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Effect of black soldier fly larvae fat inclusion in the diet on the performance and caecal bacterial diversity of broiler Japanese quails (*Coturnix japonica*)

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ABSTRACT

In this thesis, a total of 100 Japanese quails (Coturnix japonica), were randomly divided into 4 groups. A basal diet containing corn oil (CO) as added fat served as control group was tested against 3 experimental diets where CO was partially substituted by black soldier fly (BSF) larvae fat by 25%, 50% and 100% (BSFO 25, BSFO 50 and BSFO 100 respectively). The objective was to evaluate if this substitution could be beneficial to quails' growth performance, slaughter traits and caecal bacterial diversity. During the whole rearing period, there were no obvious significances in the growth performance parameters such as feed intake (FI), average weekly gain (AWG) and feed conversion ratio (FCR), with the exception of an improvement in the FCR in last week of rearing (35-42 days) for the diets in which BSFO was included but not significantly (C: 2.55; BSFO 25:2.47; BSFO 50: 2.48; BSFO 100: 2.47). For the selected internal organs weights and gastrointestinal segments lengths no significant differences were found between all dietary treatments. A total of 10 quails per group were randomly picked, and the caecal content was obtained. Bacterial structure was examined by high-throughput sequencing using the V4–V5 region of the 16S rRNA gene. The Alpha diversity measurements showed a lower diversity richness and evenness in the samples of quails fed with a total inclusion of *H. illucens* fat. Beta diversity measurements showed statistical dissimilarities between the studied groups mainly the BSFO 100 group compared to the control animals. Firmicutes were the dominant phylum in all the groups and the core bacteria at genus level was composed by *Subdoligranulum*, *Unclassified genus within family Lachnospiraceae*, *Blautia*, *CHKCI001* and *Streptococcus*. Linear discriminant analysis with effect size (LEfSe) showed significant differences between the control group and the others 3 experimental groups. The most significant differences were found by comparing the control group to the BSFO 100 group. A total of 16 significantly different taxa were found. Notably, the relative abundance of *Actinobacteria*, *Coriobacteriia*, *Coriobacteriales*, *Eggerthellaceae*, *Erysipelotrichia*, *Erysipelotrichales*, *Erysipelotrichaceae*, *Eubacterium* brachy group, *Anaerotruncus*, *Negativibacillus*, *Coprococcus* 3, *Anaerostipes*, *Lachnoclostridium* and *Bacillus* were significantly higher in the control group. On the other hand, in the BSFO 100 group, the genera *Fusicatenibacter* and *Subdoligranulum* were significantly enriched. The results suggest

that insect fat influences the caecal bacteria of quails and can substitute the conventionally used oil sources in poultry diets such as corn oil.

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INTRODUCTION

In the modern agricultural market, poultry farming ranks high both in terms of importance and expansion. Global meat consumption is expected to rise by 14% by 2031, compared to a base period average of 2018-2020, owing to rising wealth and population. Beef, swine, poultry and sheep meat nutritional availability is expected to increase 5.9%, 13.1%, 17.8%, and 15.7 percent by 2031, respectively. Meat consumption has been shifting towards poultry because in low-income developing countries, this reflects the cheaper cost of chicken compared to other meats, and in high-income countries, it demonstrates a growing preference for white meats, which are easier to prepare and considered being a healthier dietary choice. In 2031, poultry meat will account for 41% of all meat sources worldwide, compared to 34% for pig meat, 20% for beef and 5% for sheep meat (FAO, 2022).

Recently, there have been significant advances in the field of research on changing the composition of the poultry gut environment using feed additives such as pre- and probiotics, and insects as a source of protein and antimicrobial peptides (Benzertiha et al., 2019; Kierończyk et al., 2018). Insects can also be a good source of nutrients for animals in livestock production because of their high fat and amino acid content (Józefiak, Agata, et al., 2019; Józefiak et al., 2016). From a practical point of view, before September 2021, only the fat fraction from insect biomass can be used in livestock nutrition due to European Union restrictions (regulations no. 999/2001 and 1069/2009), except for live insects, which can also be provided in animal diets, but this ban was lifted by the Commission Regulation (EU) 2021/1372. Black soldier fly larvae (BSFL), as the most profitable species due to flexibility in terms of adaptation to the rearing substrate (pre-consumer wastes), favorable nutritive value and short-term production, has the highest economic potential for usage in animal diets (Józefiak et al., 2016). A small recent number of experiments have been performed to evaluate the effects of BSFL fat on the growth performance, nutrient digestibility coefficients, and select physiological traits of broilers (Kierończyk et al., 2020, 2022; Kim et al., 2020, 2021; Schiavone et al., 2017, 2018), laying hens (Heuel et al., 2021), and turkeys (Sypniewski

et al., 2020). These studies indicated that there is no limiting factor that could negatively trigger the results of bird production parameters. Furthermore, there is evidence that black soldier fly larvae (BSFL) fat may positively modulate physiological and immunological status through a high concentration of medium-chain fatty acids (MCFAs), including lauric acid (C12:0) as a dominant component (Sypniewski et al., 2020).

Also, to date, the available literature on the effect of inclusion of BSFL oil in poultry nutrition is very little, concerning specifically the caecal bacterial microbiome and there is only (Kierończyk, 2022) that have a published study in this concern but was done using of a different analysis technique than the one used in the current study (Next-generation-sequencing). Therefore, there is still a lack of enough information in the available literature about the influence of this novel energy source on caecal bacterial microbiota populations, which theoretically should have an important impact on bird health status.

The aim of the present study was to investigate the effect of partial (25%, 50%) or total (100%) replacement of commonly used corn oil (CO) by Black soldier fly larvae (BSFL) fat, on growth performance, slaughter performance focusing on carcass, internal organs weights and lengths and caecal bacterial microbiome in finisher stage of broiler quails.

OBJECTIVES

The objective of the present thesis included the description and comparison of the growth and slaughter performances in addition to the diversity of the bacterial community in the caecum of finisher broiler quails by the impact of the inclusion of black soldier fly larvae oil in substitution of commercial vegetable oil such as the corn oil. Studying the caecum in the current thesis is due to that this organ represents the highest bacterial population diversity according to the previous research results, which could play a crucial role in the immunological, growth and digestive performances. Therefore, the general target of this work is to contribute to improving the limited knowledge about the microbiology of the quail caecal bacterial microbiome and to better understand the role of this GIT segment.

1. THE DIGESTIVE SYSTEM AND MICROBIOTAL COMMUNITY OF POULTRY

It is believed that broilers genetic selection for improved production efficiency is nearing its limit and focus is lately being shifted to the digestive system microbiome in supporting immunity and production (Tallentire et al., 2018). (Yadav & Jha, 2019) stated that the effectiveness of several dietary modifications tested, is determined mainly by detecting beneficial impacts on productivity. Factors linked to immunity, and management and housing conditions, such as stocking density, light regime, source of poultry, feed, water quality, ventilation, and illnesses, can affect bird performance, increasing the difficulty to identify the impact of each component on the animals' productivity (Van Limbergen et al., 2020).

Understanding the physiology of the digestive system, how bacteria interact with the host, and what information is currently available on generally recognized bacterial species are essential before exploring the potential of bacterial connections within the gut.

Quails since many years have been employed in genetics research as an animal model substitute for chicken (Shimakura, 1940), still being a significant model in biomedical investigations, developmental biology and behavior (Minvielle, 2009). A common domesticated species of poultry bred for eggs and meat in Europe and Asia is the Japanese quail (*Coturnix japonica*), a migratory bird native to East Asia. Quail has been utilized as a model for studies on chicken due to the well-researched and confirmed genetic similarities between the two species (Mills et al., 1997). Despite having a similar evolutionary history to *Gallus gallus domesticus* and both being members of the Phasianidae family of the order Galliformes, as study model, *Coturnix japonica* offers more significant benefits. They are easy to raise and small of size, grow quickly, their life cycle is short and reach sexual maturity between 7-8 weeks from hatching (Huss et al., 2008).

1.1 The avian digestive system anatomy and physiology

The avian GIT system is notably different from mammalian GIT systems, and it contains a unique microbial environment that is tailored to its host. A meal of standard feed takes around 4 hours to pass through the gastrointestinal tract in young stock, 8 hours in laying chickens and 12 hours in broody chickens, depending on identical circumstances. Hard, intact grains take longer to digest than crushed grains, and some whole grains may pass through undetected (Poultry hub, 2022). In comparison to mammals, birds require a considerable quantity of energy to be taken quickly from food sources in order to maintain homeostasis. To avoid being washed away, the avian commensal microbiota must be able to bind to mucosal layers in the GIT, absorb energy quickly and have a high metabolite potential. The GIT of chickens is thought to include over 900 different bacterial species, each with its own unique community. (Stanley et al., 2014).

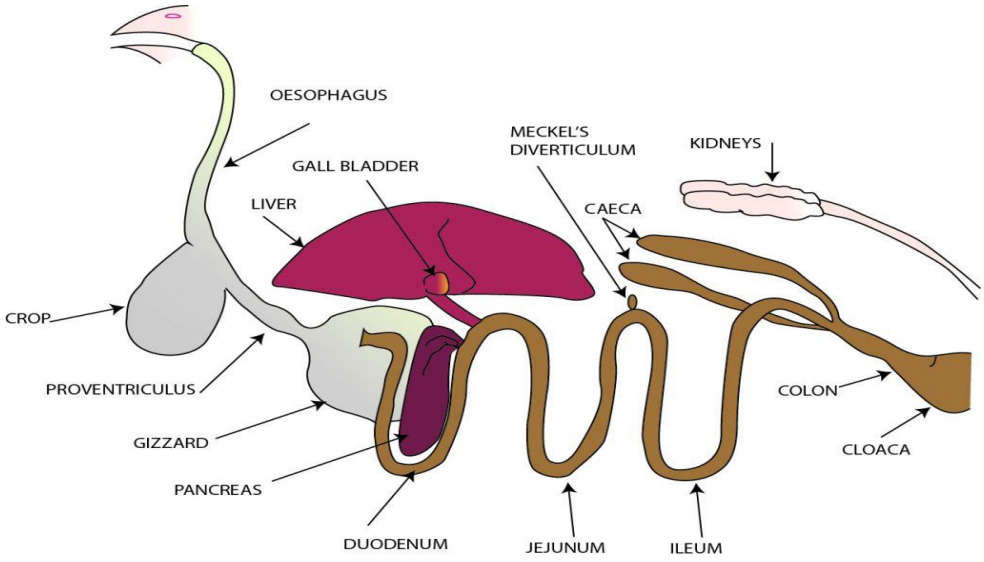

Figure 1: Anatomy of the avian digestive system (Poultry hub, 2022).

1.1.1 Mouth, Esophagus and crop

The digestive process in birds begin by prehension with the beak. Mucus is then produced in the mouth to lubrify feed, help its passage to the esophagus and also has a protective function against pathogenic bacteria and viruses (Scanes & Pierzchala-Koziec, 2014). Through the passage of feed in the esophagus, it is then deposited in the crop or straightly to the stomach by primary peristalsis in the esophagus. (Rodrigues & Choct, 2018). The crop is a swollen pouch that helps with feed fermentation and storage (Scanes & Pierzchala-Koziec, 2014). Even though the digestive enzymes released in the mouth started the digesting process, the crop just serves as a temporary storage pouch. The crop adapted for birds who are generally targeted by other predators yet should look for food in the wild. These birds may swiftly swallow big amounts of food and then go to a more secure spot to digest it. (USDA, 2022).

1.1.2 Proventriculus

When the crop wall contracts, feed is evacuated to the gizzard after having passed the proventriculus (Denbow, 2015). The proventriculus is where in digestion of proteins begins, which is helped by the production of pepsinogen and HCl (Scanes & Pierzchala-Koziec, 2014). This digestive segment has a tiny capacity, so the gizzard receive quickly the feed with the possibility of returning multiple times to the proventriculus for enzymatic digestion(Rodrigues & Choct, 2018).

1.1.3 Gizzard

The gizzard, also known as the mechanical stomach, is made up of two sets of powerful muscles that function as the bird's teeth and is protected by a thick lining (USDA, 2022). The digestive secretions from the salivary glands and proventriculus flow into the gizzard, where they are ground, mixed, and mashed into particles smaller than 0.1 mm before being transferred into the small intestine (Jacob & Pescatore, 2011).

1.1.4 Small intestines

The duodenum, jejunum and ileum make up the small intestine, which is responsible for nutrient absorption and proceeding with the digestion process before feed transfer to the caecum, which bear the responsibility for starch, cellulose, and indigestible carbohydrates fermentation and digestion (Stanley et al., 2015). The small intestine is made up of the duodenum (also known as the duodenal loop) and the lower small intestine. Most of the digestion takes place in the duodenum, and the nutrients produced are mostly absorbed in the lower small intestine. The pancreas provides digestive enzymes and bicarbonate to the duodenum (to balance hydrochloric acid from the proventriculus), whereas the liver provides bile. The pancreas' digestive fluids are largely engaged in protein digestion. Bile is a detergent that aids in lipid breakdown and fat-soluble vitamin absorption (A, D, E and K) (USDA, 2022).

1.1.5 Caecum

The caecum is composed of two sacs with blind ends in which bacteria can metabolize substances that were not digested in the small intestine (Svihus et al., 2013). More

fermentation of the digesta is allowed in caecum due to the decreased transit rate of the latter in comparison to other gastrointestinal segments, and in result, the short-chained fatty acids produced by the bacterial flora are in greater concentrations and absorption of water is higher (Rodrigues & Choct, 2018; Rehman et al., 2007; Clench & Mathias, 1995). Every 24 to 48 hours, the caecum is emptied (Clench & Mathias, 1995).

1.1.6 Colon and Cloaca

Digesta enters the colon through the ileo-caecal junction, and then either passes to cloaca or the caecum through peristaltic contraction or retrograde antiperistalsis, respectively (Duke, 1989). Absorption or digestive activities are minimal to absent in the colon. The digesta is transported from the colon into the cloaca, where it is combined with uric acid before being ejected.

1.2 Avian gut Microbiome

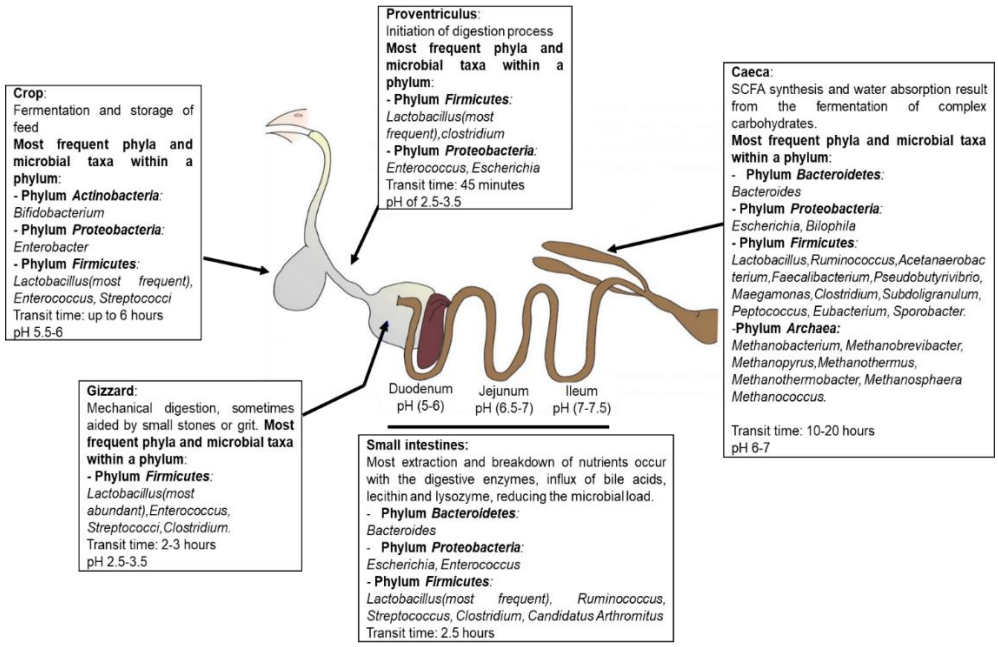

Figure 2: Microbiotal diversity in poultry GIT (Bindari & Gerber, 2022).

1.2.1 Bacterial microbiome

Firmicutes, *Tenericutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* are the five most common bacterial phyla discovered in the chicken GIT (Wei et al., 2013). Most of the bacteria in the intestines, including the ileum, gizzard and duodenum, belong to the *Lactobacillus* genus. The crop is frequently inhabitated by *Bifidobacterium* in addition to *Enterobacter* (Saxena et al., 2016; Yeoman et al., 2012). Because the stomach's acidity makes it an undesirable habitat for most germs, it contains less diversity than other parts of the gastrointestinal tract (Rehman et al., 2007).

Lactobacilli are the most common bacteria in the gizzard, with lactose-negative enterobacteria, coliforms and *Enterococci* but in lesser concentrations (Sekelja et al., 2012; Yadav & Jha, 2019;

Zoetendal et al., 2004). According to Engberg et al., (2004) The levels of *Clostridium perfringens*, as a potentially hazardous specie, inhabit the gizzard but at a low level (10^3 bac/g of gizzard content).

Poultry small intestines' most inhabitated phylum is Firmicutes (Yeoman et al., 2012), with *Lactobacillus* genus mostly prevalent, adding *Clostridium*, *Ruminococcus*, *Streptococcus* and *Candidatus Arthromitus* representing it to a lesser extent (Jin et al., 1997; Lu, Idris, et al., 2003; Munyaka et al., 2016; Wang et al., 2016; Yeoman et al., 2012). *Proteobacteria*, especially *Escherichia* and *Enterococcus*, have been discovered in the ileum.

In the chicken digestive system, the caecum has the highest bacterial population and variety (Dibner & Richards, 2005; Oakley et al., 2014; Shakouri et al., 2009). *Firmicutes* are the most common phylum, then comes next *Bacteroidetes*. *Proteobacteria* less common and the same for *Archaea*. It was found that the most common taxa in the caecum were *Faecalibacterium, Ruminococcus*, *Lactobacillus*, and *Clostridium* (Gong et al., 2002; Jin et al., 1997; Qu et al., 2008; Wang et al., 2016).

The predominant genera in cloaca and excreta are *Faecalibacterium*, *Lactobacillus*, *Bacillus*, *Clostridium*, *Ruminococcus* and *Eubacterium*; however, the cloacal mucosal and fecal microbiota is not stable and it changes quickly because different parts of the GIT empty at different times (Deusch et al., 2015; Sekelja et al., 2012).

1.2.2 Viral microbiome

Viruses causing chicken illnesses could damage the gastrointestinal tract of chicks from the first days of life, allowing dangerous bacteria and protozoa to proliferate in an environment that is favourable to their proliferation (Jindal et al., 2014; Mekuriaw Arega, 2018). Recent research, on the other hand, has switched its attention to community-based assessments of the gut microbiome. In the chicken intestine, gene analysis and sequencing of viruses showed the sequences identifiying *Geminiviridae* and *Caudovirales* viruses, as well as some unidentified viral genes (Kim et al., 2011). Concerning the overall GIT health and enteric illness syndromes in birds, there is still a lot of unknown functions to be

recognized and described about the functions of those viruses. As a result, determining the viral contents of the chicken gut, as well as identifying and characterizing these viruses, is critical (Day et al., 2015). Recently, new avian rotaviruses, astroviruses, parvoviruses, picornaviruses and caliciviruses have been discovered in chickens utilizing electron microscopy, next generation sequencing (NGS), and quick diagnostic procedures. These are tiny viruses that cause illnesses in poultry (Lima et al., 2017). The viral families *Retroviridae*, *Herpesviridae*, *Myoviridae* and *Siphoviridae* were found in broilers through the use of next-generation-sequencing technique (Devaney et al., 2016). Given the following, few informations are known about the virome profile of chicken. Some of the difficulties in identifying components of the poultry virome are likely due to the lack of shared genes among viruses and sequencing technologies employed in virome research.

1.2.3 Archaea

Dosoretz et al., (1987) discovered anaerobic methanogenesis in both fowl caeca and poultry manure, which results in the generation of CH_4. The major function of microbial fermentation in poultry caeca is the generation of volatile fatty acids. Methanogenesis began in the second month of life and escalated with age, according to (Marounek et al., 1998) research of the caecal fermentation pattern in chickens aged 1 to 4 months. (Saengkerdsub et al., (2007), on the other hand, found CH_4 in the feces of 5-day-old broilers. In the caeca, archaea of all kinds make up roughly 1% of the overall microbial population (Qu et al., 2008). In feces samples from chickens, analysis of cell wall composition using culture techniques confirmed the presence of *Methanogenium spp.* as a prominent component of the archaeal community (Miller et al., 1986). (Qu et al., 2008) discovered that Methanomicrobia the class that includes *Methanogenium spp*. accounted for 45-47 percent of the archaeal environmental gene tags (ETG) observed in the microbiome of chickens. However, no Methanogenium-related spp. sequences were found in (Saengkerdsub, Anderson, et al., 2007) investigation, which focused on using molecular techniques to find and measure methanogenic archaea in the caeca of adult chickens. *Mbb. woesei* was found to be the most prevalent species in chicken caeca. *Mbb. woesei* had an average population of

3.61 x 10^6 cells per gram wet weight, and it was found even in very young birds, indicating that it colonizes the digestive tract early in life (Saengkerdsub, Anderson, et al., 2007).

1.2.4 Fungi

Some researchers have looked at the involvement of *Aspergillus* and *Fusarium* in poultry illness (Javed et al., 2005; Kumar et al., 2003; Ortatatli et al., 2005; Steinlage et al., 2003). Among broilers, the caecum has the lowest fungal burden, followed by the proventriculus and the jejunum, at 1.6 x 10^3 to 3.9 x 10^3 cfu/g digesta (Robinson et al., 2020). Culture-dependent research has led to the discovery of *Candida*, *Saccharomyces*, *Geotrichum*, *Trichosporon* and *Rhodotorula* (Cafarchia et al., 2019; Shokri & Khosravi, 2011; Sokół et al., 2018; Subramanya et al., 2017). In contrast to these findings, (Byrd et al., 2017) detected 88 fungal species in broiler chicken ceca, with *Aspergillus* being the most common, followed by *Penicillium*, *Verticillium* and *Sporidiobolus*. Only two research have investigated the poultry mycobiome using culture-independent approaches to date. Only two *Cladosporium* species were found in the caecum of broilers fed an unmedicated diet using 454 Roche sequencing, whereas further 17 fungal species were found in animals treated with essential oils (Hume et al., 2012). *Sarocladium kiliense* was shown to be the most prevalent species in the turkey ileum, followed by *Candida*, *Dothidea* and *Cladosporium* species (Ward et al., 2019). Sequential sampling of birds from the same cage demonstrated an increase in ileal alpha diversity with time, even though the same four species were dominating (Ward et al., 2019).

1.3 GIT microbiome functions

1.3.1 Bacterial successional development

Microorganisms colonize the intestines immediately after hatching (Oakley et al., 2014). The GIT of poultry chickens is inundated by foreign bacteria shortly after hatching. The invaders and microbiota establish a foundation for the development of a stable and diversified population throughout time (Oakley et al., 2014; Pan et al., 2013). The stomach of chickens is first colonized by facultative aerobes, which are eventually replaced by anaerobes (Gong et al., 2002). Aerobic bacteria's rapid growth and oxygen used produce lowering intestinal environment conditions, and consequently encouraging the colonization and development of obligate anaerobic bacteria (Wise & Siragusa, 2007). According to Crhanova et al., (2011) the GIT microbiome composition varies as a result of the developmental growth and age of chickens, and until reaching a stable condition, the microbiotal profile undergoes several alterations. After few hours of hatching, streptococci and enterobacteria invade the caeca and spread throughout the digestive system in less than 24 hours. Bacteria including lactobacilli, streptococci and coliforms may be seen in the GIT on day three (Bagust & Ireland, 1984). During the first six weeks of life, the varied bacterial community present in the chicken caecum grows. Chickens' bacterial populations shift from *Proteobacteria*, *Bacteroidetes* and *Firmicutes* to exclusively *Firmicutes* at the age of three weeks (Oakley et al., 2016). Awad et al., (2016) discovered a significant bacterial population in caecal and jejunal mucosa from the first day of the birds' life related mainly to the increase in microorganism consumption from their surroundings after hatching. Microbiota detected in avian guts of varies in composition between young and aged birds. Proteobacteria were identified in higher concentrations during the first few days of life, then reduced. *Firmicutes* including *Ruminococcaceae*, *Lachnospiraceae*, *Lactobacillaceae* and *Clostridiaceae* were the most common in older birds (Gong et al., 2002). *Campylobacter coli*, *Clostridium perfringens* and *Lactobacillus delbrueckii* were identified in the gastrointestinal tract of 3 days old chicks, but *Streptococcus*, *Enterococcus* and *L. acidophilus* were observed in 7 until 21 days chicks (Lu et al., 2003). In their

second week of age, chicks' caecal microbiome contained Gram positive anaerobes (*Clostridium*, *Lactobacillus*, *Eubacterium*, anaerobic cocci) and Gram-negative anaerobes (*Bacteroides* and *Fusobacterium*) (Salanitro et al., 1978). Poultry older than 40 days have a plethora of bacteria in their digestive tracts, including gram-positive cocci, *E. coli* species, *Bifidobacterium* species, *Lactobacillus* species, *Clostridium* species, *Bacteroides* species, and *Streptococcus* species (Bagust & Ireland, 1984).

