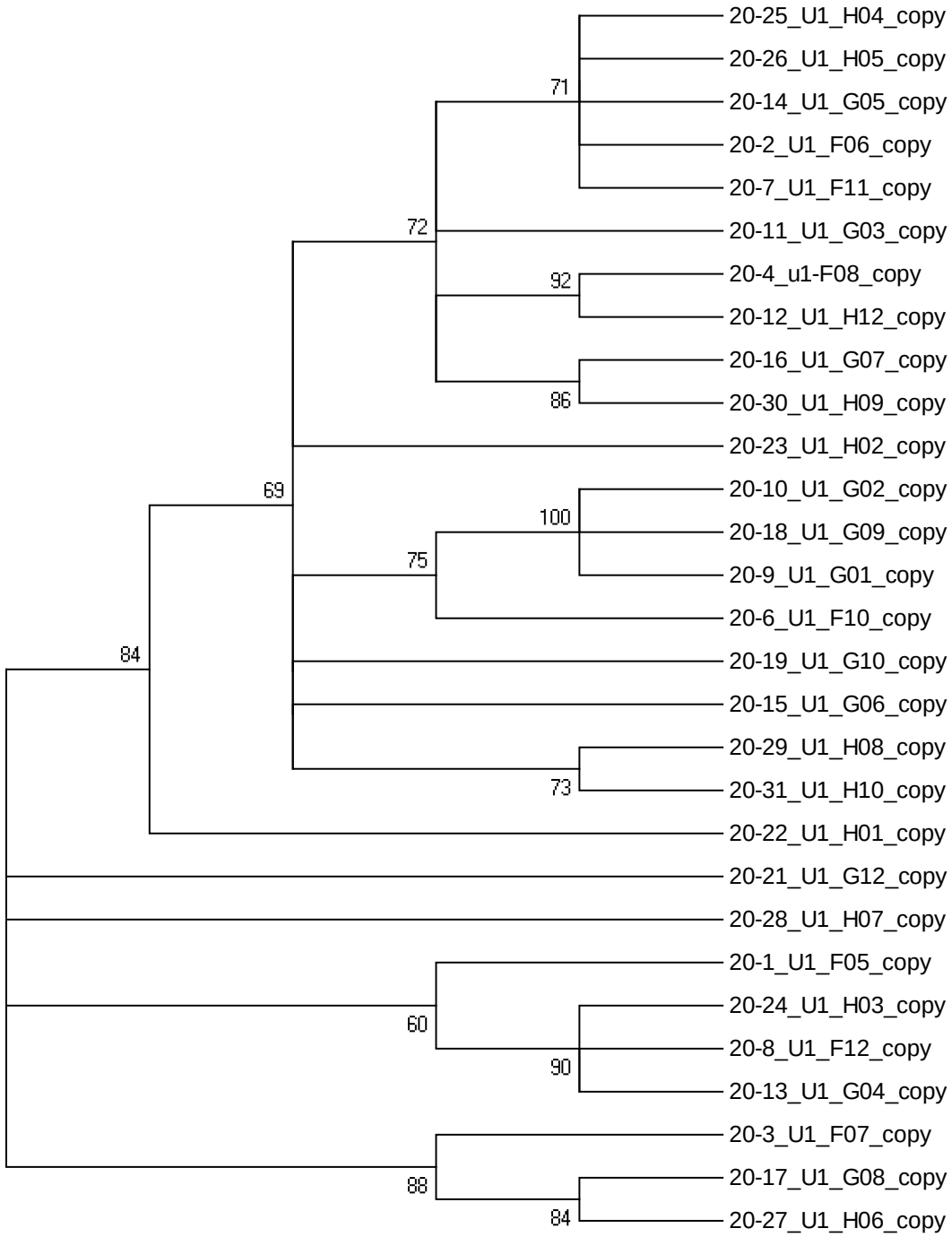

Figure 6: Molecular Phylogenetic analysis for 20%MNC fed birds microbiota profile by Maximum Likelihood method