1.3.2 Productive performance

As Stanley et al., (2016) study showed, the gastrointestinal microbiome was related with the low and high poultry performance, adding that sometimes non-reproducible or inconsistent findings were found in different experimental treatments. Even though individual taxonomic microbiota could not be decisively connected to a production performance, as evaluated by FCR, high performance poultry's microbiome was often enriched with bacterial communities having the ability to degrade starch and cellulose such as the families *Erysipelotrichaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroides fragilis*, and *Clostridium cellulosi* (Stanley et al., 2013, 2016). *Clostridium lactatifermentans* and *Ruminococcus torques* were found in the ceca, whereas the ileum contained *L. crispatus*, *L. aviarius*, and *Lactobacillus salivarius* (Torok et al., 2013). Despite that different strains of *Lactobacillus* have capacity to act as substantial source of energy by creating short-chain fatty acids, not all of them are advantageous, as indicated by the studies above. *Lactobacillus salivarius* has been linked to bile deconjugating activity, which has been linked to a reduction in chicken growth rate and *Streptococcus faecium* and *Clostridium perfringens* are two more bacteria that deconjugate bile (Knarreborg et al., 2002). While some taxa in the caecal and ileal microbiome were shown to be favorably connected with bird weight, other taxa were found to be adversely correlated with the latter (Johnson et al., 2018). Feces from low-performing poultry, as evaluated by feed conversion ratio, were enhanced by different bacterial genera than excreta of high-performing birds, according to Singh et al., (2012). However, *Bacteroides*, *Lactobacillus*, and *Enterococcus* populations in low- and high-FCR birds were shown to

be similar in terms of abundance. As previously stated, the bulk of research has focused on the relationship between the caecum and ileum bacteria and production.

1.3.3 Interaction between microbiome and health

Cells and their discharged soluble byproducts, presenting a complicated compound network, are normally involved in maintaining intestinal homeostasis in a healthy fowl gut (Kamada et al., 2013). The intestinal microbiota has a role in regulating the immune system of the host, as well as influencing proper structural and functional organ development and metabolism (Sommer et al., 2013). The immune responses of the mucosal mucosa to the resident intestinal microbiota can discriminate between commensal and pathogenic microorganisms (Kogut, 2019). The varied microbiota prevalent in the avian GIT is known to alter the T-cell receptors repertoire located in the spleen and intestins (Mwangi et al., 2010). B-cell response and immunoglobulin A (IgA) production are also influenced by the gut microbiome. By precisely binding to bacterial epitopes, IgA plays a crucial function in controlling the makeup of the gut microbiota (Suzuki & Nakajima, 2014). Microbiome present in the intestinal tract of poultry favors the synthesis of the gut mucosal layers, supplying energy in the form of SCFA and amino acids, polysaccharide degradation and the exclusion of pathogenic microorganisms (Huss et al., 2008; Shakouri et al., 2009; Dunkley et al., 2007). Any disturbance in gut health can have an impact on one or more systems (Choct, 2009). The harmful impacts that could happen include decreased fat digestibility, enzymatic degradation of the intestinal mucus, pathobionts becoming pathogens due to disruptions in microbial metabolism and the dysregulation of adaptative immune cells (Teirlynck et al., 2011). When greater availability of nutrients that are undigested in the colon, microbial populations proliferate more rapidly (Chan et al., 2013). As a result, the gut microbiota's equilibrium with the host is upset, leading to metabolic, pathogenic, or sterile inflammation (Kogut et al., 2018). In the chicken housing environment, a healthy stomach improves digestibility, minimizes nutrient excretion and reduces ammonia (NH3) and other gas emissions. These gases may be hazardous to the environment and human health (Costa et al., 2008).

1.4 Factors affecting poultry gut microbiome

There are several variables that determine intestinal microbial composition, including age, gender, and breed. This section addresses some of those aspects (Kers et al., 2018). Bird size, production phase, air currents (humidity) and temperature may all affect the health and productivity of poultry birds in the poultry farm environment (Kocaman et al., 2006). Intestinal motility and feed intake can be affected by external and internal stimuli, including variations in humidity or dryness, temperature fluctuations, and wind (Oviedo-Rondón, 2019). Condensation and litter moisture can build up in a chicken coop that is not properly aired (Dunlop et al., 2016). Stress can impair the immune systems of chickens, causing them to lose the ability to maintain a healthy microbiome in their gastrointestinal tract (GIT) (Weiss & Hennet, 2017).

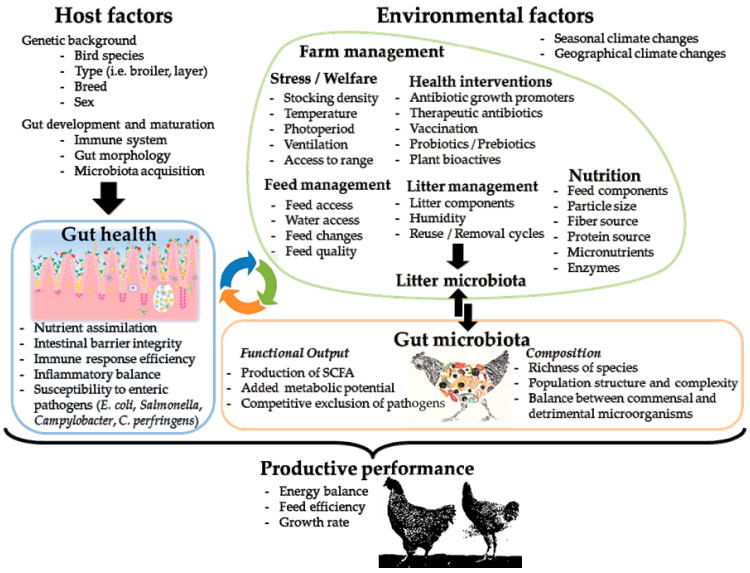

Figure 3: Important environmental and host-related variables that influence chicken gut microbiota and its relationship to gut health and productivity. (Carrasco et al., 2019).

1.4.1 Age, sex and breed

An important factor influencing GI bacteria, cell density and metabolic function is the age of the birds. More than a dozen research groups (Kers et al., 2018; Rehman et al., 2007; Stanley et al., 2014; Yeoman et al., 2012) have investigated gut microbiota taxonomic makeup using a variety of sequencing techniques. One-day-old broiler chicks have already established a microbial community in their gastrointestinal tract, according to Ballou et al., (2016) and S. Kumar et al., (2018). As the bird ages, the GIT microbiome undergoes a sequence of changes, as more stable bacterial species are replaced and established (Crhanova et al., 2011; Lu, Sanchez, et al., 2003). (Lu, Sanchez, et al., 2003) found *L. delbrueckii*, *Campylobacter coli* and *C. perfringens* in the intestinal tract of chicks during this first week of age, while *Streptococcus*, *Enterococcus* and *L. acidophilus* were more prevalent in chickens between first and third week of age. The GI tract contains *L. crispatus* at 28 and 49 days of life, but the composition is markedly different from subsequent ages (Lu, Sanchez, et al., 2003).

Despite the fact that the impact of sex becomes increasingly significant as soon as broilers achieve sexual maturity, changes were reported in younger chicks and shows a relation with sex-based variations in lipid and glycan metabolism from the caecal microbiome (Cui et al., 2020; Lee et al., 2017; Lumpkins et al., 2008). According to (Lee et al., 2017) females had more *Oscilospira* and *Tenericutes* in the caecum of Ross broilers (308) at 35 days of age, whereas males had more Bacteroides. At 35 days old, the caecum of males present higher prevalence with *Megamonas*, *Bacteroides*, *Phascolarctobacterium* and *Megasphaera*, and in females, *Akkermansia* presented higher prevalence, according to a research by Cui et al., (2020). Torok et al., (2013) discovered that sex affects the overall amount of eubacteria in the caecum, since males have had a greater *Eubacteria* prevalence than females at 22d and 42d. Lumpkins et al., (2008) demonstrated that By day 3 of age, ileal bacterial population of female and male chicks varied.

Even though this effect seems to be limited to certain taxa, the microbiome is impacted by chicken genotype (B. Kim et al., 2020; Pandit et al., 2018; Tůmová et al., 2021; Cosmin et al., 2020; Wen et al., 2019). Kers et al., (2018) looked at the results of 12 studies that sequenced the 16S rRNA in feces from the different poultry breeds. *Actinobacteria* was detected in all four investigations of Cobb

chickens and in three of the eight that have reported data on Ross broilers. *Bacteroidetes,* similarly, detected in the four trials using Cobb chickens and in six of eight studies including Ross breed. There have also been reports of large variations in the caecum microbiotal makeup between young aged Ross and Hubbard hens (Richards et al., 2019). On day zero, *Enterobacteriaceae* dominated Hubbard's caecal microbiota, while *Enterococcaceae* and *Clostridiaceae* dominated Cobb's. On day 3, Hubbard chickens had a greater quantity of *Bifidobacteriaceae* and a lower level of *Enterobacteriaceae* than Ross chickens, but by day 7, no variations in microbial composition were identified across breeds (Richards et al., 2019).

It is obvious from the foregoing data that breed, sex and age may alter the poultry's microbiota, therefore microbiome studies must include extensive information regarding these characteristics.

1.4.2 Housing and Litter

Poultry litter is a mixture of excreta, exfoliated skin and feed, feathers, bedding material and aerosolized pathogens (Just et al., 2009). *Salmonella*, *E. coli*, *Campylobacter*, and *Clostridium perfringens* are often found in litter investigations (Bennett et al., 2005). Depending on litter management procedures, poultry litter comprises a complex and dynamic microbiome of gastrointestinal and ambient bacteria (Lovanh et al., 2007; Lu et al., 2003). Chicks start consuming litter on day one, inoculating their GI tract with litter germs. Litter can affect the formation, content, and structure of poultry GI microbiota (Aktan et al., 2004; Garrido et al., 2004; Torok et al., 2009; Yin et al., 2010). Improper litter management can increase litter moisture, ammonia, pH and microorganism density and variety (primarily bacteria) (Cressman et al., 2010; Omeira et al., 2006). Reused litter hosted fewer *Salmonella* and *Clostridium perfringens* than new litter, however *Campylobacter jejuni* and *C. coli* survived longer (Kassem et al., 2010). Two studies indicated that reused litter could influence chickens' immune systems (Lee et al., 2011; Shanmugasundaram et al., 2012), showing that litter conditions may indirectly affect chickens' GI microbiota. Wang et al., (2016) found that early chicks were more affected by litter management than adult birds. Wet litter had a higher Shannon

diversity and richness of microbiota than dry litter, suggesting that litter management may influence chicken gut microbiota (Dumas et al., 2011; Oakley et al., 2013). This litter is sometimes linked to reduced chicken intestinal immunity (Collett, 2012). Although the relationship between early stomach microbial colonization and chicken productivity is unclear, the data obtained show that litter quality, type, and management affect gut microbiota composition.

1.4.3 Diet (Prebiotics, probiotics and antibiotics)

Dietary changes can produce a considerable shift in microbial composition within 24 h (Daniel et al., 2014). The abundance and variety of the ensuing microbial composition and the synthesis of subsequent metabolites can be helpful or harmful to human physiology (Montalban-Arques et al., 2015). Many studies have indicated that food impacts GI bacterial diversity (Apajalahti et al., 2001; Engberg et al., 2002, 2004). Many recent studies showed that diet composition can be advantageous or enhance pathogenicity, such as necrotic enteritis (NE) predisposition (Loh & Blaut, 2012; M'Sadeq et al., 2015; Munyaka et al., 2016; Timbermont et al., 2011; Torok et al., 2008). Deprival of feed affect intestinal immunity and microbial taxonomy, increasing Salmonellosis occurence (Burkholder et al., 2008; Lamot et al., 2014). Other than the conventional diets used in poultry nutrition (animal or vegetal sources), insects were found to be a nutrient factor and biologically active component that may have positive impact on GIT immunity by affecting microbiotal community (Benzertiha et al., 2020; Józefiak et al., 2019; Józefiak et al., 2017).

Antibiotics are not species-specific, and data shows dosage impacts commensal microbiome development and composition (Gao et al., 2017). Chlortetracycline, a tetracycline antibiotic, reduces *Proteobacteria*, whereas amoxicillin, a beta-lactam antibiotic, reduces *Lactobacillaceae* (Schokker et al., 2017). Distinct antibiotic classes may impact different taxa, possibly due to different mechanisms of action.

Probiotics, which are live bacteria supplements that act by colonizing or changing the microbiotal composition of the gastrointestinal tract, may improve performance and immunity while lowering

antibiotic use. *Lactobacillus spp*., *Bacillus subtilis* and *Streptococcus faecalis* are some of the most common probiotic species. Microbiome maturation is accelerated by supplementation with these strains; pathogen colonization is impeded; gut cellular architecture is improved; and the bird's immunological state is boosted (Gao et al., 2017; Haghighi et al., 2005).

Selectively fermentable compounds that allow certain changes in the composition and/or activity of the gastrointestinal microflora are the definition of prebiotics, which include mannan, xylo- and fructo-oligosaccharides (Gibson et al., 2004). This increases the performance of broilers as well as their immune responses (Biswas et al., 2019; Ribeiro et al., 2018). Since butyrate enhances broiler GIT immunity, xylo-oligosaccharide supplementation increased the amount of genes leading to butyrate production; this might be the mechanism of action of prebiotics. (De Maesschalck et al., 2015).

1.5 Caecal bacterial microbiome composition

In the current thesis we are focusing on the caecal bacterial microbiome and this segment of the gastrointestinal tract of avian species is inhabitated by different phyla belonging to the Bacteroides, Firmicutes and Proteobacteria. And the genera, Lactobacillus, Faecalibacterium, Ruminococcus, Clostridium and Oscillospira present higher than 50% of the avain caecal bacterial diversity (Cardenas et al., 2021).

Table 1. Studies showing prevalent caecal bacterial community of poultry.

Dominant microbiota	**Reference**
Bifidobacteria (1%), *Bacteroides* (2%), *Atopobium* (3.6%), *Gamma Proteobacteria* groups (20%), *Sporomusa* sp. (21%), *Clostridium coccoides* (27%) and *Clostridium leptum* (20%)	(Zhu et al., 2002)
E. cecorum, Clostridia, Ruminococci and *Fusobacterium prausnitzii*	(Gong et al., 2002)
Proteobacteria, Bacteroidetes and Firmicutes (> 90%) *Eubacterium, Propionibacterium, Peptostreptococcus, Clostridium* and *Bacteroides*	(Wei et al., 2013)

Bacteroides (5%), *Lactobacillus* (8%), *Fusobacterium* (14%) and *Clostridiaceae* (65%).	(Albazaz et al., 2014)
Proteobacteria and Tenericutes (up to 5%), Bacteroidetes (more than 18%), *Streptococcaceae, uncultured Clostridiales, Bacteroidaceae and Ruminococcaceae*	(Witzig et al., 2015)
In reused litter: *Anaerotruncus, Faecalibacterium* and *Blautia.* In fresh litter: *Subdoligranulum, Bacteroides*, *Lactobacillus* and *Escherichia-Shigella.*	(Wang et al., 2016)
Bacteroides (2%), *Coriobacteriaceae* (7%), *Lactobacillus* (10%), *Bifidobacterium* (10%), *Ruminococcaceae* (19%) and *Lachnospiraceae* (47%)	(Apajalahti et al., 2016)
Firmicutes (60%), Bacteroides (10-20%), Actinobacteria (2-6%), *Lactobacillus, Faecalibacterium, Bacteroidaceae and unclassifed Rikenellaceae.*	(Chen et al., 2020)
55-70 % Firmicutes, 25-30 % Bacteroidetes, *Lactobacillus, Faecalibacterium, Unclassified Ruminococcaceae, Unclassified Rikenellaceae, Unclassified Lachnospiraceae*	(Zhu, Wang, et al., 2019)
Firmicutes, bacteroidetes, *Alistipes, Faecalibacterium, Unclassified Ruminococcaceae, Unclassified Lachnospiraceae, Phascolarctobacterium*	(Wu et al., 2021)
Citrobacter, Clostridium, *Lactobacillus, Unclassified_Lachnospiraceae, Ruminococcus, Enterococcus, Unclassified_Peptostreptococcaceae and Turicibacter*	(Dabbou et al., 2021)
Firmicutes, Bacteroidetes, *Lactobacillus, Clostridium, Oscillospira, Ruminococcus, Bacteroides, Unclassified Ruminococcaceae, Unclassified Rikenellaceae, Unclassified Lachnospiraceae*	(Neijat et al., 2020)

1.5.1. Bacteroidetes

1.5.1.1. Rikenellaceae

According to Ranjitkar et al., (2016), this family is responsible for the whole phylum *Bacteroidetes*, with increasing prevalence until 29 days of age and then decreasing until 36 days. *Rikenellaceae* is considered to vary a lot between birds, and concerning poulty's productivity, their absence or presence may indicate almost nothing about the latter (Polansky et al., 2016).

1.5.1.2. Bacteroidaceae

According to Garcia et al., (2012), from this family, *Bacteroides*, Gram-positive non-sporing bacilli, accounts for around 25% of bacteria in the phylum Bacteroidetes. Their primary sources of nutrition are host-derived and glycans from plants, and their quantity is determined by the host's diet; nevertheless, there is evidence of antibiotic resistance such as ampicillin and tetracycline (Garcia et al., 2012). *Bacteroides* is the most numerous genera in the caecum, according to Xiao et al., (2017), accounting for a high percentage of the population.

1.5.2. Firmicutes

1.5.2.1. Lachnospiraceae

The prevalence of this family is connected to broilers with poor FCR values and has been discovered often in caecal digesta (Stanley et al., 2016). *Clostridium lactatifermentans*, a member of the *Clostridium* genus in the *Lachnospiraceae* family, is thought to have various favorable impacts on the

host, including lactate fermentation to short-chained fatty acids as an energy source and *Salmonella* suppression (Hijova & Chmelarova, 2007; Van Der Wielen et al., 2002). According to Ranjitkar et al., (2016), The relative quantity of *Lachnospiraceae* diminishes with age, with the biggest disparity happening throughout the first three weeks of life.

1.5.2.1 Ruminococcaceae

As *Clostridiales*-related bacterium family, *Ruminococcaceae* has been identified as one of the most frequent species in the poultry caecal microbiome. (Ranjitkar et al., 2016). Since this family is characterised by the SCFA production, its constituting bacteria have the ability of digesting and fermenting complex carbohydrates, making it vital for effective feed utilization and gut health (Stanley et al., 2015, 2016; Torok et al., 2011). Stanley et al., (2016) conducted a study in which showed increased *Ruminococcaceae* prevalence to be correlated to good FCR (low). Ranjitkar et al., (2016) showed various changes with age, indicating possible vulnerability to other taxa or environmental variables such as dietary composition. Furthermore, *Ruminococcus* capacity to breakdown cellulose might explain its widespread prevalence and abundance in the caecum (Devillard et al., 2004).

1.5.2.3. Bacillaceae

According to Nicholson et al., (2000) this Gram-positive rod family is a spore-forming bacteria that can live under challenging circumstances and include the *Bacillus* genus, thus they might spread across flocks. *Bacillus* species, particularly *Bacillus subtilis*, are frequently employed as probiotics, albeit it is uncertain whether their positive effects are achieved by bacterial colonization or through spores' consumption (Barbosa et al., 2005). Harrington et al., (2015) showed some benefits of *Bacillus* such as FCR improvement, intestinal immunity and Aliakbarpour et al., (2012) found better nutrient absorption. Important to add that the intestinal pathogenecity appears to decrease (Jayaraman et al.,

2013; Parks et al., 2018). These favorable benefits are thought to be mediated by competitiveness with pathogenic bacteria (Barbosa et al., 2005).

1.5.2.4. Enterococcaceae

Commonly known as lactic acid bacteria and are Gram-positive cocci. Age plays an important role in the modification of its relative abundance (Ranjitkar et al., 2016). This bacterial family is more highly prevalent in the ileum than the caecum since it is food-sensitive, and the food component concentrations are more present in the ileum (Lu, Idris, et al., 2003). Fasina et al., (2016) stated that this family can play a major role in reducing the bacterial concentrations of *Clostridium perfringens* and Cao et al., (2013) on the levels of *E. coli* in the caecum. Cao et al., (2013)also stated that poultry weight and intestinal villus measures were enhanced by adding *Enterococcus faecium*.

1.5.2.6. Lactobacillaceae

Gram-positive bacteria that originated in the stomach of chickens. Because of their capacity to ferment glucose to make lactic acid, they are classified as lactic acid bacteria. Animal performance could be improved by administering this bacteria as probiotics (De Cesare et al., 2017), yet the benefits vary greatly depending on the strain, resulting in a wide range of outcomes (Olnood et al., 2015). Caecal levels of *Escherichia coli* have significantly decreased when *Lactobacillus* plantarum was supplemented (Wang et al., 2017), resulting in improved immunity of poultry.

1.5.2.6. Clostridiaceae

From the genera of this family*, Faecalibacterium,* which was found to be linked to intestinal immunity, is a gram-positive non-sporing rod present in the gastrointestinal tract of many animal species, and also humans (Benus et al., 2010). Donaldson et al., (2017) showed that this genus levels rises with age. The ability of Faecalibacterium to express acetyl-CoA acetyltransferase and many enzymes involved in butyrate synthesis has been linked to their relevance (Polansky et al., 2016).

1.5.2.7. Veillonellaceae

This family cannot benefit from carbohydrates for energetic functions but is dependant from the conversion of lactate to short-chained fatty acids, namely acetate, for energy generation (Kwon et al., 1997; Stephen & Hamilton, 1971). This suggests that its existence may be dependent on the presence of lactic acid bacteria, which create lactic acid and by that, showing one the complicated interactions that occur within the caecum microbiome. The growth of *Salmonella* spp. and *Escherichia coli* is suppressed when *Veillonella* is cultivated with lactic acid bacteria such as *Lactobacillus acidophilus*, indicating that such interactions are also significant in host and human health. Hinton et al., (1995).

1.5.3. Tenericutes

In this phylum, Mollicutes class is present, in which 'cutis' means 'skin,' referring to the absence of a cell wall. *Tenericutes* are more found in the caecal microbiota than *Firmicutes* and *Proteobacteria*, albeit in smaller numbers (Awad et al., 2016; Sakaridis et al., 2018; Witzig et al., 2015). Mollicutes belong to the undiscovered order RF39; their function in health and sickness is unknown, however they were observed in greater prevalence in poultry with NE (Stanley et al., 2012) and excessive nutrient consumption (Siegerstetter et al., 2018).

1.5.4. Proteobacteria

This phylum is very commonly found in poultry GIT microbiome. Awad et al., (2016) and Ranjitkar et al., (2016) found that in young birds, this phylum's relative abundance is often large, but as they age, it declines and is substituted by Firmicutes. *Enterobacteriaceae* (class *Gammaproteobacteria*), which includes *Escherichia coli*, make up the bulk of this phylum (Lu, Idris, et al., 2003). Though commonly thought of as a commensal, *E. coli* is an opportunistic pathogen, meaning it can cause disease. However, *Escherichia spp*. high prevalence in young birds may aid in the immune system's fast growth (Awad et al., 2016). In the *Enterobacteriaceae* family, *Salmonella* presents food safety issue since it is zoonotic. The frequency of poultry-derived salmonellosis has decreased because of the relatively recent establishment of control programs, while developing antibiotic tolerance presenting a future source of worry (Antunes et al., 2016).

1.6. Identification and characterization of poultry gut microbiota

Different methods are adopted for the detection and characterization of the avian intestinal microbiome such as G+ C profiling based on culture, 16S rRNA base investigations, quantitative PCR, metaproteomic approaches, metagenome shotgun sequencing, and high-throughput sequencing (Lan et al., 2002).

Microbiota research began in the 1970s using methods dependent on cultures (Barnes & ELLA M, 1979). Some of the issues faced with these cultures were: bacterial species coexist in communities and are reliant on both one another and their hosts' environments for survival, difficulty to identify those that are present in extremely small amounts, absence of a categorization system based on phylogeny, and specific bacteria from the diverse gut microbiota could only be identified. As a result, isolating and growing in any chosen culture may differ from the host intestinal ecology (Apajalahti et al., 2004).

To identify specific bacterial species, limits and difficulties in the selective culture were overcomed by the examination of the DNA extracted with new culture- independent approaches. These techniques discovered up to 90% of the bacterial species previously unknown in the gastrointestinal tract of poultry. (Lan et al., 2002; Zhu et al., 2002).

Among these approaches, high-throughput or next-generation sequencing (NGS) of 16S rRNA gene amplicons is a strong tool for investigating the biology and ecological significance of gut microbiota (Diaz-Sanchez et al., 2013), and will be employed in the present thesis for bacterial profiling. For genomic research, NGS has evolved into a handy, quick, accurate, and low-cost approach (Park et al., 2013; Pettersson et al., 2009). 16S rRNA genes high-throughput sequencing usage was published the first time in 2013, for the examination of the poultry gut microbiotal diversity, even though this technology has been frequently employed in other domains (Diaz-Sanchez et al., 2013).

The 16S rRNA molecule is a tiny ribosome component with similarity in the sequence areas, across all microorganisms, extremely conserved. DNA amplification of the 16S rRNA genes is isolated from digestive tract or fecal samples, and polymerase chain reaction (PCR) amplification is performed using

primers targeting precised sections of these gene (Apajalahti et al., 2004). Each sequence's relative abundance represents the prevalence of that bacterium in the original sample, and sequencing of these amplified products (amplicons) may identify between bacteria at the genus or species level. (Flint et al., 2006; Weisburg et al., 1991). By specifying the kinds of bacteria present in a sample and their relative abundances, 16S rRNA gene sequencing gives a true census of a bacterial community. Due to the enormous diversity and richness of gut bacterial communities, the sequencing of DNA technology recently evolved so that now we can now thoroughly characterise these populations' diversities.