Cloning and sequence analysis

In total, 89 clones were successfully sequenced, and of these sequences, 31 clones originated from T1 i.e. 0% MNC fed chicken birds cecal microbiota which shows 10 OTUs, 30 clones from T3 i.e. 10% MNC fed birds with total 8 OTUs, and the remaining 28 clones originated from T5 i.e. 20% MNC fed birds has representation of 7 OTUs. The sequence analysis of the clones revealed 24 sequences (26.97%) with 99 to 100% identity to known bacteria, while 51 sequences (57.30%) had 95 to 98% identity to their nearest relatives. The remaining 14 sequences (15.73%) had <95% identity to any recorded entries in GenBank. Of the 89 clones that were examined for 3 treatments (0, 10 and 20% MNC), in the phylum level the ratio of Firmicutes: Bacteroides was 26:5 in control group whereas 1:1 and 1.8:1 in 10 and 20% MNC inclusion, respectively where higher ratio is directly linked with ability to harvest energy and nutrient digestion. Where in genus level, the most abundant sequences were homologous to Ruminococcus (29.03%), followed by Faecalibacterium (19.35%), Bacteriodes (16.13%), Eubacterium (10%), Clostridium (7%), Oscillibacter (7%), Romboutsia, Murimonas, Turicibacter and Gemmiger (3% each) in T1. T3 has Bacteroides (50%) as the most dominant, followed by Clostridium (20%), Ruminococcus (10%), Faecalibacterium (7%), Intestinimonas (4%), and Lactobacillus, Blautia, Bacillus (3% each). T5 sequences were dominated by Bacteriodes (35.71%), Ruminococcus (28.57%), Clostridium (14.29%), Faecalibacterium (11%), Staphylococcus (4%), and Lactobacillus and Blautia (3% each). We did not consider species of individual bacteria as most of the sequences were related to species rather than identical to the particular species. Although other part of intestine is important ceca of chicken is most important with highly diverse and concentrated as much as 10^{11} bacteria/ g of cecal excreta (Mead, 1997).

3.3 Volatile fatty acid

Table 7: Volatile fatty acid content of broiler chickens fed different treatment diets

Items, μM/L	MNC0	MNC5	MNC10	MNC15	MNC20	SEM	*p*- value Linear	*p* - value Quadratic
Acetate	22.9	16.3	21.9	11.1	15.9	3.0973	0.0514	0.5704
Propionate	1.9	1.2	3.6	1.9	1.1	0.8984	0.7864	0.2497
Butyrate	3.0	2.8	6	7.1	3.2	1.9656	0.4415	0.2156
Total VFA	46.2ab	37.7b	67.6a	40.5ab	50.3ab	6.648	0.5895	0.4153

The result for cecal VFA content are shown in Table 6. Acetate concentration is highest, followed by butyrate concentration and then propionate. Although, acetate, propionate and butyrate are not significant statistically there is numerical differences between the treatments.

Study 2: Effect of different levels of cassava root chips inclusion on the growth performance and gut health parameters of broiler chickens