Other molecular techniques are used as well, such as TRFLP (terminal restriction fragment length polymorphism), which was used to compare microbiota in the duodenum, jejunum, ileum, and ceca (Gong et al., 2002), and metagenomics shotgun sequencing, which provides a detailed understanding of microbiome activity in precised GIT segments with differentiating the treatments' effect on microbiome diversity (Marchesi & Ravel, 2015).

Further analysis of sequence data is done by Ion PGM, Illumina MiSeq, Roche 454-pyrosequencing, and HiSeq (adopted to profile the microorganisms in the experiment described in this thesis.). QIIME2 was used to make taxonomic designations, which were compared to public databases including SILVA, GreenGenes and the ribosomal database project (RDP). Following that are functional predictions using PICRUSt and Tax4Fun (Borda-Molina et al., 2018). Stanley et al., (2013) found that reliable microbiota profiling could only be accomplished in a controlled setting, beginning on the day of hatch, which effectively predicts future microbiota as well.

2. INSECTS' FAT AS FEED IN POULTRY NUTRITION

Entomologists and animal nutritionists have recently become interested in the nutritional value, quick growth, and excellent bioconversion capacities of insects used in animal feed (Pinotti et al., 2019). It is well known that insects are a great source of proteins and fatty acids, as well as a variety of bioactive substances that can help animals grow by stimulating their immunity (Benzertiha, Kierończyk, Kołodziejski, et al., 2020). This high interest in insect's production was expressed by many companies in the recent years especially in the EU countries.

Table 2: EU companies specialized in Insect Farming.

Country	Company	Insect species farmed
Netherlands	ILLUCENS PROTIX	*Hermetia illucens*
	Proti-Farm	*Alphitobius diaperinus*
Italy	BEF BIOSYSTEMS	*Hermetia illucens*
	Italian Cricket farm NUTRINSECT ALIA	*Acheta domesticus*
		Bombyx mori *Tenebrio molitor* *Acheta domesticus*
		Hermetia illucens *Bombyx mori*
U.K	AgriProtein ENTOCYCLE	*Hermetia illucens*
France	ENTOMO FARM INNOVAFEED NextAlim nextProtein MUTATEC	*Hermetia illucens*
		Tenebrio molitor
Belgium	MILLIBETER	*Hermetia illucens*
Spain	BioFlyTech MealFood	*Hermetia illucens*

Germany		*Hermetia illucens*
Poland		*Hermetia illucens*
Lithuania		*Hermetia illucens*
Danemark		*Hermetia illucens*
Ireland		*Hermetia illucens*
Bulgaria		*Hermetia illucens*

After protein, fat is the insect's second-largest component. On a dry matter basis, the fat content of insects ranges from 7.9 percent to 47 percent (Meneguz et al., 2018; Danieli et al., 2019; Finke, 2015; Nogales-Mérida et al., 2019). Nutritionists face a challenge when formulating feeds since not all insect species have the same profile of fatty acids (FAs) and, as a result, fats with the same nutritional value. Species, life stage, nutrition, and environmental variables all have a role in determining an insect's fat content (Barragan-Fonseca et al., 2018; Fontaneto et al., 2011; Oonincx et al., 2019a, 2019b). However, the fatty acid profile may also be influenced by the above-mentioned causes, resulting in a wide range of fatty acid compositions (Sosa & Fogliano, 2017). As a final point, changes in FAs in insects can occur within a few hours after exposure to a novel diet, whereas in mammals, similar changes can take weeks to develop. Using insects as a source of energy in animal nutrition has been successful, including in broiler chickens (Kierończyk et al., 2018; Schiavone et al., 2017), turkeys (Kim et al., 2020; Sypniewski et al., 2020), laying hens (Heuel et al., 2021), rabbits (Cullere et al., 2018), and many fish species (Belghit et al., 2018; S. Li et al., 2016).

The uniqueness of insect rearing and the favorable effects of insect biomass-based products on livestock animals have sparked a surge in interest in using insects commercially in animal nutrition (Benzertiha et al., 2020). However, many of the current studies on the use of insect biomass in animal feeding are focused on replacing traditional protein sources, with fat as an energy source receiving far less attention. Because of the fat content and FA profiles of insects, their use in animal nutrition should be considered.

2.1 Global regulations on the use of insects as animal feed

Insect producers are subject to EU law, which governs the conditions under which they can produce and sell their products in the EU. It is notable that EU legislators adopted a bundle of legal texts in the early 2000s that specify general principles and requirements in terms of food/feed safety. "Hygiene Package" (Regulations No 852/2004 and No 183/2005 on food and feed hygiene, respectively) and "General Food Law" (Regulation 178/2002) are the most well-known terms for these legislations. The feed that can be supplied for animals, farmed for the production of feed, food, or further processing by-products, has also been restricted by EU decision makers (e.g., wool or hides). Insects that are intended for human consumption or animal feed use are also subject to these regulations.

Concerning insects, law in the EU defines "animal by-products" such as products derived from insects, without live insects that are designed for inclusion in animal feed. According to Regulation No. 1069/2009 and its implementing Regulation No. 142/2011, known as the "EU animal by-products regulation," this qualification entails a number of producer responsibilities.

As revised by Regulation 2017/893 (Annex X), Regulation No 2001/999 (Annex IV) permits using proteins derived from the following species of insects: *Hermetia illucens* (Black soldier fly), *Alphitobius diaperinus* (Lesser mealworm), *Gryllodes sigillatus* (Banded cricket), *Musca domestica* (Common housefly), *Acheta domesticus* (House cricket), *Tenebrio molitor* (Yellow mealworm), in swine, aquaculture and poultry feed.

For the third countries meeting the requirements of Regulation (EU) No. 2017/893, they were allowed to export products from insects, and the Regulation (EU) No. 2019/1981 has set this list of requirements.

Finally, the European Union State Members legalized using insect PAPs (processed animal proteins) in pig and poultry feed in April 2021. For the European growing sector of insect farming, this proposition is significant since it is one of the crucial checkpoints in the authorization procedure. This proposal entered into force on September 7th, 2021, in accordance with EU rules. As a result of the aforementioned legislation, proteins from insects are now permitted in poultry or pig feed, as well as

pets (e.g., cats, reptiles, dogs, or birds) and mink as an example of fur animals. Whole insects and lipids generated from insects are exempt from the ban on being used in animal feeds.

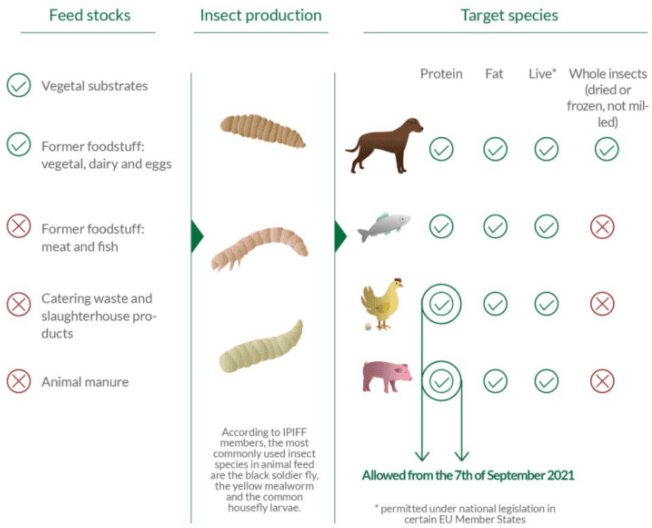

Figure 4: Feed insect proteins to animal species unlocked after the lifting of the EU 'feed ban' rules in September 2021 (https://ipiff.org/insects-eu-legislation/)

In North America, AAFCO has only defined, and approved from FDA, one insect species as a livestock feed ingredient in the US. *H. illucens* larvae, including meal (since 2018) and dried larvae (since 2016), can be used in aquaculture feed for salmon, trout, and char (Lähteenmäki-Uutela et al., 2018). Also, the CFIA (Canadian Food Inspection Agency) approved *H. illucens* product for meat chickens, salmonids, tilapia, and poultry (Lähteenmäki-Uutela et al., 2021).

Since Brazil is already the world's largest exporter of chicken, doing anything to improve the industry's long-term viability would have a major impact (Allegretti et al., 2018). The use of insects as animal feed is not yet envisioned in Brazilian feed law. Codex Alimentarius norms are generally followed in Brazil, as stated by Allegretti et al. (2018). Specific NOMs (Normas Oficiales Mexicanas) are not yet available for insect feed and food in Mexico (Lähteenmäki-Uutela et al., 2021).

In China, after approval of new feed items, they should be added to the Feed Materials Catalogue. "Safety Assurance and Quality Improvement of Feeds", issued by Japan's Ministry of Agriculture,

Forestry, and Fisheries, establishes maximal limits for heavy metals, pesticide residues, and mycotoxins. Before launching a business, feed makers, importers, and/or dealers must file notification (FAMIC, 2014). Thailand also has a major broiler and swine industry, which could benefit from insect-based feed. There are standards for product quality in Thailand, concerning animal feed, but there are none in place for insect feed. Animal feed is regulated by the Ministry of Agriculture and Cooperatives (Lähteenmäki-Uutela et al., 2021).

There is no explicit legislation regulating insect cultivation or insect foods in South Africa, while food and feed safety is addressed by a number of different government agencies across the continent. Farm feed and pet food must be registered in accordance with the Farm Stock Remedies, Agricultural Remedies, Feeds, and Fertilizers Act 1947 (Act 36 of 1947) before they can be manufactured, imported, or sold in South Africa (Gazette et al., 1947). Usman and Yusuf (2020) state that there are no existing regulations regarding insect as feed in Nigeria. All feed products, whether made or imported, are subject to NAFDAC (National Agency for Food and Drug Administration and Control) registration requirements (VMAP 003/13 and VMAP 004/13, NAFDAC recommendations). Validity of registrations is stated in years.

The FIAAA Code of Practice and the Feed Safe Standard are applicable in New Zealand and Australia to feed production (https://fiaaa.com.au/about/#bg). In all states, insects are allowed to be included in poultry diets, and as feed for fish in Victoria, Western Australia, Tasmania, ACT, and NSW. Strict regulations concerning insect feeding and insects use in animal feed are applied in Australia (DiGiacomo & Leury, 2019).

2.2 *Hermetia illucens* or Black soldier fly (BSF)

From the order Diptera, *Hermetia illucens* species belongs to the family *Stratiomyidae*. It is more generally known as the black soldier fly. Even though it was first cultivated in the Americas, it has since spread throughout the world, both in tropical and temperate zones (Čičková et al., 2015; Sheppard et al., 1994), however it cannot establish itself in places where it is not native due to its lack of cold tolerance (Spranghers et al., 2017). Only water is consumed by adults, contacting humans is very avoided, rarely if ever sting or bite, and pose no risk of spreading disease (Sheppard et al., 2002; Čičková et al., 2015). *Hermetia illucens* larvae have been shown to consume a wide range of organic substrates, distillers' grains (Webster et al., 2016), rice straw (Zheng et al., 2012), fecal sludge (Banks et al., 2014; Lalander et al., 2013), manure (Yu et al., 2009),food scraps (Green & Popa, 2012), edible internal organs of animals (Nguyen et al., 2015). They may be able to process the widest range of substrates and do so at the highest efficiency of all fly species (Kim & Mundt, 2011). There has been research into the safety of consuming BSFL. Known to have a better feed conversion ratio than both crickets and mealworms, BSFL survival rate and nitrogen and phosphorus compositions also show less variation with diet (Oonincx & de Boer, 2012). Non-feeding adults of BSF use as an energy source the dietary lipids previously stored by BSFL, and those lipids could reach a point that they could be converted to biodiesel (Li et al., 2011; Mohd-Noor et al., 2017; Nguyen et al., 2017; Wang et al., 2017). Fertilizer can be made from their leftovers and their nitrogen-rich feces (Green & Popa, 2012; Lalander et al., 2015). Their extended larval growth time of nearly three weeks compared to insects such as house flies' growth only in 5 days, means that BSF larva will consume higher amounts of substrates and pupae produced will be consequently bigger (Čičková et al., 2015). Furthermore, pre-pupa BSFL leave the substrate and travel to a high, clean spot, a behavior known as "self-harvesting," which eliminates an otherwise labor-intensive step in their farming (Diener et al., 2011; Sheppard et al., 1994). Considering all these advantages, raising BSFL is not only feasible, but might also provide a sustainable animal feed or human food source in the future.

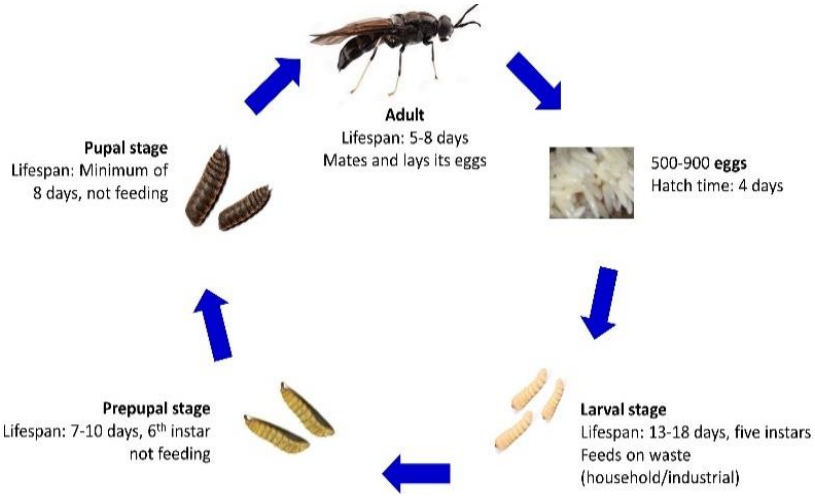

Figure 5: The Black soldier fly (Hermetia illucens) life cycle (Da Silva & Hesselberg, 2020)

2.3 Parameters affected by BSF fat inclusion in poultry ration

2.3.1 Growth performance

Several trials were conducted in the accessible literature, particularly on *H. illucens* and *T. molitor* lipids as alternatives for conventional fat sources in poultry feeding (Dabbou et al., 2021), published the most recent study, which found no effect on growth performance metrics (FCR, BWG, and FI). In addition, (Kim et al., 2020) investigated the total replacement of corn oil and coconut oil in broiler chicken nutrition and found that dietary treatments had no effect on FI (Feed Intake) and BWG (Body weight gain), but the FCR (Feed Conversion Ratio) ratio was significantly reduced in the treatment supplemented with *H. illucens* fat and coconut oil throughout the experiment (30 days). Furthermore, tests on broiler chickens were undertaken to replace soybean oil with *H. illucens* fat totally or partially, and none of the studies indicated any negative impacts on growth performance measures (Schiavone et al., 2017, 2018; Sypniewski et al., 2020). Insect fat obtained from *H. illucens* larvae can substitute traditional fat sources in broiler chicken and turkey feeding without affecting growth performance features, according to the experiments stated above. Furthermore, laying hens fed fat from *H. illucens*

larvae showed no differences in BWG, or the FI (Heuel et al., 2021). Sypniewski et al., (2020) investigated whether *H. illucens* fat could completely or partially substitute soybean oil in young turkeys. The authors found no impacts of dietary interventions on the growth performance indicators, such as BWG, FI, and FCR, during each phase of the study. Furthermore, the findings concern the inclusion of a full-fat meal derived from insect biomass, which may hint at certain biological implications associated with the use of insect lipids in animal nutrition. The use of *H. illucens* larval full-fat meal as a substitute for soybean meal, oil, or fish meal in broiler and laying quail diets had no effect on feed performance or growth performance features (Cullere et al., 2016; Dalle Zotte et al., 2018).

2.3.2 Nutrient digestibility

Higher levels of SFAs (saturated fatty acids, primarily C18:0 and C16:0) and MUFA (monounsaturated fatty acids; C 18:1, n-9) were found in insect oils (Al-Qazzaz et al., 2016). Similar to fish oil, insect fatty acids have unsaturated fatty acids, but they have more polyunsaturated fatty acids (Kierończyk et al., 2018). *H. illucens* prepupae had about 32% fat, most of which was saturated fatty acids (SFA), with lauric acid (C12:0) being the most common one (67 percent of total SFA; Surendra et al., 2016). The ratio of n-6 to n-3 is bad for *H. illucens* larvae (Cullere et al., 2019). Still, the types of fatty acids in insects depend on what they eat and the different stages of life cycle. Kierończyk et al., (2020) found that organic wastes can change the FA profile, also when administered as substrates for *H. illucens* production, wheat bran is better than cabbage or carrots at lowering lauric acid (C12:0). Most studies on insect fat substitution in broiler chicken nutrition (Benzertiha et al., 2019; Kierończyk et al., 2018, 2020) found no effects on the coefficients of the apparent digestibility of crude fat and crude protein or the metabolizable energy. Furthermore, after *H. illucens* oil supplementation in the meal, (Sypniewski et al., 2020) found no impacts on the coefficients of nutrient digestibility of crude fat and crude protein, nor on the metabolizable energy in turkeys.

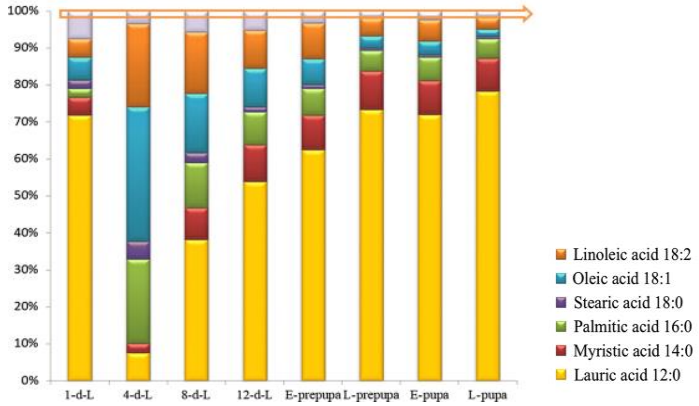

Figure 6: FA profile of BSF at different developmental stages (Zhu et al., 2019)

2.3.3 Gut morphology and microbiome

The inclusion of BSF larva fat as feed supplements had no effect on intestinal morphology, according to a recent study by (Dabbou et al., 2021), as well as the progression of morphological features along the various intestinal segments showing that the poultry's GIT in this experiment, including the control, was in good condition. In addition, it was identified that, passing from the duodenum into the ileum, the morphometric indices have a diminishing gradient, suggesting that the physiologic gut growth and absorption processes seen in chickens were preserved. (Biasato et al., 2018). In the same manner, the absence of notable histological abnormalities in birds fed BSF fat indicates that different dietary treatments with inclusion of *Hermetia illucens* larvae fat did not damage the health of animals. Previous studies by Sypniewski et al., (2020) and Schiavone et al., (2018) corroborate these results when BSF fat was substituted for conventional oils used in the diets of broiler chickens and turkeys.

Although SFAs are the key elements of the FA profile in BSFL fat, the comparatively high concentration of lauric acid can affect the microbiota composition. Lauric acid has well-known antibacterial, antiviral, and antiparasitic effects (Dayrit, 2015; Ushakova et al., 2016). (Kierończyk, 2022) observed that BSFL fat in turkey diets significantly altered specific microbial populations in the

ceca, such as the *Bacteroides* - *Prevotella* cluster, which showed decreased growth in the digesta. No difference was found in *Lactobacillus*, *Bifidobacteria*, *Enterobacteriaceae*, *Campylobacter jejuni*, or *E. coli* in the jejunal digesta of broilers fed diets high in lauric acid (Zeitz et al., 2015). Furthermore, (Timbermont et al., 2010) stated that lauric acid has the most potent inhibitory activity against *C. perfringens* and can be utilized to prevent necrotic enteritis in poultry flocks. The *Enterobacteriaceae* family was reduced as a result of BSFL fat (Sypniewski et al., 2020). Dabbou et al., (2020) found that BSFL fat was more effective (in vitro) than *T. molitor* oil against *Pasteurella multocida*, *Yersinia enterocolitica* and *Listeria monocytogenes* due to the higher lauric acid level (790 vs. 250 g/kg SFA content). As a result, the lauric acid level is critical in determining the antibacterial characteristics and quality of the BSFL fat.

The positive effect of BSFL fat incorporation on serum IL-6 levels was detected. It is well known that IL-6 is a mediator of the acute phase response (Akira, 2000), and its levels are elevated following *E. coli* lipopolysaccharide (LPS) (Nakamura et al., 1998), *Salmonella typhimurium* LPS (Xie et al., 2000), and *Clostridium perfringens* (Zhang et al., 2019) exposures. Furthermore, Bacteroides LPS promotes the expression of IL-6 (Leite et al., 2017). This was consistent with the caecal microbiota analysis, which revealed that BSFL fat addition suppressed the *Bacteroides-Prevotella* cluster.

2.3.4 Carcass and meat quality

These parameters can be characterized by chemical composition and physical properties (pH value, colour, tenderness; (Benzertiha, Kierończyk, Rawski, et al., 2020). Several studies looked at how adding insect fat to poultry diets affected the quality of the final product (Cullere et al., 2019; B. Kim et al., 2020; Kierończyk et al., 2018, 2020; Benzertiha et al., 2019;); (Cullere et al., 2019) did not find any changes in the chemical and physical-chemical properties of the thigh meat caused by the different diets. The high amount of SFAs in the birds' food may have something to do with these effects. Kim et al. (2020) found that the pH value of the breast meat of 30-day-old broiler chickens was significantly affected by their diets, with *H. illucens* fat and corn oil having higher values than coconut oil. Also, the color was more yellow when *H. illucens* fat or coconut oil was used instead of corn oil. However, some

authors said that the color, pH, and for cooking losses of thigh and breast meat, they felled within the permissible range of meat qualities. Adding 15 % *H. illucens* full-fat meal to the broiler quail diets increased cooking loss and tenderness and decreased the pH value and redness of the breast meat compared to the group that added soybean meal and oil (Cullere et al., 2016, 2018; Zotte et al., 2019). Also, the BSF full-fat meal changed the composition of FA in breast meat by making it have more SFA. With a higher rate of *H. illucens* larvae, the proportion of SFA in broiler chicken breast meat grew at the expense of the PUFA fraction. PUFA do many things in the body and are the building blocks of molecules that help cells work (Abdulla et al., 2019). It's important to note that in the case of insect full-fat meal, other components like chitin may have a big effect on the results seen (Benzertiha et al., 2020).

Table 3: Studies on the inclusion of the *Hermetia illucens* fat in poultry feed.

Species	Insect	Ingredients substituted	Substitution level (%)	Treatment period	Traits and Impact	References
Broiler chicken	*Hermetia illucens* oil	SO	50, 100	1-35 days	-BWG: NS -FI: NS -FCR: NS -Blood traits and immune system: NS	(Schiavone et al., 2017)
Broiler chicken	*Hermetia illucens* oil	SO	50, 100	21-48 days	-BWG: NS -FI: NS -FCR: NS -Blood traits and immune system: NS - GIT health: NS	(Schiavone et al., 2018)
Broiler chicken	*Tenebrio molitor* oil	Poultry fat and palm oil	100	7-30 days	-BWG: NS -FI: NS -FCR: NS	(Benzertiha et al., 2019)
Broiler chicken	*Hermetia illucens* oil	CO and coconut oil	100	1-30 days	-BWG: NS -FI: NS	(Kim et al., 2020)

					-FCR: P<0.05 -Blood traits and immune system: P<0.05 -GIT microbiota: NS	
Broiler chicken	*Hermetia illucens* oil	SO	25, 50, 75, 100	1-35 days	-BWG: NS -FI: NS -FCR: NS -FCR and FI: Decreased in HI groups in comparison to soybean oil until 14 d	(Kierończyk et al., 2020)
Turkey	*Hermetia illucens* oil	SO	50, 100	7-28 days	-BWG: NS -FI: NS -FCR: NS - Blood traits and immune system: P<0.05 -GIT microbiota: P<0.05	(Sypniewski et al., 2020)
Broiler chicken	*Hermetia illucens oil*	SO	BSF, MBSF1, MBSF2	33 days	-BWG: NS -FI: NS -FCR: NS - Blood traits and immune system: NS -GIT microbiota: P<0.05	(Dabbou et al., 2021)

Broiler chicken	*Hermetia illucens* oil	SO	50, 100	1-35 days	-Blood traits and immune system: P<0.05 -GIT health: P<0.05 -GIT microbiota: P<0.05	(Kierończyk, 2022)

SO: soybean oil; CO: Corn oil; BWG: Body weight gain; FI: Feed intake; FCR: Feed conversion ratio; BSF: Black soldier fly; MBSF: Modified black soldier fly; GIT: Gastro-intestinal tract; NS: No significance.

3. MATERIALS AND METHODS

The animals were treated in accordance with the EC Directive 63/2010/EEC on the protection of the animals used for experimental and other scientific purposes. The experimental procedures were approved by the Ethical Animal Care and Use Committee of the Department of Veterinary Medicine and Animal Production of the University of Napoli Federico II, Italy (prot. N. 2017/0017676). The trial was carried out on a private quail's farm in Sardinia (Italy).

3.1 Birds, Diets, and Experimental Design

In the experiment, a total of 100, 7 days old Japanese quails (*Coturnix japonica*; average weight 36.01 g ± 0.36), were randomly divided into 4 groups (5 replicates/ group). The replicates were housed in galvanized metal cages (100cm length × 50cm depth × 25 cm height) and reared for 5 weeks (until 42 days of age). Experimental diets, in mash form, were formulated by mixing a corn and soybean meal-based diet according to NRC (1994) with 2 different oils (corn oil, and BSFL oil) to reach 50 g per kg of diet. A basal diet containing corn oil (CO) as added fat served as control group (C) and was tested against 3 experimental diets where CO was partially substituted by BSF larvae fat by 25%, 50% and 100% (BSFO25, BSFO50 and BSFO100 respectively). The BSF larvae fat is the fat extracted from BSF larvae (cold press extraction technique), as commercially available from PROTIX (Dongen, The Netherlands). The analysis of the diets' contents was performed according the AOAC (2005) methods: for crude protein (method 978.04), ether extract (method 920.39), crude fiber (method 978.10), dry matter (2001.12) and ash (method 930.05). Based on the composition of the diet's constituents, the content of amino acids was estimated. From the diets' chemical composition, their metabolisable energy (ME) was calculated based on the NRC, (1994) equations. Detailed information about the experimental diets and their nutritional characteristics are shown in Table 4. To perform a fatty acids (FAs) transmethylation analysis, a base-catalysed procedure was used as reported by Christie et al, (1982) and modified by Chouinard et al, (1999). A gas chromatograph (Agilent technologies, model 5890, CA, USA), equipped with a fused SP-2560 silica capillary column L × I.D. 100 m × 0.25 mm,

df 0.20 μm (Supelco, Inc., Bellefonte, PA, USA) were used for FAs methyl esters quantification. Helium was used as carrier gas, with 280 kPa of constant pressure, 50 mL/min splitting flow, and 1 μL of injection volume. Concerning column parameters, for 15 min, the column temperature was sustained at 170 ◦C, then increased up to 240 ◦C by adjustment of 5 ◦C/min. The overall execution duration was 64 minutes. FAs peaks were categorised through comparison of the retention times of commercial standard comprising 37 methyl esters of FAs (Sigma-Aldrich, MO, USA). Controlling of the CLA isomers retention time, was done by the elution of the commercial standard (Larodan AB, Solna, Sweden) of these fatty acids. By applying a percentage calculation to the overall area of the eluted peaks, the area of each specific FA found in the sample was determined. The fatty acid profile analysis of *H.illucens* fat, corn oil and the diets used in this experiment are shown in Table 5.The experiment was designed to compare the growth and slaughter performance, in addition to the sampling and analysis of caecal microbiome bacterial diversity.

Feed and fresh water were administered ad libitum. At the beginning of the trial the infrared heat lamps (150 W) were at 25 cm from the floor of the cages and have been set to ensure a temperature of 32-35 °C at bird level; thus, the temperature was gradually decreased to reach 23-24 °C at 28 days of age. The lighting program was 16:8 h light:dark. The trial was carried in the period August-September 2021.

Table 4: Chemical and nutritional composition of experimental diets (as-fed).

Diets	**Control**	**BSFO 25**	**BSFO 50**	**BSFO 100**
Ingredients g/100g as fed				
Corn 8.8	48.1	48.1	48.1	48.1
Soybean meal 44	43	43	43	43
BSF fat	0	1.25	2.5	5
Corn Oil	5	3.75	2.5	0
Calcium carbonate	1	1	1	1
Dicalcium phosphate	2	2	2	2
Salt	0.3	0.3	0.3	0.3
Methionine	0.1	0.1	0.1	0.1
[1] Premix Vit-Min	0.5	0.5	0.5	0.5
Chemical composition				
Dry matter %	90.2	89.9	90.1	89.8
Ash, %	6.52	6.43	6.47	6.39

Crude protein, %	23.1	23.1	23.1	23.1
Crude fiber, %	9.32	9.15	9.43	9.24
Ether extract, %	7.73	7.86	8.04	7.92
Lysine, %	1.41	1.41	1.41	1.41
Calcium, %	0.96	0.96	0.96	0.96
Phosphorus, %	0.44	0.44	0.44	0.44
Methionine+ Cysteine, %	0.92	0.92	0.92	0.92
[2] ME (kcal/kg)	2900	2900	2900	2900

1: Provided per kg ofproduct: Fe, 50,000 mg; Co, 200 mg; Cu, 8,500 mg; Mn, 75,000 mg; Zn, 70,000 mg; Se, 250 mg; I, 1,500 mg; folic acid, 500 mg; pantothenic acid, 13.5 g; niacin, 30 g; vit. A, 10,000,000 IU; cholecalciferol 50,000 mg; vit. K3, 4,000 mg; vit. B2, 5,000 mg; vit. B6, 2,000 mg; vit. B12, 10,000 mg; vit. E (dl-a-tocopheryl acetate), 21,978 IU. 2: Metabolisable energy.

Table 5: Fatty acid profile of the dietary fats and experimental diets (% total FA).

Fatty acids	Dietary Fats		Experimental diets			
	CO	BSFO	Control	BSF25	BSF50	BSF100
C4:0	0.15	1.57	0.008	0.025	0.043	0.078
C6:0	-	0.01	-	0.0002	0.0003	0.0006
C10:0	-	0.41	0.116	0.121	0.126	0.136
C12:0	-	37.71	-	0.471	0.943	1.885
C13:0	-	0.08	-	0.001	0.002	0.004
C14:0	-	10.46	0.084	0.215	0.346	0.607
C16:0	10.85	17.11	11.122	11.201	11.279	11.435
C16:1	-	2.89	-	0.036	0.072	0.145
C18:0	1.51	2.79	2.681	2.697	2.713	2.746
C18:1 trans 9	0.12	0.34	0.006	0.009	0.011	0.017
C18:1 CIS9	30.40	11.97	23.027	22.797	22.566	22.106
C18:1 CIS11	-	0.06	-	0.001	0.001	0.003
C18:2 cis ω6 (LA)	54.90	12.13	52.748	52.214	51.679	50.610
C20:0	0.54	0.21	0.758	0.753	0.749	0.741
C20:1	-	0.04	0.043	0.044	0.044	0.045
C18:3 ω3 (ALA)	1.07	0.68	4.623	4.618	4.613	4.604
CLA1 (C18:2 c9-t11)	-	0.67	-	0.008	0.017	0.034
C20:2 ω6	0.01	-	0.0007	0.0005	0.0003	-
C22:0	0.13	0.05	0.006	0.005	0.004	0.002
C20:3 ω3	-	-	0.17	0.17	0.17	0.17
C20:3 ω6	-	0.03	-	0.0003	0.0007	0.0013
C22:1	0.04	0.38	0.002	0.006	0.010	0.019

C24:0	0.17	-	0.008	0.006	0.004	-
C20:5 ω3 (EPA)	0.02	-	0.0009	0.0006	0.0004	-
C22:4 ω6	0.05	-	0.002	0.002	0.001	-
C22:5 ω3 (DPA)	0.03	-	0.002	0.001	0.001	-
Saturi (SFA)	13.35	70.41	14.839	15.553	16.266	17.693
Monoinsaturi (MUFA)	30.56	15.68	23.078	22.892	22.706	22.334
Polinsaturi (PUFA)	56.08	13.51	57.545	57.013	56.481	55.417
omega 6	54.96	12.16	52.752	52.217	51.681	50.611
omega 3	1.12	0.68	4.794	4.788	4.783	4.772
CLA	-	0.67	-	0.008	0.017	0.034
Rapporti						
PUFA/SFA	4.20	0.19	3.753	3.703	3.652	3.552
omega 6/omega 3	49.26	17.89	11.004	10.905	10.805	10.606
LA/ALA	51.40	17.85	11.41	11.307	11.203	10.993

CO = corn oil fatty acid profile; BSF = Black soldier fly; LA =linoleic acid; ALA = alpha linoleic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

3.2 Growth performance

Weekly, the live weight and the feed intakes of the quails were recorded per replicate. The feed intake was calculated by weighing each day an amount of feed to offer and the amounts of feed scattered and refused by the animals. According to these measurements, the body weight gain (BWG) and the feed conversion ratio (FCR) were calculated for each week of the trial and for the entire experimental period. The mortality rate was recorded along the experimental period.

3.3 Sampling and slaughter performance

At 42 days of age, all the quails were slaughtered in a specialized slaughterhouse. After the bleeding and the removal of the feathers, the whole digestive tract, head and legs, the carcasses were weighed (dressed carcasses) to evaluate the dressing percentage. The internal viscera (liver, spleen and heart, the whole gastro-intestinal tube, from which the stomach and the caeca were separated) were removed and weighed and their weights were expressed as a percentage of the live weight. The yield of the eviscerated carcass was calculated. The full intestinal length and caecal length were measured and

expressed as percentage of the quail live weight. From each carcass the legs were separated and weighed.

Then, the caecum was dissected in order to obtain its contents. The portion of caecal samples was gently squeezed from 10 birds per group (n = 40), and immediately packed, sealed in sterilized Microcentrifuge tubes 2.0 ml, Eppendorf®. They were stored at −80 ˚C for one week then freeze-dried using Heto powerdry LL3000 freeze dryer (Thermo Fisher Scientific, USA). The dried samples were transported to the Institute of Animal Physiology and Genetics of the Czech Academy of Sciences (Prague, Czech Republic) for analyses of the microbial populations by next-generation sequencing (NGS).

3.4 DNA Extraction

The DNA extraction from caecal samples was carried out using the DNeasy® PowerSoil® Pro Kit (Figure 7).

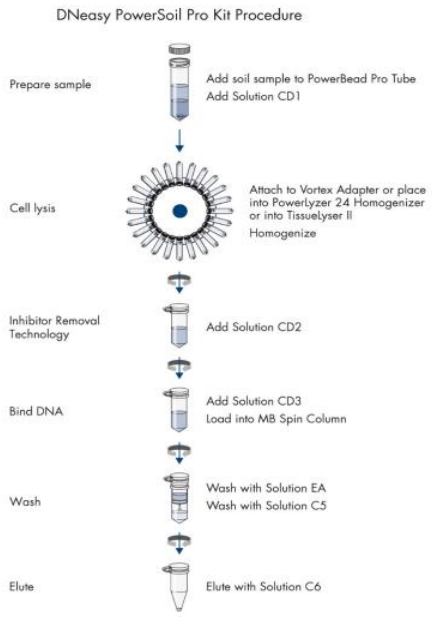

Figure 7: DNeasy PowerSoil Kit procedure.

The detailed description of DNA isolation procedure is described below:

1. Spin the PowerBead Pro Tube briefly to ensure that the beads have settled at the bottom. Add the whole amount of caecal dried sample (weights are listed in Table 6) and 800 µl of Solution CD1. Vortex briefly to mix.

2. Heat the tubes at 65°C for 5 min.

3. Secure the PowerBead Pro Tube horizontally on a Vortex Adapter for 1.5–2 ml tubes (cat. no. 13000-V1-24). Vortex at maximum speed for 5 min.

4. Repeat step 2 and 3.

5. Centrifuge the PowerBead Pro Tube at 15,000 x g for 1 min.

6. Transfer the supernatant to a clean 2 ml Microcentrifuge Tube (provided).

7. Add 200 µl of Solution CD2 and vortex for 5 s.

8. Centrifuge at 15,000 x g for 1 min at room temperature. Avoiding the pellet, transfer up to 700 µl of supernatant to a clean 2 ml Microcentrifuge Tube (provided).

9. Add 600 µl of Solution CD3 and vortex for 5 s.

10. Load 650 µl of the lysate onto an MB Spin Column and centrifuge at 15,000 x g for 1 min.

11. Discard the flow-through and repeat step 8 to ensure that all of the lysate has passed through the MB Spin Column.

12. Carefully place the MB Spin Column into a clean 2 ml Collection Tube (provided). Avoid splashing any flow-through onto the MB Spin Column.

13. Add 500 µl of Solution EA to the MB Spin Column. Centrifuge at 15,000 x g for 1 min.

14. Discard the flow-through and place the MB Spin Column back into the same 2 ml Collection Tube.

15. Add 500 µl of Solution C5 to the MB Spin Column. Centrifuge at 15,000 x g for 1 min.

16. Discard the flow-through and place the MB Spin Column into a new 2 ml Collection Tube (provided).

17. Centrifuge at up to 16,000 x g for 2 min. Carefully place the MB Spin Column into a new 1.5 ml Elution Tube (provided).

18. Add 50 µl of Solution C6 to the center of the white filter membrane.

19. Centrifuge at 15,000 x g for 1 min. Discard the MB spin column and collect eluted DNA solution.

The concentration and quality of the nucleic acids were measured using NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, U.S.A) and the DNAs were kept at -20 ºC until needed for the analysis.

The dried weights and DNA concentrations are listed in table 6.

Table 6. Dried weights and DNA concentrations of the samples.

Samples	**Dried weight**	**DNA Concentration**	**A260/A280**
C1	20 mg	526.344 ng/µl	1.879
C2	76 mg	656.281 ng/µl	1.871
C3	46 mg	187.902 ng/µl	1.835
C4	33 mg	395.873 ng/µl	1.861
C5	129 mg	652.374 ng/µl	1.853
C6	55 mg	542.163 ng/µl	1.887
C7	24 mg	67.242 ng/µl	1.796
C8	42 mg	746.042 ng/µl	1.881
C9	35 mg	380.719 ng/µl	1.853
C10	114 mg	897.776 ng/µl	1.856
H25-1	95 mg	381.904 ng/µl	1.842
H25-2	45 mg	578.281 ng/µl	1.867
H25-3	84 mg	981.143 ng/µl	1.87

H25-4	56 mg	243.82 ng/µl	1.813
H25-5	63 mg	802.56 ng/µl	1.871
H25-6	104 mg	846.823 ng/µl	1.857
H25-7	33 mg	530.906 ng/µl	1.87
H25-8	35 mg	793.039 ng/µl	1.868
H25-9	70 mg	905.89 ng/µl	1.873
H25-10	92 mg	125.431 ng/µl	1.692
H50-1	60 mg	1553.108 ng/µl	1.862
H50-2	40 mg	865.442 ng/µl	1.857
H50-3	120 mg	650.747 ng/µl	1.855
H50-4	79 mg	437.083 ng/µl	1.839
H50-5	41 mg	666.463 ng/µl	1.879
H50-6	48 mg	988.895 ng/µl	1.874
H50-7	36 mg	903.561 ng/µl	1.871
H50-8	50 mg	869.566 ng/µl	1.857
H50-9	110 mg	113.296 ng/µl	1.648
H50-10	56 mg	878.975 ng/µl	1.855
H100-1	51 mg	853.736 ng/µl	1.874
H100-2	109 mg	213.076 ng/µl	1.74
H100-3	76 mg	532.379 ng/µl	1.872
H100-4	54 mg	1086.944 ng/µl	1.852
H100-5	85 mg	437.059 ng/µl	1.848
H100-6	82 mg	417.057 ng/µl	1.895
H100-7	62 mg	403.768 ng/µl	1.877
H100-8	49 mg	1173.209 ng/µl	1.876
H100-9	98 mg	425.78 ng/µl	1.855

H100-10	76 mg	797.091 ng/µl	1.88

3.5 PCR Amplification of 16S rRNA fragment

3.5.1 PCR program and primers used

Reagents	**Volume (µl)**
EliZymeTM HS FAST MIX Red Master Mix	15
Bact BF	1
Bact BR	1
ddH_2O	11
DNA template	2
Total	**30**

Each DNA sample was diluted 10 times in nuclease-free water, and 2 µl of the diluted solutions were used as template for PCR reaction. The variable V4-V5 region of the bacterial 16S rRNA gene was amplified using the particular primer pair BactBF (GGATTAGATACCCTGGTAGT) and BactBR (CACGACACGAGCTGACG) and EliZymeTM HS FAST MIX Red Master Mix (Elisabeth Pharmacon, Czech Republic), as described by (Fliegerova et al., 2014). The PCR assay was performed using the thermocycler Biometra TAdvanced (Biometra, Germany), as follows: a denaturation step at 95°C for 5 minutes, 25 cycles consisting of 30 seconds at 95°C, 30 seconds at 57°C, 30 seconds at 72°C and final elongation step at 72°C for 5 minutes.

3.5.2 Agarose gel electrophoresis

The size and quality of each PCR amplicon were determined using agarose gel electrophoresis. Generally, 1.5% agarose gels prepared in 1 X TBE buffer were used. The agarose was dissolved in the

buffer and boiled, then, ethidium bromide (0.5 µg/ml) was added into the gel for staining. The gels were poured into plastic trays with a plastic comb attached to create sample wells, before hardening. The gels were run using the cleaver Scientific's horizontal gel electrophoresis with a running buffer of 1x TBE. A volume of 5 µl of 200-1500 kb DNA marker (Top-Bio, Czech Republic) was loaded in the first well as a molecular size marker, then 4 µl of the DNA samples were added in the following wells. The gels were run at 110 V for 30 minutes. The DNA was visualized using Azure 200 - Gel Doc (Azure Biosystems, USA). The length of the amplicon fragments was approximately 300 bp as shown in Figure 8.

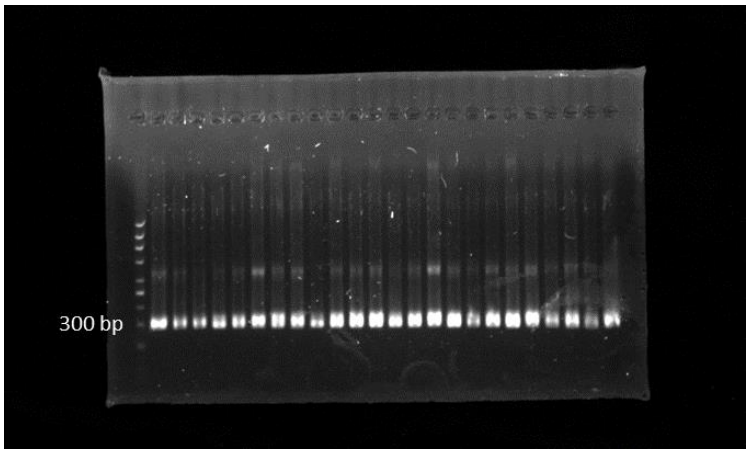

Figure 8: Agarose gel electrophoresis showing amplicons quality and size.

3.5.3 Purification and concentration of the PCR products

The purification of the PCR products was done using the Monarch® PCR & DNA Cleanup Kit (New England Biolabs, USA). The detailed description of the procedure is described below:

Before the beginning:

Add 4 volumes of ethanol (> 95%) to one volume of DNA Wash Buffer.

Add 0.36 volumes of isopropanol to one volume of DNA Cleanup Binding Buffer.

Protocol steps:

1. Dilute the samples (x 5) with DNA Cleanup Binding Buffer, mix well by pipetting up and down or flicking the tube. Do not vortex.

2. Insert column into collection tube and load sample onto column and close the cap. Spin for 1 minute, then discard flow-through.

3. Re-insert column into collection tube. Add 200 µl of DNA Wash Buffer and spin for 1 minute. Discarding flow-through is optional.

4. Repeat washing step (Step 3).

5. Transfer column to a clean 1.5 ml microcentrifuge tube.

6. Add 20 µl of DNA Elution Buffer to the center of the column. Wait for 1 minute, then spin for 1 minute to elute DNA.

The concentration and quality of the nucleic acids were measured using NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, U.S.A).

3.6 Library preparation for next-generation sequencing

The first step in the sequencing process is library preparation, in which the 16S rDNA fragment that was amplified is converted into a format compatible with the sequencing technology (Head et al., 2014). New England Biolabs (USA) Ion TorrentTM Nebnext® Fast DNA Library Prep Set was used for this research.

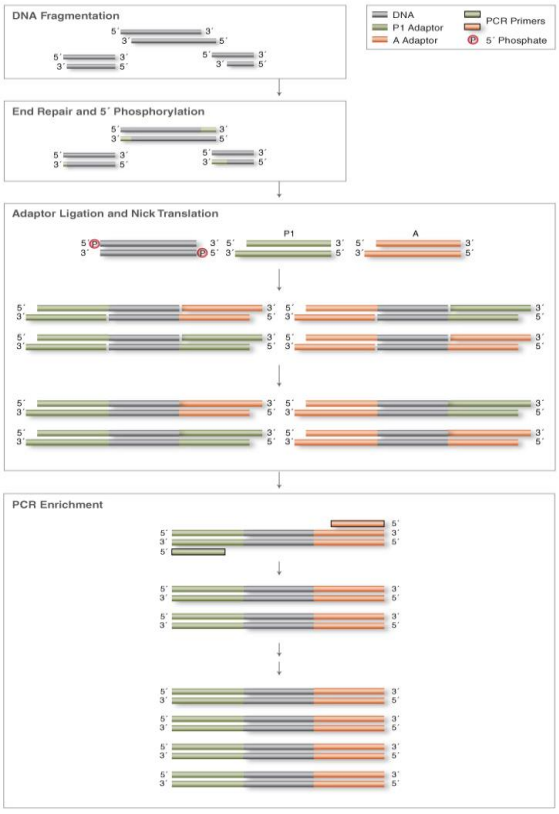

Figure 9: DNA Library Preparation Workflow for Ion Torrent

3.6.1 End repair

Because DNA fragmentation does not result in homogeneous, blunt-ended fragments, end repair is needed to ensure that each molecule is free of overhangs and contains 5′ phosphate and 3′ hydroxyl

groups. Libraries to be used in blunt-ended adaptor ligation, including Ion Torrent™ library construction, can be used directly in the ligation step.

The original reaction is performed in 60 µl, half volume was used.

Reagents used in the mixture with a total volume of 30 µl were the following: NEBNext End Repair Reaction Buffer (3 µl), NEBNext End Repair Enzyme Mix (1.5 µl), Free nuclease H2O (18.5 µl) and DNA amplicons (7 µl).

The following PCR conditions were used: 20 minutes at 25°C, followed by 10 minutes at 70°C, hold at 8°C using the thermocycler Biometra TAdvanced (Biometra, Germany) to complete the End repair reaction.

Figure 10: Biometra TAdvanced Thermocycler (Biometra, Germany).

3.6.2 Adaptor ligation

The DNA fragments undergo a process called blunt-end ligation, which attaches short adaptor sequences to both ends of the fragments. These adapters integrate fragment-specific sequences in order to facilitate the sequencer's identification process. Each sample was equipped with a P1 adaptor in addition to a variety of barcoded adaptors.

Reagents used in the mixture with a total volume of 19 µl were the following: T4 DNA Ligase Buffer for Ion Torrent (5 µl), P1 adaptor (0.5 µl), Bst 2.0 WarmStart DNA Polymerase (0.5 µl), T4 DNA Ligase 2.5 µl) and Free nuclease H_2O (10.5 µl).

The following PCR conditions were used: 15 minutes at 25 °C, 5 minutes at 65 °C and hold at 8°C, using the thermocycler Biometra TAdvanced (Biometra, Germany) to complete the End repair reaction (Figure 10).

3.6.3 Purification

The elimination of small fragments of DNA, such as primers, dimers and adaptors from the DNA library is the goal of DNA clean-up performed with magnetic beads.

Ampure beads AMPure XP (Beckman Coulter, USA) should be added to each well at a concentration of 1.2 x volume (for a 50 µl volume, add 60 µl) and pipetted up and down several times to combine (Figure 11(A)). It takes around 2 to 5 minutes of incubation at room temperature to bind the DNA to the beads. Then, the plate is placed on a magnet and left for 1–2 minutes until the liquid is clear (Figure 11 (B)) to later on remove and discard the supernatant. After that, the plate is wash twice with being kept attached to the magnet: 180 µl of 70% ethanol should be added, followed by a 1-minute before removing and discarding the ethanol. Ethanol should be removed as much as possible from the beads after the two washes (Figure 11 (C)). When the ethanol has completely evaporated (dry the beads 2 to 3 minutes), the plate is took off the magnetic surface and 50 µl of 10 mM Tris-HCl should be added with pipetting up and down. A 2-minute incubation period is required to elute the DNA from the beads. The DNA can be extracted from the beads by placing the plate again on a magnet. The liquid will clean up in a minute or two. Supernatant (DNA) should be transferred to a new plate.

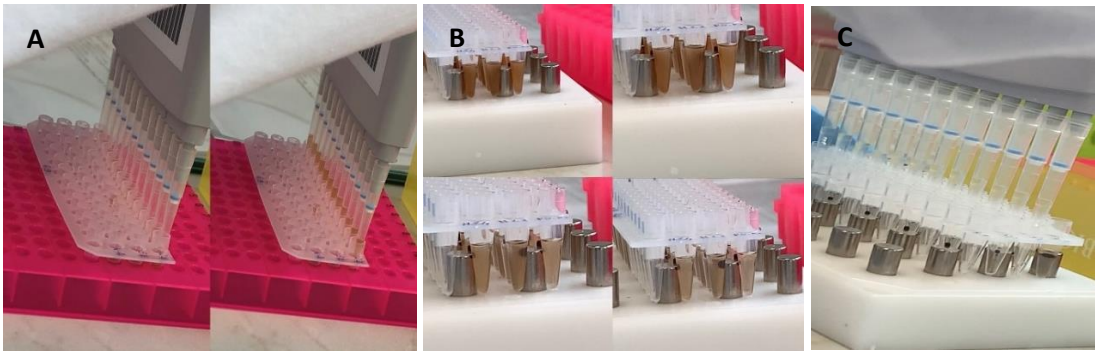

Figure 11 (A), (B), (C): Purification of DNA with Ampure beads.

3.6.4 PCR amplification

In order to obtain an appropriate library suitable for sequencing, amplification is carried out with a high-fidelity polymerase NEBNext® Ultra™ II Q5® Master Mix (New England Biolabs, USA) with a purpose of being able to increase the concentration of library and select molecules with the appropriate adaptors at both ends.

Reagents used in the mixture with a total volume of 27 µl were the following: NEBlibF primer (1 µl), NEBlibR primer (1 µl), NEBNext® Ultra™ II Q5® Master Mix (25 µl). This mixture was added into wells of a new 96 well plate, then 23 µl of the purified samples were added.