3.4 Growth performance

Table 8: Growth performance parameter of different treatments

Items	Control	CSV12.5	CSV25	CSV37.5	CSV50	SEM	*P*- value Linear	*P*- value Quadratic
BW, g								
Starter	730.67a	664.33ab	659.83ab	640.50b	583.83b	21.584	**<0.0001**	0.9560
Finisher	1813.17	1697.00	1734.33	1637.00	1540.33	67.815	0.0092	0.7093
Overall	2543.83a	2361.33ab	2394.33ab	2277.67ab	2124.17b	80.538	**0.0013**	0.7635
ADFI, g								
Starter	47.83	46.17	47.00	44.83	44.83	0.914	0.0178	0.9231
Finisher	144.50	147.67	148.33	151.83	138.50	3.908	0.5319	0.0496
Overall	96.17	97.17	97.83	98.17	91.67	2.008	0.2193	0.0519
ADG, g								
Starter	34.67a	31.67ab	31.67ab	30.50ab	27.83b	1.014	**<0.0001**	0.8962
Finisher	86.33	80.83	82.50	77.83	73.17	3.226	0.0081	0.7023
Overall	60.67a	56.00ab	57.00ab	54.33ab	50.67b	1.936	**0.0016**	0.8199
FCR								
Starter	1.37b	1.47ab	1.51ab	1.47ab	1.61b	0.048	**0.0043**	0.9779
Finisher	1.67b	1.84ab	1.81ab	1.95a	1.89ab	0.061	**0.0084**	0.2273
Overall	1.59b	1.73ab	1.73ab	1.82a	1.82a	0.045	**0.0009**	0.2546

Inclusion of cassava in different levels has significant effect on different indicators of the growth performance of broiler chickens.

3.5 Gut histology

Table 9: Gut histology parameter of different treatments

Items	Control	CSV12.5	CSV25	CSV37.5	CSV50	SEM	*P*- value Linear	*P*- value Quadratic
Villus height, μm	930.78	929.43	947.77	886.3	861.76	65.2744	0.3884	0.6099
Crypt depth, μm	107.03	110.29	102.81	108.74	117.9	9.3321	0.5002	0.4773
Villus: Crypt	8.70	8.43	9.22	8.15	7.31	------	-------	------
Villus surface area, μm^2	125454	110282	105940	91788	84802	12281	0.0165	0.8876

Dietary treatments did not significantly affect the ileum histology parameters such as villus height, crypt depth, villus to crypt ratio or villus surface area. There are numerical differences between the treatments as villus height and surface area are decreasing whereas crypt depth is increasing which could be due to faster turnover of the mucosa, which also requires higher maintenance energy and the overall growth performance is compromised.

3.6 Volatile fatty acid

Table 10: Volatile fatty acid parameter of different treatments,

Items, μM/ L	CRC0	CRC12.5	CRC25	CRC37.5	CRC50	SEM	*P* - value Linear	*P* - value Quadratic
Acetate	4.1181	3.0102	4.5370	5.3970	5.2935	0.8730	0.0985	0.6847
Propionate	0.6986	0.7970	0.9230	1.1200	0.8546	0.2041	0.3345	0.3980
Butyrate	4.2442	0.9443	1.2264	1.9569	0.2540	0.9030	0.0956	0.3803
Total VFA	22.6227	12.5611	16.0192	18.6123	18.0570	4.4595	0.8289	0.2872

Dried cassava root chips are low in protein, deficient in carotene and high fiber (insoluble fiber) which limits its use in higher quantity in the feed of poultry (Aro et al., 2008). Cassava chips contains around 40.91% resistant starch. Resistant starch is starch and starch degradation

products that escape digestion in the small intestine. Raw cassava contains about 75.38% resistant starch (Onyango et al., 2006) which is due to comparatively longer chain length.

Chapter 4: Discussion

In chapter 3, results showed significant differences between treatments for growth performance of broiler chickens. This section further investigates the possible differences among treatments due to VFAs concentration or microbiota profile.

Study 1: Effect of different levels of MNC inclusion on the growth performance and gut health parameters of broiler chickens

4.1 Growth performance

MNC is a coproduct of macadamia nut oil industry. High in protein, energy and fiber content. MNC can be partial alternative to both corn and SBM. MNC being high in insoluble fiber, other studies have shown the effect of insoluble fiber in the diet of chicken and found that if included in moderate concentration, performance of bird is not affected instead there is decrease in the nutrient concentration of the diet (Hetland et al., 2002). A study by Ryssen et al., (2014), using macadamia nut cake (0%, 10% and 50% inclusion) and wood ash to compare with feed lime as a calcium source and its effect on the bone characteristics of chicken. Authors found that the growth was not compromised with 10% MNC inclusion in agreement with our study, whereas 50% MNC inclusion depressed the Ca and P concentration in the bone. Acheampong et al., (2016) conducted research using 600 ross broiler chickens fed with inclusion of MNC to replace soybean oil cake (SOC) at 0, 25, 50, and 100%. Authors concluded that MNC can substitute SOC only up to 25% as the abdominal fat increases with 50% inclusion and the growth parameters such as feed intake, final body weight and weight gain significantly reduces with 100% substitution of SOC. MNC inclusion is limited due to the high residual lipid present and the high fiber which reduces the palatability and the feed intake when included in higher percentage. In cattle, inclusion of 20% MNC in feed has increased the FCR (Acheampong et al.,

2008) which is similar to our study. As MNC is cheap replacement to corn and soybean it would still be beneficial to include up to 15% without any compromise in the growth performance. Although MNC is high in fiber content still there is no retardation in growth, the reason could be due to well-balanced gut microbiota, energy provided from their metabolites and high lipid content.

4.2 Microbiota profiling:

The result of microbiota profiling is in accordance with other studies, which have shown diet modify the microbial communities (Lan et al., 2005; Hird, 2014; Waite and Taylor, 2015). According to Apajalahti et al., (2001), birds fed similar diet has more closely aligned microbiota profiles. From the clone sequences, we found that all 3 treatments have the diverse and normal flora with is in accordance to finding of other researches. Wei et al. (2013) found that cecal microbiota is dominated by Firmicutes, Ruminococcus, Clostridium,Eubacterium, Fecalibacterium, Blautia, Butyrivibrio, Lactobacillus, Megamonas, Roseburia, Ethanoligenes, Hespellia, Veillonella, and Anaerostipes. In a research by Pan and Yu (2014), dominant microbiota are Firmicutes, Bacteroidetes, and Proteobacteria (>90%) Peptostreptococcus, Propionibacterium, Eubacterium, Bacteroides, and Clostridium, which is similar to our result when MNC is included in 10-20%. Apajalahti and Vienola (2016) found Lachnospiraceae (47%), Ruminococcaceae (19%), Bifidobacterium (10%), Lactobacillus (10%), Coriobacteriaceae (7%), Bacteroides (2%) and others (5%) in the ceca of commercial farm birds, whereas in our result the Bacteroides increases with decrease in Ruminococcaceae and lactobacillus and did not show presence of Lachnospiraceae. In contrary to our finding, Wang et al., (2016) found that Escherichia/Shigella, Lactobacillus, Bacteroides, and Subdoligranulum are the dominant bacteria in ceca of chicken kept in fresh litter. Albazaz and Bal (2014), found Clostridiaceaen (65%) and

Bacteroides up to 5% whereas, in our study the birds fed MNC either 10 or 20% has substantial Bacteroides count.

4.3 Volatile fatty acid

Common VFAs that are produced in mono-gastric animal ceca includes: acetate, propionate, butyrate, lactate, valerate, and isovalerate (Jamroz et al., 1998). VFAs production is low in the initial stage of life and then stabilize after 2 weeks. Also, the VFAs production is increased in birds fed plant protein based diet (Tsukahara and Ushida, 2000). In ceca non-starch polysaccharides (NSP), and undigested carbohydrates are fermented to produce short chain fatty acid also known as VFA and other gases.

We did not find any significant differences between the high fiber included diet and control diet fed birds. This could be due to the absence of digestive enzyme for fiber digestion and the relatively short digestive tract and transit time of digesta within the GIT of broiler chickens (Iji et al., 2001). In contrast to our result, Walugembe et al., (2015) found that butyrate was significantly lowered when dietary fiber ingredients were increased. Other VFA were not different among treatments fed MNC or corn-soybean fed control diet. This result is not surprising as MNC contains high amount of insoluble fiber which is not highly fermented in the chickens. All the indigestible components of dietary fiber cause insignificant fermentation and unchanged VFA production in ceca of chicks (Angkanaporn et al., 1994). Danayrolles et al., (2007) found relatively low acetic acid concentration and significantly increased butyrate when birds were fed diet with increasing insoluble fiber. This is somewhat similar to our result except there were no any significant difference in VFA production although there is numerical decrease in acetate and increase in butyrate. This insignificant could also be due to only up to 20% MNC inclusion which was not enough to produce significant VFA. The results would have been

different with diet fed higher soluble fiber which increase microbial proliferation and increase VFA concentration.