The following PCR conditions were used: 30 seconds at 98°C, 7 cycles consisting of 10 seconds at 98°C, 30 seconds at 58°C and 30 seconds at 72°C, then 5 minutes at 72°C and hold at 8°C using the thermocycler Biometra TAdvanced (Biometra, Germany) to complete the End repair reaction.

3.7 Quantification and quality control of NGS libraries

The quantification and quality control of libraries is important to ensure that the DNA is of the appropriate size and concentration, free of contaminants. The main purpose is to determine the final library concentration for careful loading onto the sequencer, avoiding over or under loading.

3.7.1 Dilution of libraries

In order to have the proper concentrations that fall within the assay's range, libraries must be diluted before quantification.

Dilution	DNA library (µl)	10 mM Tris-HCl (µl)
1:20	5	95
1:1000	2	98
1:4000	25	75

3.7.2 Quantification with qPCR

In order to improve the quality of NGS data, accurate library quantification is essential. The KAPA Library Quantification Kit (Roche, USA) was used in this study.

Below is a detailed description of the process:

A total of 6 µl of KAPA SYBR FAST qPCR Master Mix (2X) were used for each sample with 1 µl of Primer Premix (10X) and 0.2 µl of 50X ROX Low. Overall, 6.2 µl of the mixture was dispensed into each well of a 96-well plate, 4 µl of each dilution of libraries was added in the wells, 4 µl of nuclease free H2O was added to all negative control wells and finally 4 µl of each DNA Standard was added into the appropriate wells, from the most diluted (Standard 6) to the most concentrated (Standard 1).

The qPCR run was performed using the QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, USA) with the following cycling protocol:

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	5 minutes	1
Denaturation	95°C	30 seconds	35
Annealing/Extension/ Data acquisition	60°C	45 seconds	
Melt curve analysis	60-65-95°C		

For a consistent concentration, the standard curve should meet the following requirements:

Reaction efficiency	Between 90-110%
Slope of the standard curve	Between -3.1 and -3.6
R2	≥ 0.99

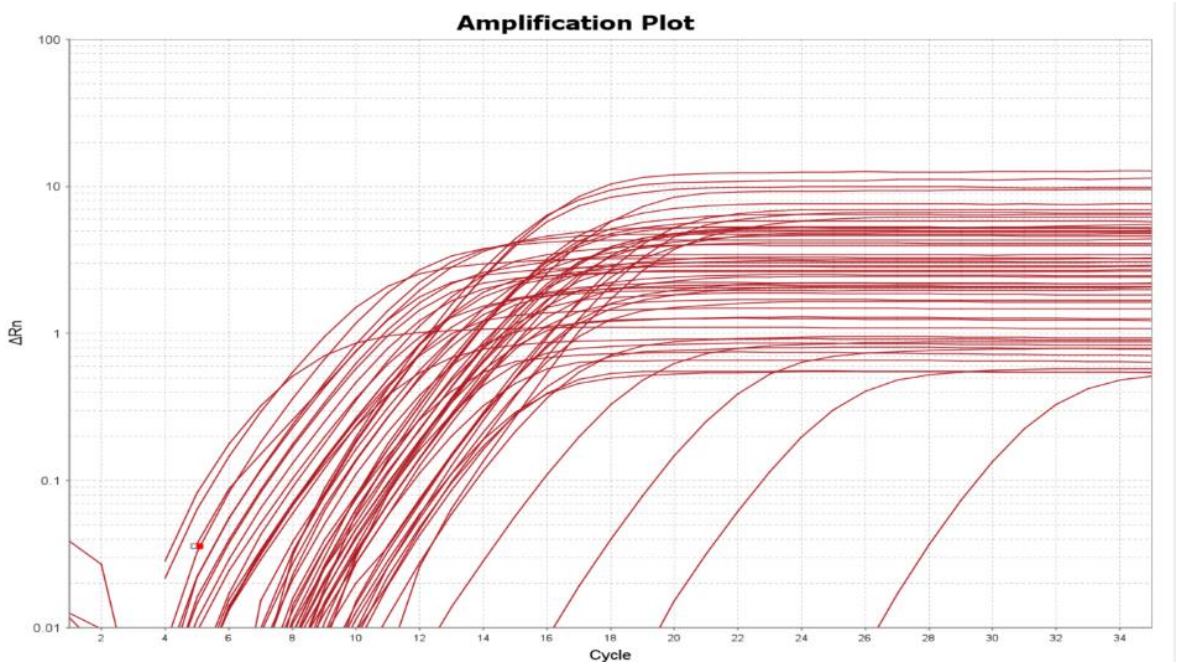

Figure 12: Amplification plot of qPCR showing the concentrations of the samples.

3.7.3 Quality control of libraries

Besides quantification, the quality control of the libraries is important for successful NGS results. Such analysis can detect impurities and determine the lengths of DNA fragments and the library size distribution.

DNA libraries were analysed using the 2100 Bioanalyzer instrument (Agilent, USA).

Agilent High Sensitivity DNA Kit components:

Agilent High Sensitivity DNA Chips	**Agilent High Sensitivity DNA Reagents**
10 High Sensitivity DNA Chips	High Sensitivity DNA Ladder
1 Electrode Cleaner	High Sensitivity DNA Markers 35/10380 bp (4 vials)
Syringe Kit	High Sensitivity DNA Dye Concentrate (1 vial)
1 Syringe	High Sensitivity DNA Gel Matrix (2 vials)
	2 Spin Filters

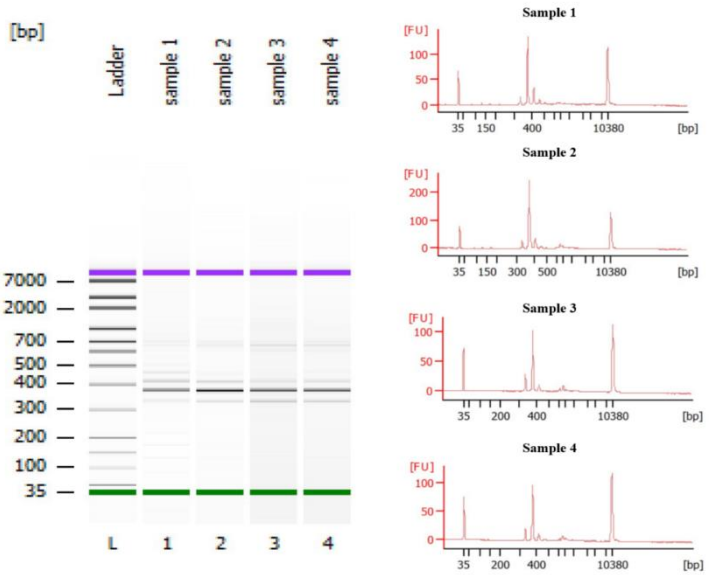

Figure 13. Electropherogram summary of Agilent 2100 Bioanalyzer for DNA samples

The following characteristics are essential for a successful High Sensitivity DNA sample run:

1. Sample peak appear between the lower and upper marker peaks.

2. Baseline is flat.

3. Baseline readings at least 5 fluorescence units.

4. Marker readings at least 3 fluorescence units higher than baseline readings.

3.7.4 Final dilution

The final dilution is performed to equalize all DNA library concentrations in order to guarantee an equal read distribution and appropriate read depth for all samples.

Following qPCR, the concentrations of each library were determined for each dilution. All libraries were diluted to 30 pM before being volumetrically pooled; 1 µl of each normalized library was dropped in a microcentrifuge tube. The content was softly mixed up and down.

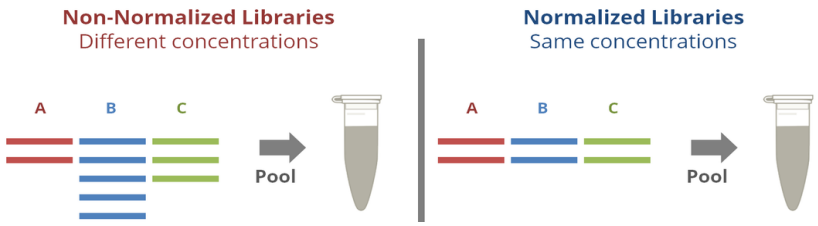

Figure 14: DNA library normalization

3.8 Template preparation

The primary goal is to create numerous copies of the same amplicon to ensure a strong detectable signal during sequencing.

Template amplification and enrichment was done using the Ion OneTouch™ 2 System (Thermo Fisher Scientific, USA), which comprises the Ion OneTouch™ 2 Instrument and the Ion OneTouch™ ES Instrument (Thermo Fisher Scientific, USA).

3.8.1 Clonal Amplification

In this process, DNA sequences are attached to Ion Sphere™ particles (ISPs) using an oil emulsion. Then PCR amplification occurs in order to produce clonal colonies for each DNA template

Figure 15: Clonal amplification - The Ion OneTouch™ 2 Instrument

1. Setting up of the instrument

A total of 150 µl of the Ion OneTouch™ Breaking Solution is dispensed into each of the two Ion OneTouch™ recovery tubes, the Ion OneTouch™ amplification plate is installed as well as the Ion OneTouch™ oil on the left front port and the Ion OneTouch™ recovery solution on the right front port. Amplification solution:

Ion PGM™ Hi-Q™ View Reagent	**Volume**
Ion PGM™ Hi-Q™ View Reagent Mix	800 µl
Nuclease-free Water	25 µl
Ion PGM™ Hi-Q™ View Enzyme Mix	50 µl
Diluted library (26pM)	25 µl
Ion PGM™ Hi-Q™ View ISPs	100 µl
Total	**1000 µl**

The entire volume of the amplification solution is added to the Ion OneTouch™ Reaction filter. Then, 850 µl of the Ion OneTouch™ Reaction Oil is added two times and the Reaction filter is installed. At the end of the run the sample is centrifuged, the supernatant is removed but only 100 µl of the solution is left. A total of 500 µl of the Ion OneTouch™ Wash Solution is added to each recovery tube and the suspension is combined into one new 1.5 ml Eppendorf tube. The ISPs is

centrifuged for 2.5 min at 15,500 × g and then supernatant is removed but only 100 µl of the solution is left and ready for enrichment.

3.8.2 Enrichment of the Template-Positive ISPs

After clonal amplification, the non-templated ISPs are removed from the templated ISPs. Magnetic beads are used to separate Ion Sphere™ particles that are template positive.

Figure 16: Clonal enrichment - The Ion OneTouch™ ES Instrument

- Solutions preparation

A melt-off solution is prepared using 280 µl of the Tween™ Solution and 40 µl of 1 M NaOH.

A total of 13 µl of Dynabeads™ MyOne™ Streptavidin C1 Beads is dispensed to an Eppendorf tube, which is placed on a magnet for 2 minutes. The supernatant is discarded than 130 µL of My One™ Beads Wash Solution is added.

- Flat Bottom 8-well strip preparation

Well number	Reagent to dispense in well
Well 1	100 µl Template positive ISPs
Well 2	130 µl Dynabeads™ MyOne™ Streptavidin C1 Beads+ My One™ Beads Wash Solution
Well 3	300 µl of Ion OneTouch™ Wash Solution
Well 4	300 µl of Ion OneTouch™ Wash Solution
Well 5	300 µl of Ion OneTouch™ Wash Solution
Well 6	Empty
Well 7	300 µl of freshly prepared Melt-Off Solution
Well 8	Empty

Figure 17: Flat Bottom 8-Well Strips

- Instrument preparation

A new tip is added on the tip arm of the instrument. A total of 10 µl of Neutralization Solution is added to a PCR tube, placed in the base of the tip loader. The eight-well strip is placed in right side of slot.

3.9 Sequencing

The Ion torrent PGM™ Sequencer (Thermo Fisher Scientific) was used for this step and the Ion 316™ Chip v2 BC was adopted.

Figure 18: (A) Ion 316™ Chip v2 BC, (B) Ion torrent PGM™ Sequencer.

3.9.1 Initialisation

Before initialisation, the Ion Torrent should be cleaned using 250 ml of 18 MΩ water. Then the wash bottles are prepared: Wash 1 (350 µl 100 mM NaOH), Wash 2 (2 liters of 18 MΩ water + entire bottle of W2 solution + 70 µl of NaOH) and Wash 3 (50 ml of W3 solution). A total of 20 µl of each dNTP is added to conical tubes and initialization begins.

3.9.2 Sample preparation and run

A total of 5 µl of Control ISPs is added to the template-positive ISPs and then centrifuged for 2 minutes at 15,500 × *g*. The supernatant is removed but only 15 µl of the solution is left, and 12 µl of Sequencing Primer are added to the ISPs. The microcentrifuge tube is placed in the thermal cycler: 95°C for 2 minutes and then 37°C for 2 minutes. Then 3 µl of Ion PGM™ Hi-Q™ View Sequencing Polymerase is added and the mixture is incubated at room temperature for 5 minutes.

Before the run, the old chip is replaced with a new one, and it is checked by the instrument. Then the entire sample (~30 µl) is loaded into the chip following the protocol. When the run is complete, a cleaning step should be done.

3.9.3 Sequencing run assessment

A run's quality is determined by several factors:

- The chip's loading: better results are obtained when the loading density is larger.
- The quality and quantity of the DNA libraries, with a high proportion of final libraries and a low proportion of polyclonal.
- The size distribution, which includes a tight distribution with a limited population of short fragments and a range of sequences that is adequate.

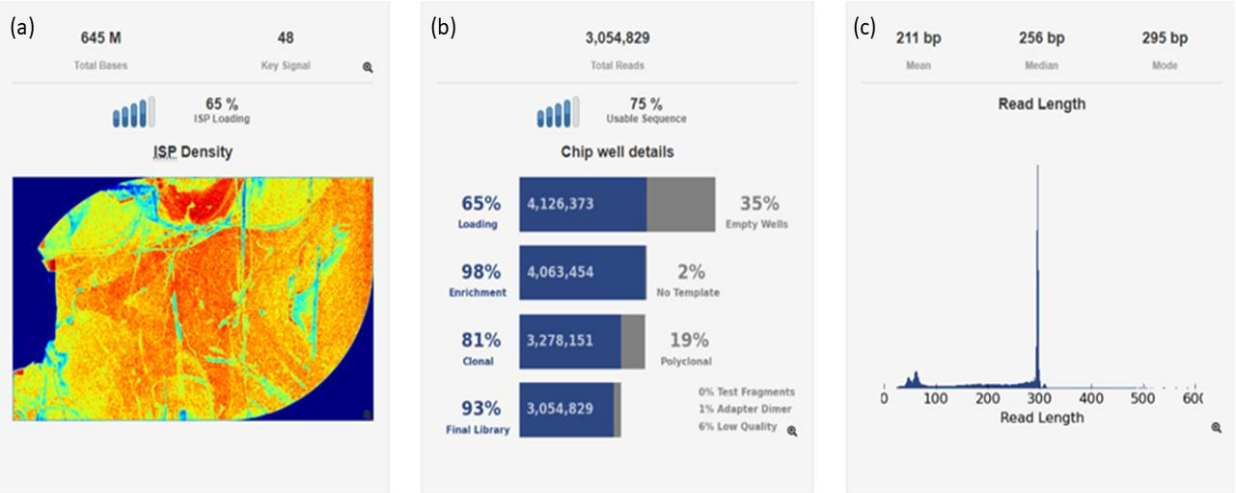

Figure 19: Sequencing results (a) Loading density on the Ion 316™ (b) Chip Quality and quantity of the templated library (c) Read length histogram.

3.10 Bioinformatics and Statistical Analysis

The statistical analysis, regarding the quails' growth and slaughter performances, was done using the IBM SPSS Statistics package (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA: IBM Corp.). The Shapiro–Wilk test and the Levene test were performed to assess the normality and the homogeneity of variances and data distribution, respectively. The data collected from animals of the different groups were analyzed using the GLM procedure (General Linear Model) and multiple comparisons using Tukey's HSD test, when variances among groups were homogeneous, and Games-Howell test, when variances were not homogeneous were done accordingly. Significance was declared at $p < 0.05$ and the results were expressed as mean and pooled standard error of the mean (SEM).

Regarding high throughput sequencing (HTS) bioinformatics, the Ion Torrent Software filtered the raw sequencing reads in order to remove low quality and polyclonal sequences. Bacterial 16S rRNA gene sequences were saved in FASTQ format for the analysis with QIIME 2 pipelines (Bolyen et al., 2019). DADA2 was used for sequence quality as a filter and for the removal of chimeric sequences (Callahan

et al., 2016). The sequences were then clustered into Amplicon Sequence Variants (ASVs) using VSEARCH and the taxonomy classification was performed using the SILVA database (version 132) with 97% threshold (Rognes et al., 2016). The bacterial diversity was assessed through alpha diversity indices such as Chao1, Observed Species, Faith Phylogenetic Diversity, Pielou Evenness and Shannon index. Beta diversity was assessed using Bray Curtis distance matrix, Jaccard distance matrix and both weighted and unweighted UniFrac distance matrix. Principal coordinate analysis (PCoA) was used for the visualization of the community associations and the results were plotted using EMPeror (Vázquez-Baeza et al., 2013). Linear discriminant analysis (LDA) with an effect size (LefSe) algorithm (Segata et al., 2011) was performed in the Galaxy Web module (http://huttenhower.sph.harvard.edu/galaxy/ (accessed on 15 August 2022) for biomarker identification.

The non-parametric Kruskal-Wallis H test was used to compare alpha diversities. Beta diversities were examined using 999 permutations of the permutational multivariate analysis of variance PERMANOVA. In addition, the PERMDISP test was performed to assess the homogeneity of dispersions across animal groups. The factorial Kruskal-Wallis and pairwise Wilcoxon tests were used to identify the taxa with significant differences in abundance among the groups. The alpha value was 0.05, with a threshold value of 2.0 for the logarithmic LDA scores of discriminative features.

4. RESULTS

4.1 Growth performance

No statistical significance (P > 0.05) differences between treatments were recorded in the case of live weight (LW), average weekly gain (AWG), feed intake (FI), and feed convertion ratio (FCR) during the different rearing periods of the experiment.

Table 7: Effects of corn oil replacement with *Hermetia illucens* larvae fat (BSFO) on the growth performance parameters of broiler quails.

	Diets				p-value	SEM
	Control	BSFO 25	BSFO 50	BSFO 100		
LW, g						
7	36.05	35.83	35.44	36.7	0.668	0.36
14	55.372	54.94	55.25	55.12	0.975	0.34
21	106.27	105.46	104.99	106.02	0.955	0.86
28	155.54	155.62	154.93	157.07	0.966	1.49
35	193.8	195.18	194.29	195.84	0.977	1.71
42	220.48	223.9	221.42	223.34	0.964	2.6
AWG, g						
7-14	19.32	19.11	19.81	18.42	0.692	0.41
15-21	50.9	50.53	49.74	50.9	0.960	0.86
22-28	49.27	50.15	49.93	51.04	0.954	1.09
29-35	38.26	39.56	39.36	38.77	0.978	1.14
36-42	26.67	28.72	27.14	27.51	0.960	1.37
FI, g						
7-14	55.88	55.8	55.9	55.8	0.998	0.23
15-21	113.85	113.14	113.24	113.24	0.519	0.18
22-28	239.61	240.7	240.89	240.94	0.067	0.21

29-35	372.13	377.72	377.24	378.34	0.148	1.08
36-42	542.88	544.37	545.99	545.14	0.540	0.74
FCR, g/g						
7-14	1.01	1.02	1.02	1.01	0.950	0.01
15-21	1.08	1.08	1.07	1.07	0.905	0.01
22-28	1.56	1.55	1.56	1.5	0.990	0.01
29-35	1.94	1.95	1.95	1.96	0.984	0.02
36-42	2.55	2.47	2.48	2.47	0.743	0.03

BSFO: Black soldier fly oil; SEM, standard error of the mean; LW, live weight; AWG, average weekly gain; FI, feed intake; FCR, feed conversion ratio; (n = 25/group).

4.2 Slaughter performance

No significant differences (P > 0.05) were noticed in the case of the organs' lengths or weights selected.

Table 8: Effects of corn oil replacement with *Hermetia illucens* larvae fat (BSFO) on viscera, carcass and legs weights (g) and length of the gastrointestinal segments (cm) of broiler quails (42 days).

	Diets				p-value	SEM
	Control	BSFO 25	BSFO 50	BSFO 100		
Weight (g)						
Carcass	159.74	167.05	167.28	161.34	0.383	1.91
Viscera	31.6	32.59	31.5	32.28	0.935	0.69
Heart	2.15	2.22	2.13	2.12	0.540	0.03
Liver	3.65	3.83	3.69	3.77	0.910	0.09
Spleen	0.12	0.15	0.13	0.14	0.150	0.004
Legs	4.56	4.54	4.65	4.33	0.108	0.05
Length (cm)						
Duodenum	13.38	13.1	12.6	12.74	0.181	0.12
Jejunum	20.64	21.28	21.56	22.56	0.130	0.29
Ileum	19.58	20.8	20.78	18.92	0.069	0.30
Caecum	8.12	8.38	8.52	8.14	0.215	0.08

Colorectum with Cloaca	7.06	7.7	7.12	7.52	0.217	0.13

BSFO: Black soldier fly oil; SEM, standard error of the mean; (n = 25/group).

4.3 Sequencing Analysis

A total of 40 samples of caecal content were subjected to sequence analysis. Bacterial 16S rRNA gene V4-V5 region sequencing generated a total of 1,368,982 reads of high-throughput sequencing. After passing through quality filtering (HTS), 669,753 reads were left. The sampling depth was rarefied to 5000 reads per sample. The rarefactions curves reached a plateau, indicating that the sequencing depth was enough to determine the caecal core bacterial population for all groups (Figure 20). The sequencing length of all libraries was 225 bp. The total number of identified amplicon sequence variants (ASVs) was 837. The sequencing analysis details are recapped in the table 9.

Table 9: Sequencing analysis summary

Number of samples	40
Number of reads	1,368,982
Number of reads passed filtring	669,753
Rarefaction depth	5000
Number of ASVs	837
Total frequency	629,775
Sequencing length (bp)	225

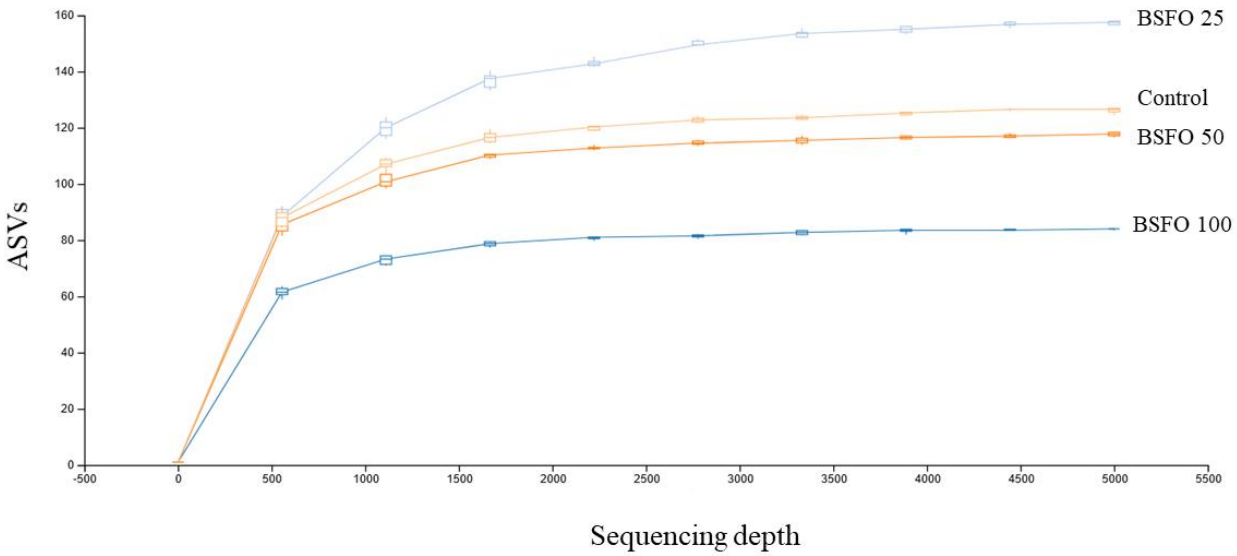

Figure 20: Rarefaction curves representing the sequencing depth (number of reads) and the number of ASVs (sequence variants) found in the caecal content of quails.

4.4 Alpha Diversity

Alpha diversity describes the species richness and eveness in a certain ecosystem using several indices (Thukral, 2017).

In this study, the bacterial diversity of quails' ceca was determined using the Shannon entropy, Chao1 index, Pielou Evenness index, Faith Phylogenetic Diversity and the Amplicon Sequence Variants (ASVs). Shannon entropy estimates the richness and evenness based on the abundance of species (Shannon, 1948). Chao1 estimates the overall richness relative abundances (Chao & Chiu, 2016). Pielou Evenness index indicates how equally abundant the species are in each sample, revealing whether one species dominates the samples or whether the abundances of all the species have equal abundance (Pielou, 1966). Faith Phylogenetic Diversity estimates the feature diversity, relying on the phylogenetic tree for species (Faith, 1992).

Alpha diversity metrics were calculated in each sample and mean values are listed in the table 10.

The analysis revealed that diversity indices were lower in the BSFO 100 group, indicating a reduced diversity richness and evenness in the samples of quails fed with a total inclusion of *H. illucens* fat. Mainly, the Shannon entropy revealed a significant difference in richness between the control group and the BSFO 100 group ($p = 0.03$), also between the BSFO 25 group and the BSFO 100 group ($p = 0.03$). Similarly, Pielou evenness index showed a significant difference between the control group and the BSFO 100 group ($p = 0.02$), also between the BSFO 50 group and BSFO 100 group ($p = 0.04$). However, chao1 index and faith phylogenic diversity, showed no significant differences between the groups. The results of the alpha diversity assessment are shown in the table 11. Alpha diversity boxplots showing the different indices are illustrated in figures 21-25.

Table 10: Summary of alpha diversity indices of bacterial community in ceca of quails (means ± SD).

Index	**Control**	**BSFO 25**	**BSFO 50**	**BSFO 100**
Shannon Entropy	5 ± 1.2	4.8 ± 0.8	4.8 ± 0.8	4.1 ± 0.6
Pielou Evenness	0.7 ± 0.1	0.7 ± 0.09	0.7 ± 0.08	0.6 ± 0.06
Faith pd	11.6 ± 2	11.4 ± 2.2	10.9 ± 1.3	10 ± 1.8
Chao1	133.4 ± 53	145.5 ± 68.1	111.1 ± 32.4	93.6 ± 37.5
ASVs	131.6 ± 51.3	140.3 ± 62.5	110.8 ± 32	93 ± 37

Table 11: Effects of diet on diversity indices of bacterial community in ceca of quails (p-values).

		Shannon Entropy	Pielou Evenness	Faith pd	Chao1	ASVs
Diet		**p-value**	**p-value**	**p-value**	**p-value**	**p-value**
Control	BSFO 25	0.6	0.4	0.8	0.7	0.8
Control	BSFO 50	0.45	0.5	0.3	0.4	0.4
Control	BSFO 100	0.03*	0.02*	0.1	0.07	0.05
BSFO 25	BSFO 50	0.9	0.6	0.5	0.3	0.3
BSFO 25	BSFO 100	0.03*	0.1	0.2	0.1	0.1
BSFO 50	BSFO 100	0.05	0.04*	0.2	0.2	0.2

ASV1, Amplicon sequence variant.

* Significant difference ($p < 0.05$)

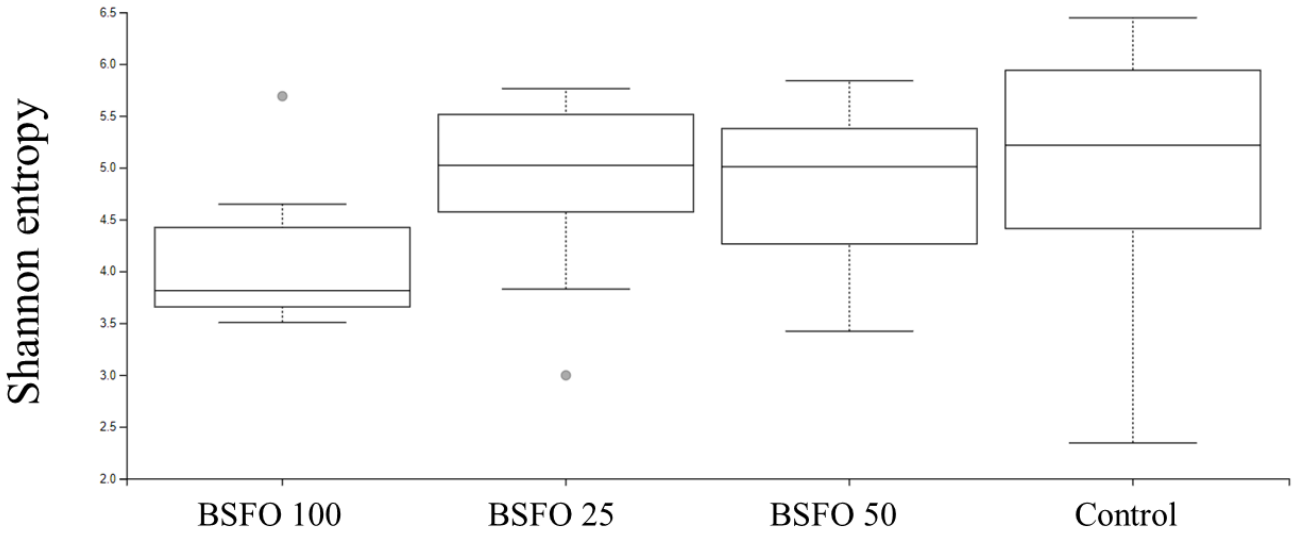

Figure 21: Comparison of alpha-diversity indices in ceca of quails. Shannon entropy index.

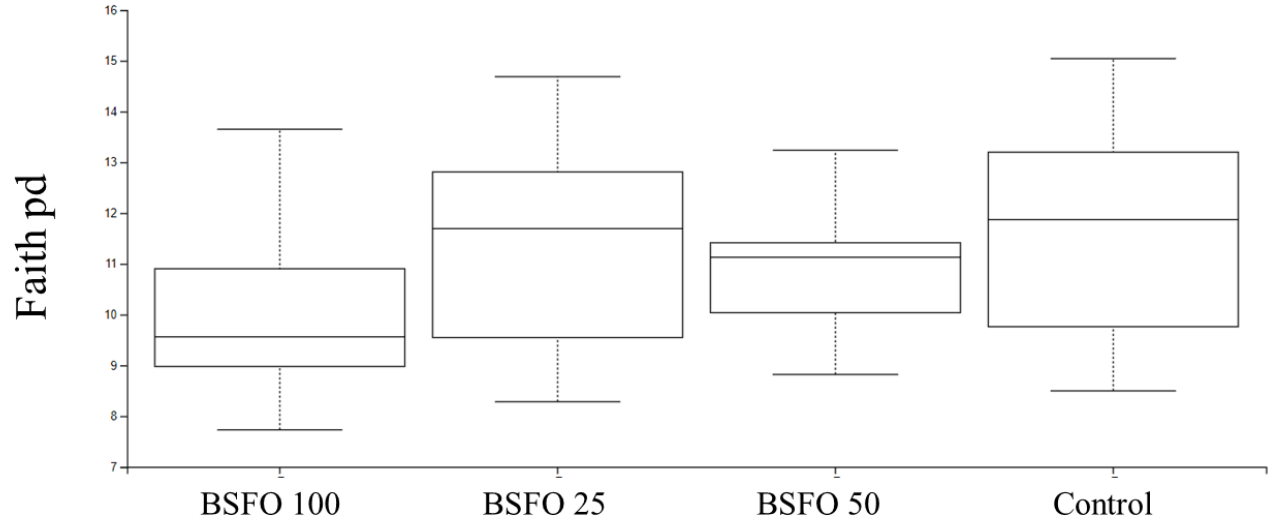

Figure 22: Comparison of alpha-diversity indices in ceca of quails. Faith Phylogenetic Diversity.

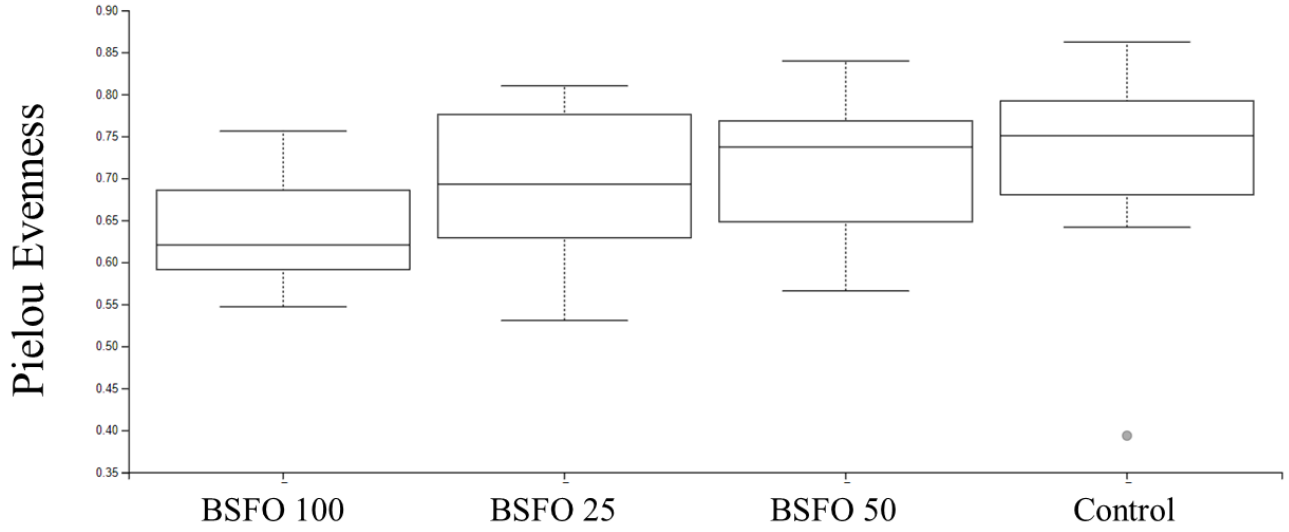

Figure 23: Comparison of alpha-diversity indices in ceca of quails. Pielou Evenness index.

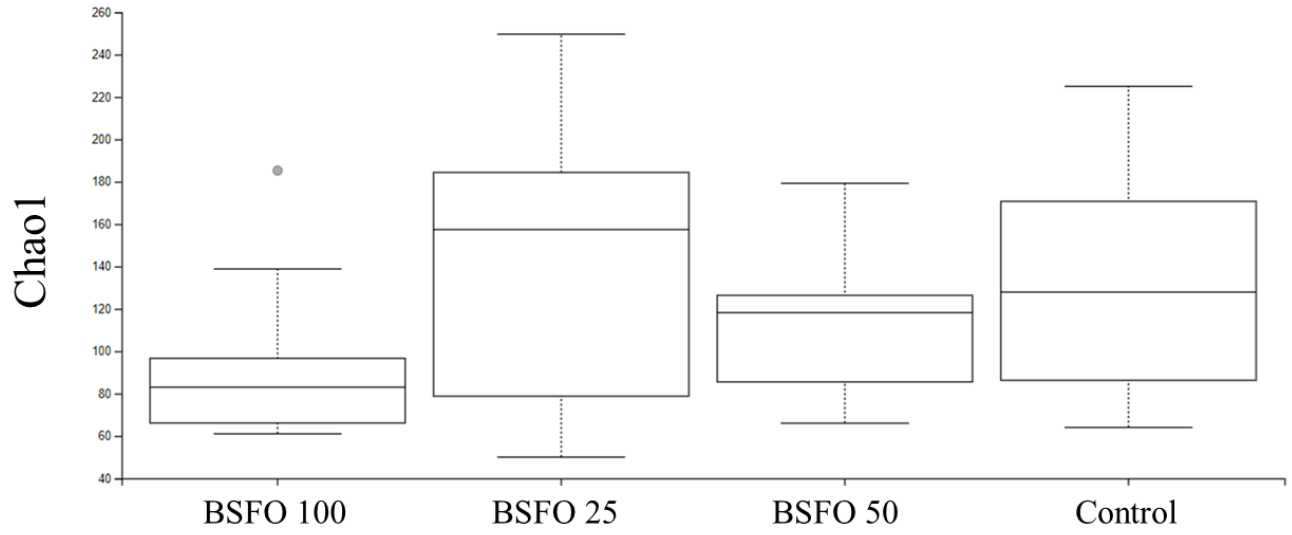

Figure 24: Comparison of alpha-diversity indices in ceca of quails. Chao1 index.

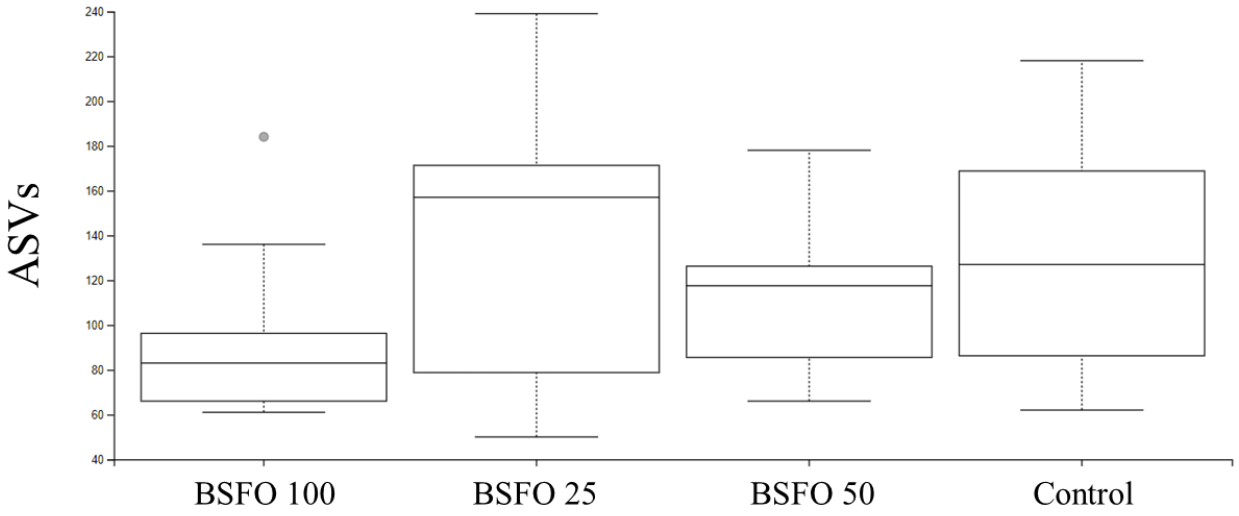

Figure 25: Comparison of alpha-diversity indices in ceca of quails. Observed ASVs.

4.5 Beta diversity

Beta diversity assesses the change in diversity of species from one ecosystem to another using several algorithms. Bray Curtis distance matrix is used to calculate the compositional dissimilarities between two different sites, based on counts at each site. Jaccard distance matrix is used to calculate the shared and distincts microorganisms between two different groups. UniFrac distance matrices are used to calculate the dissimilarities between populations while incorporating the phylogenetic distances information.

In the results, Beta diversity was assessed by Bray Curtis distance matrix, Jaccard distance matrix and both weighted and unweighted UniFrac distance matrix. Results are illustrated by PCoA plots. Permutational multivariate analysis of variance (PERMANOVA) based on the same similarity matrix was done to verify whether there is a significant difference in the distance between the groups of animals. In addition, permutational analysis of multivariate dispersions (PERMDISP) was done to test the homogeneity of dispersions between the groups.

PCoA plots based on Bray Curtis distance matrix (figure 26), Jaccard distance matrix (figure 27) and both weighted and unweighted UniFrac distance matrix (figure 28 and 29, respectively) showed the spatial separation of the samples. Even if the four group clusters are not very well separated, statistical analysis revealed significant differences between some groups, p-values are listed in tables 12-15.

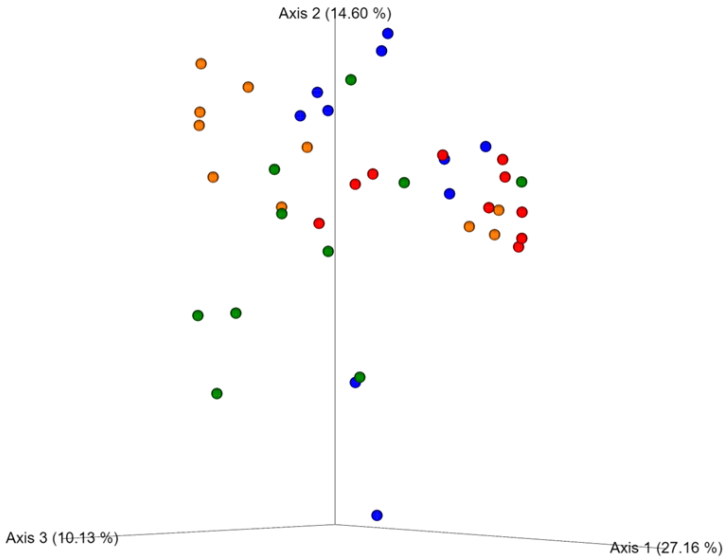

Figure 26: Principal Coordinate Analysis (PCoA) of bacterial communities in ceca of quails. The PCoA plot was constructed using Bray Curtis method.

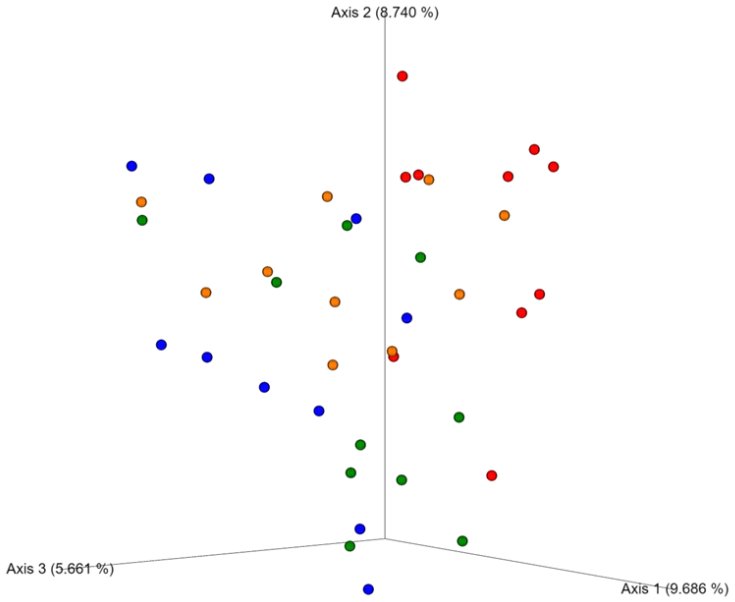

Figure 27: Principal Coordinate Analysis (PCoA) of bacterial communities in ceca of quails. The PCoA plot was constructed using Jaccard distance method

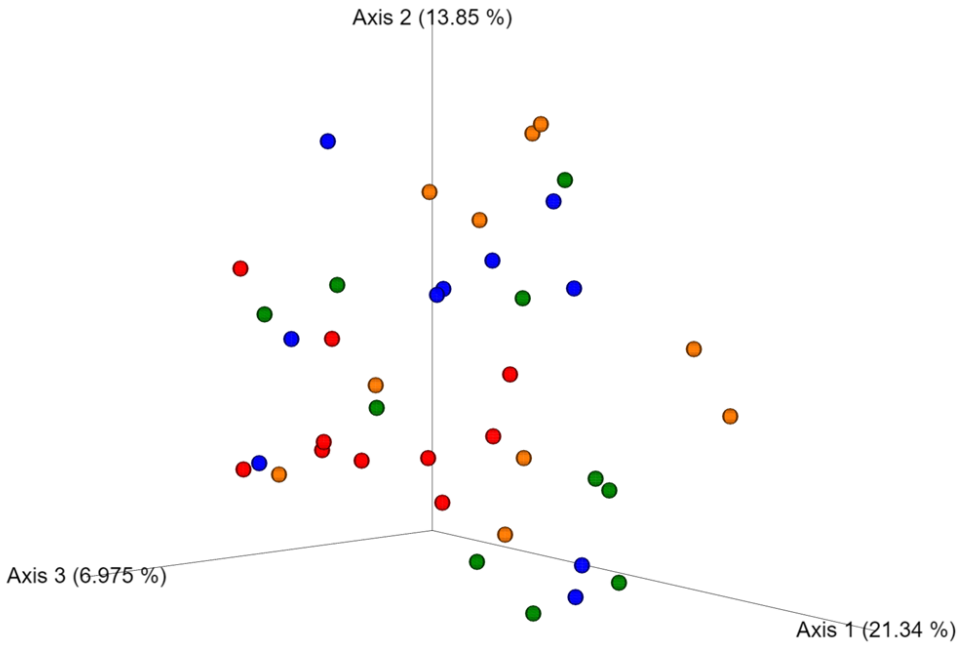

Figure 28: Principal Coordinate Analysis (PCoA) of bacterial communities in ceca of quails. The PCoA plot was constructed using unweighted UniFrac distance matrix.

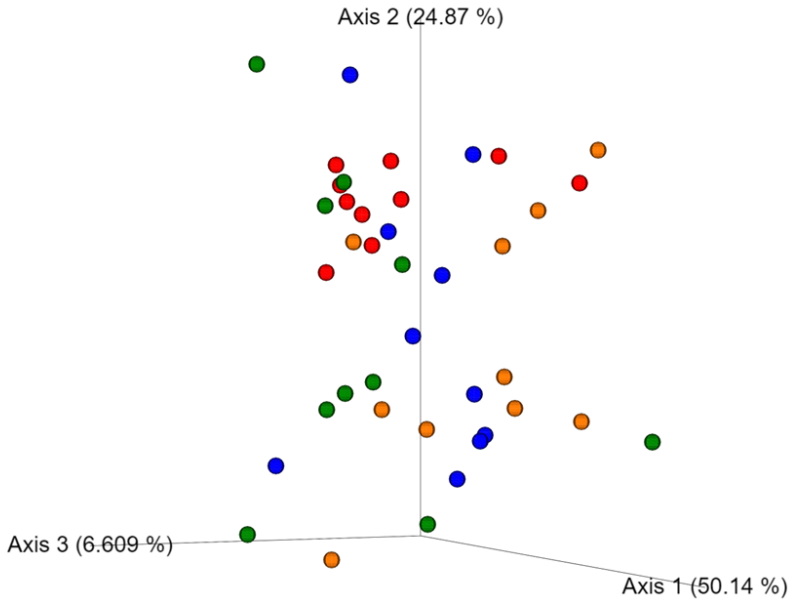

Figure 29: Principal Coordinate Analysis (PCoA) of bacterial communities in ceca of quails. The PCoA plot was constructed using weighted UniFrac distance matrix.

Table 12: Permutational multivariate analysis of variance (PERMANOVA) and dispersions (PERMDISP) based on Bray Curtis distance matrix.

		Bray Curtis distance matrix	
		PERMANOVA p-value	**PERMDISP p-value**
Control	BSFO 25	0.355	0.177
Control	BSFO 50	0.089	0.053
Control	BSFO 100	0.002*	0.005*
BSFO 25	BSFO 50	0.142	0.437
BSFO 25	BSFO 100	0.003*	0.017*
BSFO 50	BSFO 100	0.009*	0.039*

* Significant difference ($p < 0.05$)

Table 13: Permutational multivariate analysis of variance (PERMANOVA) and dispersions (PERMDISP) based on Jaccard distance matrix.

		Jaccard distance matrix	
		PERMANOVA p-value	**PERMDISP p-value**
Control	BSFO 25	0.092	0.699
Control	BSFO 50	0.003*	0.068
Control	BSFO 100	0.004*	0.413
BSFO 25	BSFO 50	0.002*	0.176
BSFO 25	BSFO 100	0.001*	0.736
BSFO 50	BSFO 100	0.004*	0.261

* Significant difference ($p < 0.05$)

Table 14: Permutational multivariate analysis of variance (PERMANOVA) and dispersions (PERMDISP) based on Weighted Unifrac distance matrix.

		Weighted Unifrac distance matrix	
		PERMANOVA p-value	**PERMDISP p-value**
Control	BSFO 25	0.201	0.396
Control	BSFO 50	0.006*	0.043*
Control	BSFO 100	0.043*	0.159
BSFO 25	BSFO 50	0.156	0.454
BSFO 25	BSFO 100	0.106	0.506
BSFO 50	BSFO 100	0.011*	0.812

* Significant difference ($p < 0.05$)

Table 15: Permutational multivariate analysis of variance (PERMANOVA) and dispersions (PERMDISP) based on Unweighted Unifrac distance matrix

		Unweighted Unifrac	
		PERMANOVA p-value	PERMDISP p-value
Control	BSFO 25	0.234	0.534
Control	BSFO 50	0.119	0.044*
Control	BSFO 100	0.053	0.849
BSFO 25	BSFO 50	0.185	0.383
BSFO 25	BSFO 100	0.028*	0.510
BSFO 50	BSFO 100	0.022*	0.054

* Significant difference ($p < 0.05$)

4.6 Taxonomical composition

A total of 5 phyla, 8 classes, 13 orders, 24 families and 76 genera were detected in the samples. Some of the sequences were unassigned, they accounted for 10.9 ± 6.2% (mean ± standard deviation) in the control group, 9.7 ± 4.7% in the BSFO 25 group, 9.3 ± 3.9% in the BSFO 50 group and 7.5 ± 2.7% in the BSFO 100 group. Firmicutes were detected the dominant phylum in the four groups (87.3 ± 6.3% C – 89.1 ± 5% BSFO 25 – 89.4 ± 4.1% BSFO 50 – 91.5 ± 2.8% BSFO 100) followed by a minor population of Actinobacteria, Tenericutes, Proteobacteria and Bacteroidetes as shown in the figure 30.

The Firmicutes phylum was mainly dominated by the class Clostridia and a low abundance of Bacilli followed by a minor population of Coriobacteriia, Mollicutes, Erysipelotrichia, Gammaproteobacteria, Bacteroidia and Actinobacteria as shown in the figure 31.

On the order level, Clostridia was mainly dominated by the order Clostridiales and Bacilli were represented by the order Lactobacillales, rare orders were also present and shown in figure 32.

On the family level, Clostridiales were represented by *Ruminococcaceae* and *Lachnospiraceae*, while Lactobacillales were represented by *Streptococcaceae* and *Lactobacillaceae*, as shown in figure 33. Bacterial taxa with relative abundance lower than 1% are summarized as “Others”.