Many of the metabolic products of putrefaction are specifically found as end-products of protein, and not of carbohydrate, fermentation. Straight-chain volatile fatty acids (VFAs) are produced in both fermentation types, while branched chain fatty acids (BCFAs) are only produced in protein fermentation, specifically when branched-chain amino acids are fermented. The amino acids valine, leucine and isoleucine are converted to isobutyrate, 2-methyl-butyrate and isovalerate, respectively (Smith and Macfarlane, 1998). Thus, the presence of these BCFAs indicates ongoing protein fermentation activity. Unlike many other protein fermentation products, BCFAs are not known to be toxic (Apajalahti and Vienola., 2016).

Study 2: Effect of different levels of cassava root chips inclusion on the growth performance and gut health parameters of broiler chickens

4.4 Growth performance:

Body Weight Gain:

Body weight gain is significantly higher in control diet than in diet with 37.5 and 50% inclusion of cassava ($P=0.002$) in the starter phase whereas there is no any significant difference in the finisher stage of life ($P>0.05$). Reason for this could be presence of high fiber, anti-nutritional factor presents in the cassava root meal with peel and dusty nature of cassava mash feed which cause irritation and difficult to consume specially for chicks (Ngiki et al., 2014). Also, corn is completely replaced by the cassava in starter diet and shows retarding growth with increasing cassava level. This is in accordance with other researches done by Onjoro et al. (1998). In overall body weight is significantly different between T1 and T5 where T1 control diet has higher weight than T5 50 % cassava fed diet ($P<0.05$). We can conclude that around 50% replacement of corn as in T3 can be fed to chicken without any negative effect on weight gain (Kana et al., 2012). The retardation in overall weight with higher inclusion of cassava was expected as already mentioned by Ochetim (1991) and Tang et al. (2012).

Average Daily Feed Intake:

Although average daily feed intake is highest in T1 in starter stage and T4 in finisher as well as overall period, it is also believed that viscous nature of cassava, at high temperature, could cause reduced feed intake as cassava material may create a gut feeling effect, leading to reduced appetite, but it remained unchanged among treatments ($P>0.05$). The feed was balanced as per the requirement of the starter and finisher broiler along with special consideration for

including cassava in the ration such as supplementation of lysine and methionine along with addition of fatty acid to decrease the dustiness of the cassava, birds consumed far or more same without significant difference (Garcia and Dale, 1999).

Average Daily Gain:

Average daily gain is linearly decreasing with the treatments where highly significant ($P<0.01$) difference can be seen in the starter period in T1 than in T5. Although there is no any significant difference in the finisher stage, in overall period there is significant higher average daily gain ($P<0.05$) in T1 than T5. This differences in the gain between corn based diet and cassava based diet could be due to less efficient utilization of cassava compared to higher expected utilization and absorption of corn based diet. This drawback of less nutrient utilization of cassava can be improved by using enzyme supplementations (Midau et al., 2011; Bhuyian et al., 2012 and Kana et al., 2014)

Feed Conversion Ratio:

Feed conversion ratio is the ratio of feed consumed to the weight gain so lower FCR value is desirable which also indicate efficient utilization of feed. In the starter stage, T1 has the significantly lower FCR value compared to T5 ($P<0.05$). Whereas, in finisher phase T4 have the significantly higher FCR than T1 ($P<0.05$). In overall period, again T4 and T5 has significantly higher FCR value compared to T1 ($P<0.01$). As there is no significant difference in the feed consumption the difference in FCR could be because of weight gain. As mentioned corn is highly digestible and utilized by the body on the other side cassava inclusion in the higher percentage like 40-60% or replacing corn with cassava by 90-100% has retardation in growth

rate and affect the morphology of intestinal villus leading to decrease absorption (Onjoro et al., 1998).