At genus level, the most abundant genera were *Subdoligranulum*, *Unclassified genus within family Lachnospiraceae*, *Blautia*, *CHKCI001* and *Streptococcus,* they represented together more than half of the sequences in the four groups of quails. Less abundant genera with a meaningful relative abundance (>1%) were *unclassified genus within family Lachnospiraceae, Eubacterium* hallii group*, Ruminococcus* torques group*, Ruminococcaceae* UCG-014*, Lactobacillus, Butyricicoccus, Sellimonas, unclassified genus within family Ruminococcaceae, Ruminococcaceae* UCG-005, as shown in the figure 34. Bacterial taxa with relative abundance lower than 1% are summarized as “Others”.

The relative abundances (means ± SD) of bacterial taxa for all levels are listed in table 16.

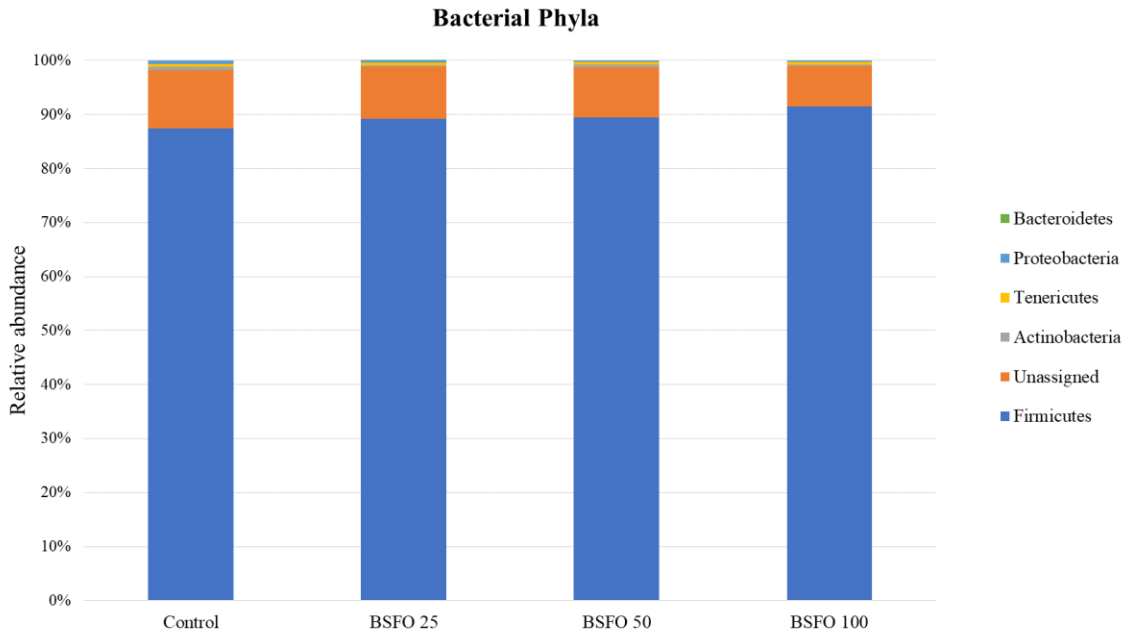

Figure 30: Taxonomical level comparison of the caecal bacteria of quails. The relative abundance is illustrated at phylum level.

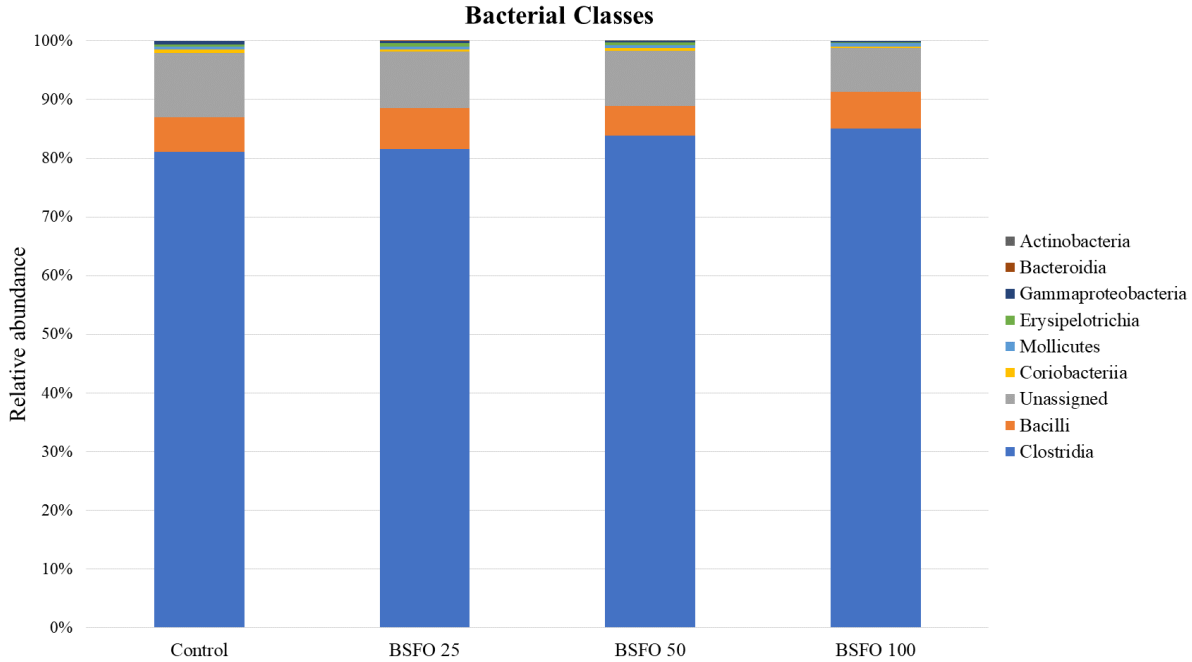

Figure 31: Taxonomical level comparison of the caecal bacteria of quails. The relative abundance is illustrated at class level.

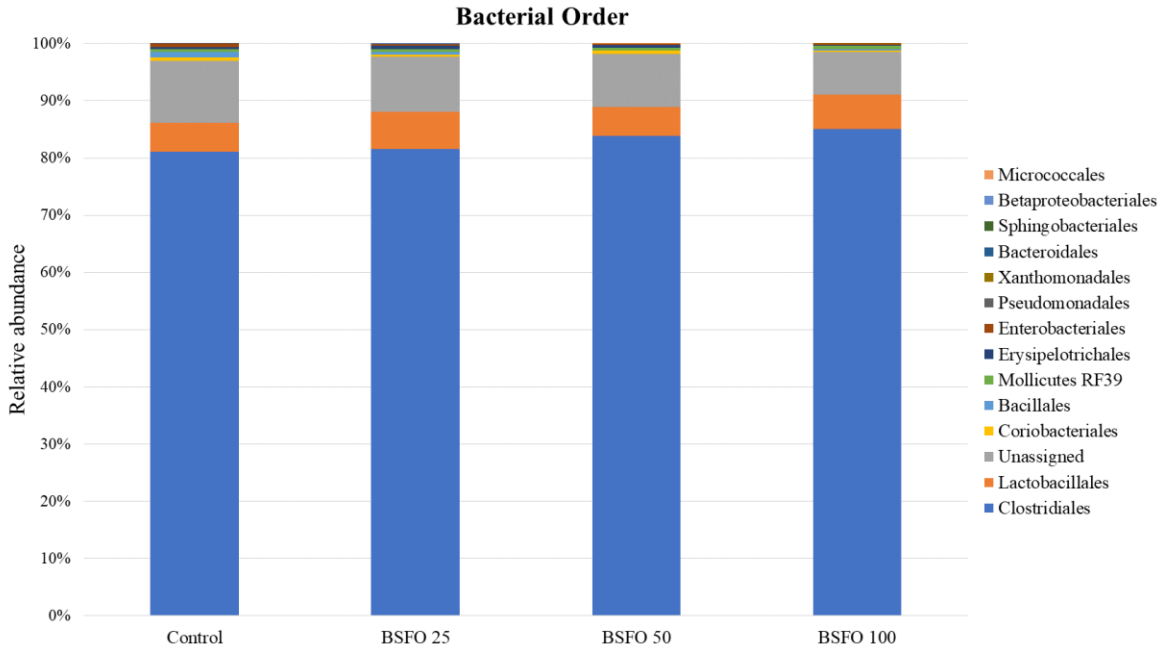

Figure 32: Taxonomical level comparison of the caecal bacteria of quails. The relative abundance is illustrated at order level.

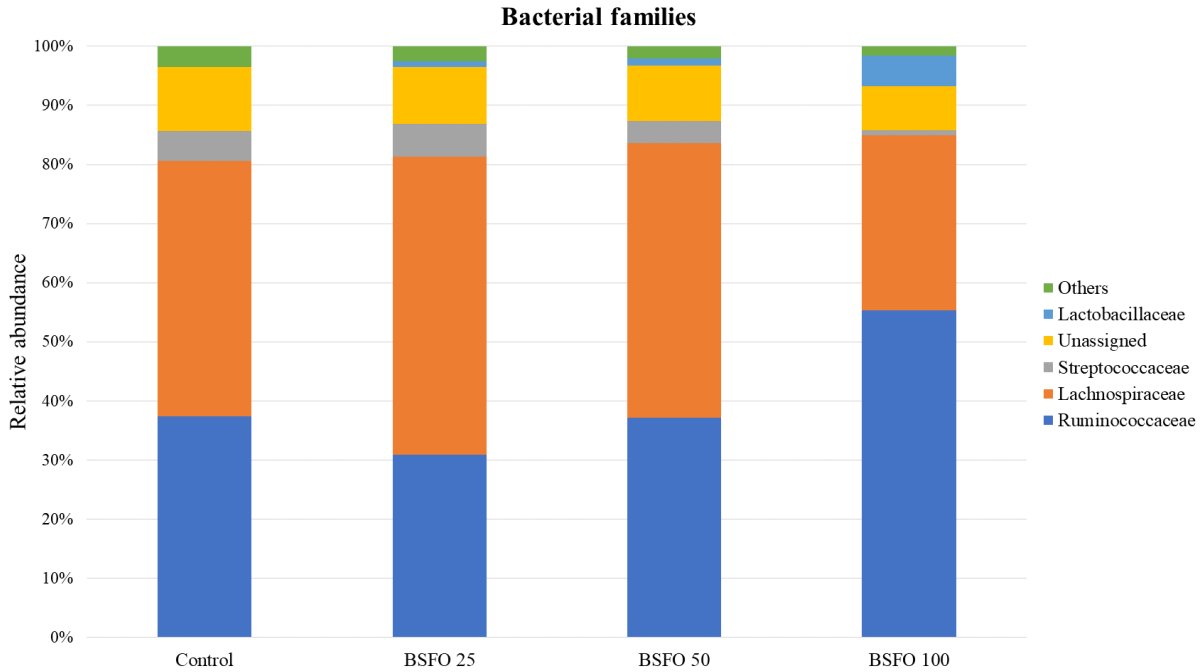

Figure 33: Taxonomical level comparison of the caecal bacteria of quails. The relative abundance is illustrated at family level. The taxa with a relative abundance lower than 1% are summarized as “Others”.

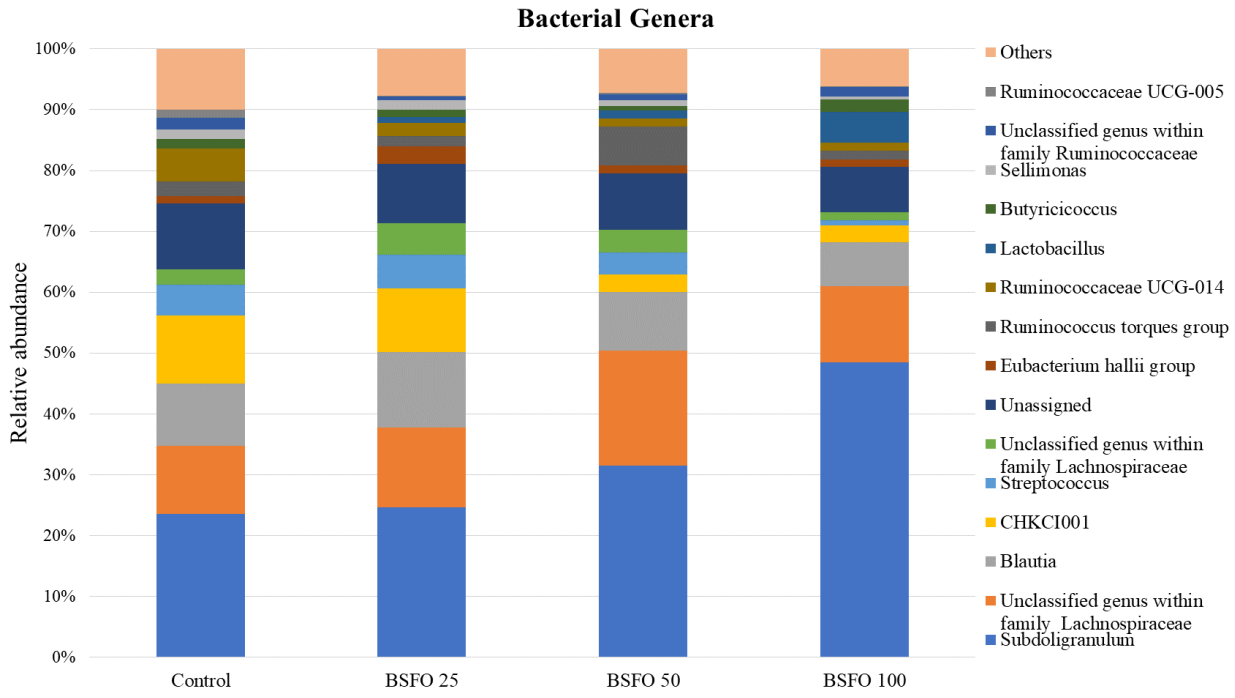

Figure 34: Taxonomical level comparison of the caecal bacteria of quails. The relative abundance is illustrated at genus level. The taxa with a relative abundance lower than 1% are summarized as "Others".

Table 16: Relative abundance (means ± SD) of bacterial taxa at phylum, class, order, family and genus levels.

Taxonomic levels	**Control**	**BSFO 25**	**BSFO 50**	**BSFO 100**
Phylum				
Firmicutes	87.3 ± 6.3%	89.1 ± 5.0%	89.4 ± 4.1%	91.5 ± 2.8%
Actinobacteria	0.6 ± 0.3%	0.3 ± 0.3%	0.5 ± 0.2%	0.2 ± 0.2%
Tenericutes	0.5 ± 0.5%	0.4 ± 0.3%	0.5 ± 0.5%	0.5 ± 0.6%
Proteobacteria	0.6 ± 0.8%	0.4 ± 0.4%	0.2 ± 0.5%	0.2 ± 0.3%
Bacteroidetes	0	0.004 ± 0.004%	0	0
Class				
Clostridia	81.1 ± 11.1%	81.6 ± 5.6%	83.9 ± 8.1%	85.1 ± 9.3%
Bacilli	5.9 ± 9.0%	7 ± 8.0%	5 ± 7.5%	6.2 ± 8.6%

Coriobacteriia	0.6 ± 0.3%	0.3 ± 0.3%	0.5 ± 0.2%	0.2 ± 0.2%
Mollicutes	0.5 ± 0.6%	0.4 ± 0.3%	0.5 ± 0.5%	0.5 ± 0.7%
Erysipelotrichia	0.4 ± 0.2%	0.6 ± 0.3%	0.5 ± 0.3%	0.2 ± 0.2%
Gammaproteobacteria	0.6 ± 0.8%	0.4 ± 0.4%	0.2 ± 0.5%	0.2 ± 0.3%
Bacteroidia	0	0.004 ± 0.004%	0	0
Actinobacteria	0	0	0.002 ± 0.01%	0
Order				
Clostridiales	81.1 ± 11.1%	81.6 ± 5.6%	83.9 ±8.1%	85.1 ± 9.3%
Lactobacillales	5 ± 9.3%	6.5 ± 8.2%	4.9 ± 7.4%	6 ± 8.4%
Coriobacteriales	0.6 ± 0.3%	0.3 ± 0.3%	0.5 ± 0.2%	0.2 ± 0.2%
Bacillales	0.9 ± 0.9%	0.5 ± 0.6%	0.06 ± 0.13%	0.2 ± 0.3%
Mollicutes RF39	0.5 ± 0.6%	0.4 ± 0.3%	0.5 ± 0.5%	0.5 ± 0.7%
Erysipelotrichales	0.4 ± 0.2%	0.6 ± 0.3%	0.5 ± 0.3%	0.2 ± 0.2%
Enterobacteriales	0.6 ± 0.8%	0.3 ± 0.4%	0.2 ± 0.5%	0.2 ± 0.3%
Pseudomonadales	0.003 ± 0.012%	0.1 ± 0.1%	0.02 ± 0.07%	0
Xanthomonadales	0	0.01 ± 0.01%	0.002 ± 0.007%	0.002 ± 0.009%
Bacteroidales	0	0.003 ± 0.003%	0	0
Sphingobacteriales	0	0.001 ± 0.001%	0	0
Betaproteobacteriales	0	0.001 ± 0.001%	0	0
Micrococcales	0	0	0.003 ± 0.006%	0
Family				
Ruminococcaceae	37.4 ± 21.6%	30.9 ± 16.9%	37.1 ± 15.3%	55.3 ± 10.6%
Lachnospiraceae	43.3 ± 17.3%	50.4 ± 20.9%	46.5 ±18.4%	29.6 ± 8.2%
Streptococcaceae	5 ± 9.3%	5.5 ± 6.6%	3.7 ± 7.1%	0.8 ± 1.3%
Lactobacillaceae	0.007 ± 0.03%	1 ± 1.8%	1.2 ± 4.3%	5.2 ± 8.6%
Eggerthellaceae	0.6 ± 0.3%	0.3 ± 0.3%	0.5 ± 0.2%	0.2 ± 0.2%
Bacillaceae	0.9 ± 0.9%	0.5 ± 0.6%	0.06 ± 0.1%	0.2 ± 0.3%
Unclassified family within order Mollicutes RF39	0.5 ± 0.5%	0.4 ± 0.3%	0.5 ± 0.5%	0.5 ± 0.7%

Erysipelotrichaceae	0.4 ± 0.2%	0.6 ± 0.3%	0.5 ± 0.3%	0.2 ± 0.2%
Enterobacteriaceae	0.6 ± 0.8%	0.3 ± 0.4%	0.2 ± 0.5%	0.2 ± 0.3%
Christensenellaceae	0.3 ± 0.3%	0.2 ± 0.2%	0.1 ± 0.1%	0.1 ± 0.2%
Eubacteriaceae	0.04 ± 0.05%	0.04 ± 0.03%	0.02 ± 0.03%	0.01 ± 0.02%
Moraxellaceae	0.003 ± 0.012%	0.1 ± 0.1%	0.02 ± 0.07%	0
Unclassified family within order Clostridiales	0.02 ± 0.04%	0	0.1 ± 0.2%	0
Family XIII	0.06 ± 0.06%	0.02 ± 0.02%	0.03 ± 0.04%	0.03 ± 0.05%
Defluviitaleaceae	0.03 ± 0.04%	0.007 ± 0.015%	0.01 ± 0.01%	0.01 ± 0.02%
Unclassified family within order Mollicutes RF39	0.03 ± 0.04%	0.01 ± 0.01%	0.009 ± 0.018%	0.01 ± 0.06%
Xanthomonadaceae	0	0.01 ± 0.01%	0.002 ± 0.007%	0.002 ± 0.009%
Clostridiales vadinBB60 group	0.02 ± 0.03%	0	0	0.002 ± 0.003%
Clostridiaceae 1	0.003 ± 0.01%	0	0	0.01 ± 0.02%
Pseudomonadaceae	0	0.003 ± 0.003%	0	0
Bacteroidaceae	0	0.003 ± 0.003%	0	0
Sphingobacteriaceae	0	0.001 ± 0.001%	0	0
Burkholderiaceae	0	0.001 ± 0.001%	0	0
Dermabacteraceae	0	0	0.003 ± 0.006%	0
Genus				
Subdoligranulum	23.6 ± 24.8%	24.7 ± 19.2%	31.5 ± 16.9%	48.5 ± 11.2%
Unclassified genus within family Lachnospiraceae	11.2 ± 4.4%	13.1 ± 5.4%	19 ± 9.9%	12.5 ± 4.5%
Blautia	10.3 ± 14.8%	12.4 ± 11.5%	9.6 ± 4.3%	7.2 ± 3.6%
CHKCI001	11.2 ± 8.8%	10.5 ± 9.1%	2.9 ± 1.5%	2.8 ± 2.4%
Streptococcus	5 ± 9.3%	5.5 ± 6.6%	3.7 ± 7.1%	0.86 ± 1.3%
Unclassified genus within family Lachnospiraceae	2.5 ± 3.7%	5.2 ± 3.4%	3.7 ± 3.8%	1.2 ± 0.7%
Eubacterium hallii group	1.1 ± 1%	2.9 ± 2.4%	1.3 ± 0.6%	1.2 ± 0.8%

Ruminococcus torques group	2.5 ± 1.9%	1.8 ± 1.4%	6.3 ± 5.6%	1.5 ± 1.3%
Ruminococcaceae UCG-014	5.3 ± 5.1%	2.1 ± 1.5%	1.4 ± 0.8%	1.3 ± 1.2%
Lactobacillus	0.007 ± 0.03%	1 ± 1.8%	1.2 ± 4.3%	5 ± 8.4%
Butyricicoccus	1.6 ± 1.5%	1.1 ± 1.1%	0.7 ± 0.6%	2 ± 1.5%
Unclassified genus within family Eggerthellaceae	0.6 ± 0.3%	0.3 ± 0.3%	0.5 ± 0.2%	0.2 ± 0.2%
Unclassified genus within family Bacillaceae	0.6 ± 0.6%	0.3 ± 0.4%	0.03 ± 0.1%	0.1 ± 0.2%
Sellimonas	1.6 ± 1.5%	1.6 ± 1.5%	1 ± 0.9%	0.4 ± 0.6%
Anaerostipes	1 ± 0.8%	0.8 ± 0.6%	0.5 ± 0.4%	0.4 ± 0.3%
Fusicatenibacter	0.08 ± 0.16%	0.7 ± 0.7%	0.8 ± 0.7%	0.8 ± 0.9%
Ruminiclostridium 5	0.7 ± 0.9%	0.7 ± 0.5%	0.7 ± 0.5%	0.4 ± 0.2%
Unclassified genus within family Ruminococcaceae	1.9 ± 1.8%	0.6 ± 0.6%	1 ± 0.7%	1.6 ± 1%
Unclassified genus within order Mollicutes RF39	0.5 ± 0.5%	0.4 ± 0.3%	0.5 ± 0.5%	0.5 ± 0.7%
Lachnoclostridium	0.4 ± 0.6%	0.3 ± 0.5%	0.3 ± 0.3%	0.06 ± 0.1%
Ruminococcaceae UCG-005	1.3 ± 2.2%	0.04 ± 0.1%	0.2 ± 0.5%	0.2 ± 0.3%
Bacillus	0.3 ± 0.3%	0.2 ± 0.2%	0.03 ± 0.05%	0.07 ± 0.1%
Eubacterium coprostanoligenes group	1 ± 0.8%	0.7 ± 0.5%	0.5 ± 0.3%	0.5 ± 0.5%
Unclassified genus within family Erysipelotrichaceae	0.2 ± 0.2%	0.4 ± 0.2%	0.1 ± 0.2%	0.1 ± 0.1%
Ruminiclostridium 9	0.8 ± 1.0%	0.2 ± 0.3%	0.3 ± 0.3%	0.2 ± 0.2%
GCA-900066575	0.3 ± 0.4%	0.1 ± 0.2%	0.2 ± 0.2%	0.1 ± 0.2%
Ruminococcaceae UCG-013	0.2 ± 0.3%	0.2 ± 0.2%	0.3 ± 0.2%	0.2 ± 0.1%
Escherichia-Shigella	0.6 ± 0.9%	0.3 ± 0.4%	0.2 ± 0.5%	0.2 ± 0.3%
Negativibacillus	0.4 ± 0.6%	0.1 ± 0.2%	0.4 ± 0.6%	0.1 ± 0.2%
Ruminococcus gauvreauii group	0.3 ± 0.3%	0.6 ± 0.3%	0.4 ± 0.2%	0.8 ± 0.9%
Christensenellaceae R-7 group	0.3 ± 0.3%	0.2 ± 0.2%	0.1 ± 0.1%	0.09 ± 0.2%

Unclassified genus within family Ruminococcaceae	0.2 ± 0.2%	0.2 ± 0.1%	0.1 ± 0.1%	0.2 ± 0.2%
Erysipelatoclostridium	0.1 ± 0.1%	0.2 ± 0.1%	0.3 ± 0.2%	0.07 ± 0.08%
Lachnospiraceae FCS020 group	0.3 ± 0.3%	0.02 ± 0.02%	0.08 ± 0.11%	0.2 ± 0.2%
Eisenbergiella	0.08 ± 0.12%	0.1 ± 0.2%	0.2 ± 0.4%	0.05 ± 0.1%
UC5-1-2E3	0.09 ± 0.15%	0.02 ± 0.03%	0.03 ± 0.1%	0.08 ± 0.1%
Ruminococcus gnavus group	0.01 ± 0.05%	0.06 ± 0.11%	0.03 ± 0.08%	0.01 ± 0.03%
Eubacterium ventriosum group	0.04 ± 0.1%	0.08 ± 0.08%	0.04 ± 0.04%	0
Ruminococcus 2	0.1 ± 0.2%	0.02 ± 0.02%	0	0
Anaerotruncus	0.2 ± 0.2%	0.004 ± 0.004%	0	0.01 ± 0.03%
Roseburia	0.02 ± 0.05%	0.005 ± 0.005%	0.04 ± 0.06%	0.08 ± 0.09%
Shuttleworthia	0.09 ± 0.15%	0.03 ± 0.04%	0.02 ± 0.02%	0.009 ± 0.025%
Anaerofustis	0.04 ± 0.05%	0.04 ± 0.03%	0.02 ± 0.03%	0.01 ± 0.02%
Acinetobacter	0.003 ± 0.012%	0.1 ± 0.1%	0.02 ± 0.07%	0
Pediococcus	0	0	0.007 ± 0.024%	0.1 ± 0.2%
Unclassified genus within order Clostridiales	0.02 ± 0.04%	0	0.1 ± 0.2%	0
Coprococcus 3	0.06 ± 0.12%	0.02 ± 0.04%	0	0
Eubacterium brachy group	0.05 ± 0.06%	0.02 ± 0.02%	0.02 ± 0.03%	0
UBA1819	0.008 ± 0.03%	0.02 ± 0.05%	0	0.03 ± 0.06%
Oscillibacter	0.02 ± 0.08%	0.02 ± 0.04%	0.02 ± 0.03%	0
Defluviitaleaceae UCG-011	0.03 ± 0.04%	0.007 ± 0.015%	0.01 ± 0.01%	0.01 ±0.02%
Flavonifractor	0.01 ± 0.03%	0.01 ± 0.01%	0.05 ± 0.08%	0.03 ±0.18%
Unclassified genus within order Mollicutes RF39	0.03 ± 0.04%	0.01 ± 0.01%	0.009 ± 0.018%	0.01 ± 0.06%
Lachnospiraceae UCG-004	0.04 ± 0.07%	0	0	0
Epulopiscium	0.01 ± 0.03%	0.02 ± 0.2%	0.003 ± 0.006%	0.01 ± 0.02%
Tyzzerella 3	0	0.01 ± 0.03%	0	0.006 ± 0.011%
Eubacterium nodatum group	0.004 ± 0.01%	0.005 ± 0.013%	0.01 ± 0.02%	0.007 ± 0.015%

Unclassified genus within family Lachnospiraceae	0	0.02 ± 0.04%	0	0
Family XIII AD3011 group	0.005 ± 0.01%	0	0	0.02 ± 0.05%
Klebsiella	0.02 ± 0.09%	0	0	0
Stenotrophomonas	0	0.01 ± 0.01%	0.002 ± 0.007%	0.002 ± 0.009%
Lachnospiraceae FE2018 group	0.003 ± 0.01%	0.01 ± 0.02%	0	0
Clostridium innocuum group	0	0.009 ± 0.009%	0.008 ± 0.012%	0
Merdibacter	0.01 ± 0.02%	0.002 ± 0.002%	0.009 ± 0.013%	0
Ruminococcaceae UCG-009	0.02 ± 0.03%	0	0	0
Lachnospiraceae UCG-010	0	0.01 ± 0.02%	0	0
Unclassified genus within family Clostridiales vadinBB60 group	0.02 ± 0.03%	0	0	0.002 ± 0.003%
Clostridium sensu stricto 1	0.003 ± 0.01%	0	0	0.01 ± 0.02%
Pseudomonas	0	0.003 ± 0.003%	0	0
ASF356	0	0	0.009 ± 0.018%	0
Harryflintia	0.009 ± 0.02%	0	0	0
Bacteroides	0	0.003 ± 0.003%	0	0
Unclassified genus within family Sphingobacteriaceae	0	0.001 ± 0.001%	0	0
Delftia	0	0.001 ± 0.001%	0	0
Unclassified genus within family Christensenellaceae	0	0.001 ± 0.001%	0	0
Brachybacterium	0	0	0.003 ± 0.006%	0

4.7 Determination of Taxonomic Biomarkers

Linear discriminant analysis effect size (LEfSe) was performed to determine the bacterial taxa with significantly different levels of abundance between the control group and the other groups in which the *H. illucens* was included.