4.5 Gut histology

Although gut histology did not change significantly there was some numerical differences among the treatments. The difference could be because of the anti-nutritive factor present in cassava and high fiber, whereas no any significant difference may be because of the similar amount of starch and other nutrient of cassava as corn. As mentioned above, due to decreasing villus height there is compromised in nutrient absorption. The longer villi in the control group is the result of activated cell mitosis (Samanya and Yamauchi, 2002), which occurs at the crypt area where stem cells divide to permit renewal of the villi. So, large crypt area indicates more intense cell production (Xia et al., 2004). We did not find any significant difference in the villus height: crypt depth ratio, where many researches have shown that it is correlated with the epithelial cell turnover and nutrient absorption. Promthong et al. (2007) found that neither corn or cassava has any effect on the thickness of the small intestine, which can be inferred from our finding.

4.6 Volatile fatty acid

The dietary carbohydrate is not just digested in the intestine but also is utilized by the cecal bacteria especially the resistant starch or non-starch polysaccharides and some amount of incompletely digested starch, to produce volatile fatty acid such as acetate, propionate, and butyrate, which benefits host in different ways (Bird et al., 2000). Our finding is similar to the finding of Promthong et al. (2007), who found that there was no any significant different in the VFA production between corn and cassava based diet. The reason behind this could be rapid

absorption of VFAs for utilization by the host body (Eliot and Shronts, 1992). Although there was no significant difference between treatments, the result shows numerical difference which indicates the presence of different bacteria ecology and the differential fermentation in the ceca. As these VFAs are utilized quickly the comparative quantification of VFAs in the gut and the systemic blood in further studies will help understand the actual physiology and differences caused by dietary ingredients will be elucidated.

4.7 Conclusions and recommendations for both studies

Our studies revealed that addition of alternative feedstuffs such as macadamia nut cake and cassava root chips can be successfully used to fully or partially replace conventional feedstuffs. Also, the price and availability of conventional feedstuffs are very fluctuating in market place. So, conventional feed based intensive chicken production is not feasible and sustainable in places like Hawaii. It's better to integrate both commercial feed in some proportion along with use of locally grown non-conventional feedstuffs such as macadamia nut cake and cassava or its products. Thus, chicken can get approximately required amount of protein, carbohydrates and lipids along with minerals and vitamins.

Due to selective growth of useful microbiota, energy from their metabolites (VFA), increased feed intake and high lipid residue in MNC 15% can be included in broilers diets without compromise in the growth performance and increased butyrate is plus to GIT health. Although inclusion of cassava in very high percentage or replacing corn completely produces some harmful effect on the growth performance, still cassava can be included in the corn-SBM based diet up to the inclusion level of 25% and below 37.5%. Inclusion of up to 25% or replacement of around 50% of corn in starter phase and up to 50% inclusion in finisher has no any disadvantages regarding body weight, feed intake, weight gain and FCR. Instead, it could be more beneficial from economic perspective and sustainable production of chicken.

Further research using macadamia nut cake and cassava root chips along with enzyme supplementation will help to optimize feed formulation, use of appropriate ingredients in proper ratio to fulfil requirements, and to improve feed intake, daily gain, feed conversion and overall performance of the birds. Positive results with inclusion of by-products from agro-based industries will encourage local poultry production and saves disposal of such products and

contribute to cleaner environment. Although we cannot replace corn and SBM completely using MNC or CRC, but partial replacement to the extent growth performance is not compromised that is inclusion around 15% MNC throughout life and CRC 25% in starter and 50% in finisher would provide attractive economic return.

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