The analysis comparing the control group to BSFO 25 (figure 35) resulted in 5 differentially abundant bacterial taxa (LDA score > 2.0). Only 1 taxon, *Anaerotruncus* (family *Ruminococcaceae*), had significantly higher relative abundance in the control group (green bars) and 4 taxa had significantly higher relative abundance in the BFSO 25 group (red bars), including *Fusicatenibacter* (family *Lachnospiraceae*), *Lactobacillaceae*, *Lactobacillus* and *Eubacterium* hallii group (family *Lachnospiraceae*).

The analysis comparing the control group to BSFO 50 (figure 36) showed 6 differentially abundant bacterial taxa (LDA score > 2.0). Five taxa had significantly higher relative abundance in the control group (green bars), including 3 phylotypes of bacilli (Bacillales, *Bacillaceae* and *Bacillus*) as well as *Anaerotruncus* (family *Ruminococcaceae*), and *Coprococcus* 3 (family *Lachnospiraceae*). However only 1 taxon, *Fusicatenibacter* (family *Lachnospiraceae*), had significantly higher relative abundance in the BSFO 50 group (red bars).

Finally, the analysis comparing the control group to BFSO 100 (figure 37) resulted in much more taxa with significantly different relative abundances. A total of 16 differentially abundant bacteria (LDA score > 2.0) were found. Fourteen taxa had significantly higher relative abundance in the control group (green bars) and only 2 were significantly more abundant in the BSFO 100 group (red bars). In the control group, the increased phylum Actinobacteria (LDA score > 3.0) included several coriobacterial phylotypes (Coriobacteriia, Coriobacteriales and *Eggerthellaceae*). Increased class Erysipelotrichia included several Erysipelotrichales phylotypes. The genera *Eubacterium* brachy group (Family XIII), *Anaerotruncus* and *Negativibacillus* (belonging to *Ruminococcaceae* family), *Coprococcus* 3, *Anaerostipes* and *Lachnoclostridium* (belonging to *Lachnospiraceae* family) and finally *Bacillus* (family *Bacillaceae*) were also significantly higher

in the control group. On the other hand, in the BSFO 100 group, the genera *Fusicatenibacter* (family *Lachnospiraceae*) and *Subdoligranulum* (family *Ruminococcaceae*) were significantly enriched (LDA score > 3.6).

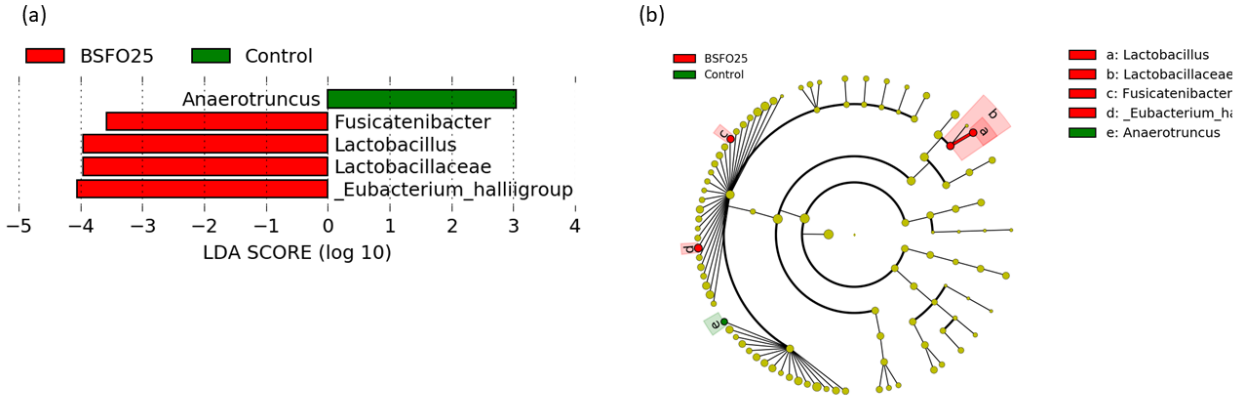

Figure 35: Linear discriminant analysis (LDA) scores. (a) The bar plots represent the significantly differential taxa between the control (green) and the BSFO 25 group (red). (b) Cladogram showed the differences in enriched taxa in the control (green) and the BSFO 25 group (red).

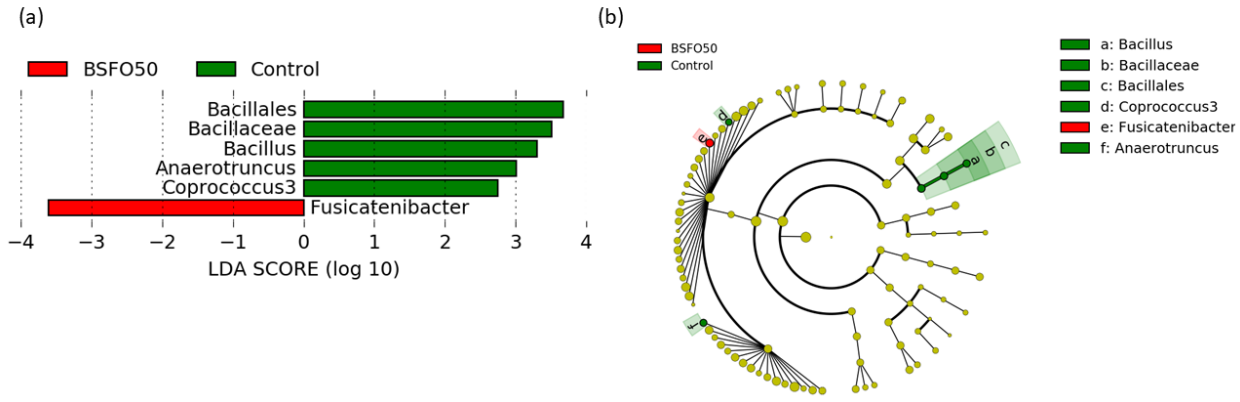

Figure 36: Linear discriminant analysis (LDA) scores. (a) The bar plots represent the significantly differential taxa between the control (green) and the BSFO 50 group (red). (b) Cladogram showed the differences in enriched taxa in the control (green) and the BSFO 50 group (red).

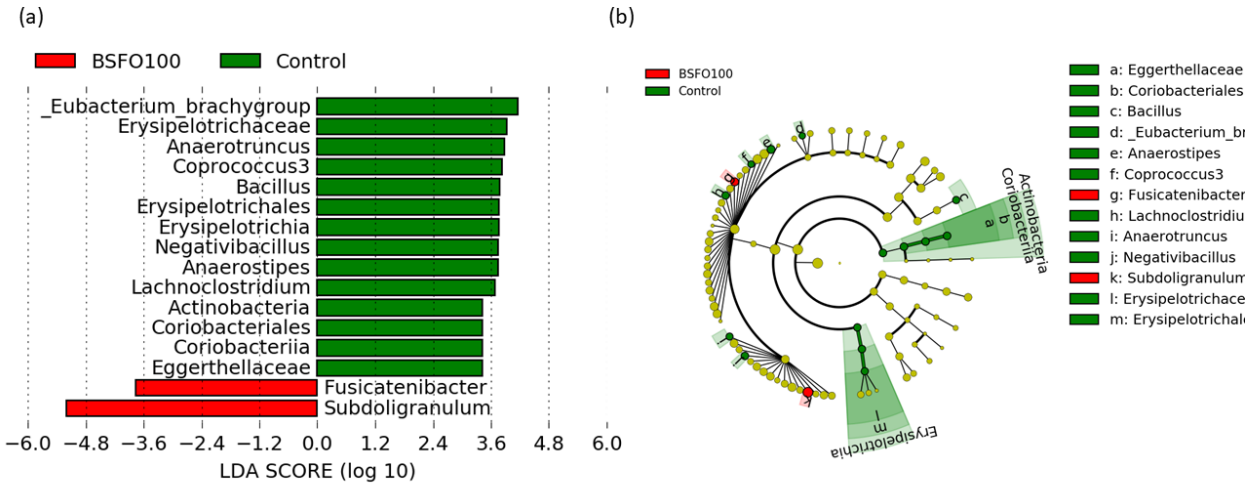

Figure 37: Linear discriminant analysis (LDA) scores. (a) The bar plots represent the significantly differential taxa between the control (green) and the BSFO 100 group (red). (b) Cladogram showed the differences in enriched taxa in the control (green) and the BSFO 100 group (red).

5. DISCUSSION

The poultry industry is currently one of the most successful and fastest growing sectors of livestock production. One of the major concerns is ensuring enough meat to satisfy the needs of the growing world population (Dabbou et al., 2021). Consequently, nutrition experts are focusing on the accurate use of nutrients to enhance growth performance in broilers (Riva & Panisello Monjo, 2020). Fats are the main source of energy because they have a high caloric content among other nutrients. Therefore, the inclusion of additional fats in feed formulation is a common method of meeting the energy needs and essential fatty acid requirements of the animal (Dabbou et al., 2021).

Recently, insect fats are used as an energy source in animal nutrition. Since insects are rich in proteins, fat, and minerals, they have been introduced effectively into animal diets as a source of several nutrients, including energy and essential fatty acids (Benzertiha et al., 2020). Specifically, Black soldier fly (*Hermetia illucens* L.; HI) has emerged as a particularly promising species due to its protein profile, lipid and calcium content (Cullere et al., 2016; Schiavone et al., 2017). However, the majority of the studies done on the use of insect biomass in animal nutrition, are focused on the replacement of traditional protein sources with far less emphasis dedicated to fat as an energy resource (Benzertiha et al., 2020). Also, there is relatively limited knowledge on the influence of the Black soldier fly larva (BSFL) fat inclusion in poultry feeding, especially on the caecal bacterial microbiota.

In the present thesis, we investigated the effect of partial and total replacement of the commonly used corn oil by Black soldier fly larvae fat, on growth performance, slaughter performance, and caecal bacterial microbiome in broiler quails. The animals were offered 4 different diets, the basal diet containing corn oil (CO) and 3 experimental diets in which the corn oil was substituted with BSF larvae fat by 25%, 50% and 100% (BSFO 25, BSFO 50 and BSFO 100 respectively).

In this study, the growth performance parameters were not significantly affected (FI, AWG and FCR) between the control group with corn oil and the 3 treatments in which BSF oil was included at different levels as a substituent of corn oil (25%, 50% and 100%). Generally, broilers growth

performance, nutritional digestibility coefficients, or meat quality were not adversely affected by the use of insect fat in place of different vegetable oils (Benzertiha et al., 2019). The disagreement between all the research could be brought about by variations in age, breed, trial settings and management, feed composition, and the wide range in FA contents of insect oils (Danieli et al., 2019; Gao et al., 2019; Kawasaki et al., 2019). Chen et al., (2022) published the most recent study, which found no effect on growth performance metrics (FCR, BWG, and FI). In addition, (Kim et al., 2020) found that the dietary treatments had no effect on FI and BWG, but the FCR ratio was significantly reduced in the treatment supplemented with *H. illucens* fat and coconut oil in later stages of rearing. In this experiment, between 35-42 days, there was an improvement in the FCR of the groups with insect fat inclusion but not statistically significant. According to this result, it would not be enough to totally agree with the hypothesis of that the high levels of lauric and myristic acid acid have a positive effect in improving feed:gain ratio (Zeitz et al., 2015). Feed intake was unaffected by fat sources is consistent with other research (Khatun et al., 2018; Poorghasemi et al., 2013), which found that feed intake was unaffected in chickens fed various fat sources in diets. No obvious influence of dietary insect oils or fats rich in medium-chain fatty acids, such as coconut oil or BSFL oil (Baltić et al., 2017; Spranghers et al., 2017), on growth performance in broiler chickens was reported, which is consistent with the current study's findings (Schiavone et al., 2017; Shokrollahi et al., 2014; Wang et al., 2015; Zeitz et al., 2015). The disparity between the earlier results and the findings of this study lacks a concrete explanation. Furthermore, the majority of previous tests on broiler poultry species were undertaken to replace soybean oil with *H. illucens* fat totally or partially, and none of the studies indicated any negative impacts on growth performance measures (Dabbou et al., 2021; Hartinger et al., 2022; Schiavone et al., 2017, 2018; Sypniewski et al., 2020). Insect fat obtained from *H. illucens* larvae can substitute traditional fat sources in broiler chicken and turkey feeding without affecting growth performance features, according to the experiments stated above. Laying hens fed fat from *H. illucens* larvae showed no differences in BWG, or the FI (Heuel et al., 2021). Furthermore, the findings concern the inclusion of a full-fat meal derived from insect biomass, which may hint at certain biological implications associated with the use of insect lipids in animal nutrition. The use of *H. illucens* larval full-fat meal as a substitute for soybean meal, oil, or fish meal in broiler and

laying quail diets had no effect on feed performance or growth performance features (Cullere et al., 2016; Schiavone et al., 2018).

Concerning the slaughter traits, the inclusion of different levels of BSF fat substituting corn oil did not result in any significant variation of the carcass and selected internal organs weights and the gastrointestinal segments lengths. The result is in accordance with the study of Kim et al., (2020), which stated that, the inclusion of coconut oil or BSF fat in substitution of corn oil resulted in no significant difference of the intestinal segments lengths and internal organs weights. Dietary BSFL did not affect intestinal length and weight in broiler chickens (Cutrignelli et al., 2018). In comparison to other commercially adopted vegetable oil source in poultry diets, *H. illucens* fat inclusion also showed no significance concerning selected slaughter traits (Kierończyk, Sypniewski, et al., 2022; Schiavone et al., 2018; Sypniewski et al., 2020).

In relation to the microbial composition, the bacterial community in the caecal samples was dominated by Firmicutes, regardless of the type of diet, which is in agreement with the previous studies (Andreani et al., 2020; Biasato et al., 2020; Cardenas et al., 2021; Costa et al., 2017; Danzeisen et al., 2011; Józefiak et al., 2020; Moula et al., 2018; Wei et al., 2013; Wilkinson et al., 2016; Yeoman et al., 2012; Zou et al., 2018). At the family level, the most dominant bacteria were *Ruminococcaceae*, *Lachnospiraceae*, they represented together 80.7% of the sequences in the control group, 81.3% in the BSFO 25 group, 83.7% in the BSFO 50 group and 84.9% in the BSFO 100. *Streptococcaceae* and *Lactobacillaceae* were also present in lower but meaningful relative abundances. The total inclusion of BSF oil caused a decrease in *Streptococcaceae* (5 ± 9.3% control group compared to 0.8 ± 1.3% BSFO 100 group), as well as an increase in *Lactobacillaceae* (0.007 ± 0.03% control group compared to 5.2 ± 8.6% BSFO 100 group. Previous studies also showed the dominance of *Ruminococcaceae* and *Lachnospiraceae* in caecal bacterial flora (Andreani et al., 2020; Biasato et al., 2020; Danzeisen et al., 2011; Józefiak et al., 2020; Moula et al., 2018; Wei et al., 2013; Wilkinson et al., 2016) and *Lactobacillaceae* presence is in agreement with Biasato et al., (2020) and Cardenas et al., (2021). Regardless of the diet, the abundance of *Clostridiaceae* and *Bacteroidaceae* families was found to be low, which is in disagreement with previous studies on quails (Du *et al*., 2020) and chickens (Biasato *et al*., 2020; Cardenas *et al*.,

2021). This discrepancy could be attributed to various factors. Previous research has indicated that bacterial species from the *Clostridiaceae* family have been associated with negative performance outcomes (Bindari et al., 2022; Stanley et al., 2016). Therefore, chickens experiencing poor performance often show an increase in species belonging to this family. Furthermore, the mucosa of low-weight chickens frequently exhibits a high occurrence of *Clostridiaceae* family (Abdel-Kafy *et al*., 2022). On the other hand, species belonging to *Bacteroidaceae* family are known to harbor genes involved in the degradation of complex polysaccharides, leading to the production of acetate, propionate, or succinate (Rychlik, 2020). Their prevalence can be influenced by various factors. A recent study highlighted that the geographical origin of chickens can impact the composition of the cecal microbiota and alter the relative abundance of several families, including *Bacteroidaceae* (Pin Viso *et al*., 2021). Additionally, the diet plays a crucial role in shaping the cecal microbiota, and several studies have revealed changes in the relative abundance of *Bacteroidaceae* family associated with variations in the chicken's diet (Bi *et al*., 2020; Zhou *et al*., 2022). *Lachnospiraceae* and *Ruminococcaceae* improve feed efficiency through SCFA production, degrading cellulose and other host-indigestible polysaccharides (Biddle et al., 2013; Broom, 2018; Wilkinson et al., 2016; Yang et al., 2017). *Streptococcaceae* is a source of streptococcosis in poultry but according to this study's analysis the inclusion of BSF larvae oil led to the reduction of its prevalence in the BSFO 100 group. *Lactobacillaceae* helps in improving the intestinal health and growth performance of poultry (Chateau et al., 1993). At the genus level, the core bacterial genera were *Subdoligranulum*, *Unclassified genus within family Lachnospiraceae*, *Blautia*, *CHKCI001* and *Streptococcus*, they represented together 61.2% of the sequences in the control group, 66.1% in the BSFO 25 group, 66.6% in the BSFO 50 group and 71.9% in the BSFO 100. This is in good agreement with previous studies which found a predominance of *Subdoligranulum* and *Blautia* in the caecal microbiota of broiler chicken (Clavijo & Flórez, 2018; Hou et al., 2016; Ijaz et al., 2018). Most of the core bacteria were previously reported in the literature as being part of the major genera in the microbiome of chicken caecum (Biasato et al., 2020; Cardenas et al., 2021; Polansky et al., 2016).

Regarding the influence of the diet on the diversity of the caecal microbiome, the richness of the bacterial community, represented by the Shannon entropy, was significantly lower in the BSFO 100 group compared to the control animals and to the BSFO 25 group. Also, the equity in bacterial species abundance, represented by the Pielou evenness index, resulted in a significantly lower species evenness in the BSFO 100 group compared to the control animals and to the BSFO 50 group. In contrary, the Chao1 index and the bacterial phylogenetic diversity were not affected by the animal's diet. The calculation of the distance between the studied quails resulted in a separation between the studies group. The Principal Coordinate Analysis (PCoA) based on Bray Curtis, Jaccard, and both weighted and unweighted UniFrac distance matrices, showed the spatial separation of the samples, even though the clusters were not very well separated, statistical analysis revealed significant differences between the groups, mainly the BSFO 100 group compared to the control animals. This is agreement with Biasato et al., (2020) that showed lower alpha diversity in diets with inclusion of *H.illucens* in broiler chickens and significant beta diversity (using the weighted UniFrac distance matrix) between birds fed HI diets and control diet fed a maize, corn gluten and soybean meals (Biasato et al., 2020) . Also, the study of Dabbou et al., (2021) showed a clear separation of the control samples fed with soybean oil vs. BSF oil group diets in beta diversity which is also already approved for insect-fed poultry species. (Biasato et al., 2018; Borrelli et al., 2017; Dabbou et al., 2021). Our results are also in accordance with Chen *et al*. (2022) who found a lower richness in alpha diversity in the chickens fed different levels of BSF fats, compared to the control birds, however the PCoA based on OTUs revealed no significant difference in beta diversity between the studied groups (Chen *et al*., 2022).

The LEfSE analysis identified taxa with significantly different abundances in each animal group. The common features between the three analyses performed were the increased relative abundance of both *Anaerotruncus* (family *Ruminococcaceae*) in the control group and *Fusicatenibacter* (family *Lachnospiraceae*) in the groups in which the *H. illucens* oil was included. *Anaerotruncus* may enhance the absorption of volatile fatty acids to increase the energy utilization of recipients, leading to an increased muscle fiber diameter and decreased drip loss (Lei et al., 2022). *Fusicatenibacter* are SCFA-producing taxa which produce formate and acetate, and it can protect

the equilibrium of the intestinal microbial community (Qiu et al., 2020; Takada et al., 2013), suppresses the intestinal inflammation (Takeshita et al., 2016), reduces diarrheal symptoms, (C. Yang et al., 2021) and regulates colonic motility (Zhang et al., 2021). As well, *Subdoligranulum*, which was the dominant genus among the four groups, was significantly enriched in the BSFO 100 group compared to the control group (LDA > 4.8). Its increase leads to positive outcomes since this genus promotes the development of intestinal epithelial cells, which minimizes *Salmonella* invasion and colonization (Eeckhaut et al., 2008). Danzeisen et al., (2011) stated that the use of antibiotics in poultry feed increased the prevalence of *Subdoligranulum* leading to an increased approval of the hypothesis that the inclusion of BSF oil can be a substitute for conventional antibiotic usage and most probably due to the high lauric acid content in its fatty acid composition (Boyen et al., 2008). The relative abundance of *Lactobacillus* is higher in BSFO 25 compared to control group and this genus provides beneficial effects on the health and performance by producing antimicrobial substances (Oakley et al., 2014), short chain fatty acids, exopolysaccharides and additional sources of energy (Pajarillo et al., 2015). Also the *Eubacterium halii* group (family *Lachnospiraceae*) was enriched in BSFO 25 group which is a butyrate producing genus (Duncan et al., 2004). The diminished abundance of *Bacilli* phylotypes (*Bacillales, Bacillaceae and Bacillus*) in BSFO 50 group could have a negative impact on nutrient absorption (Aliakbarpour et al., 2012). Also, the diminished abundance of *Coprococcus3* genus in BSFO 50 and BSFO 100 compared to the Control group is a butyrate-producing member of the *Lachnospiraceae* family (Pryde et al., 2002). In the BSFO 100 treatment compared to Control group, *Eubacterium* brachy group, *Lachnoclostridium* and *Anaerostipes* known as butyrate producing members of the *Lachnospiraceae* family decreased in abundance (Pryde et al., 2002; Ríos-Covián et al., 2016) and also Coriobacterial phylotypes relative abundance decreased in this group (*Coriobacteriia, Coriobacteriales and Eggerthellaceae*) involved in the conversion of bile salts and steroids as well as the activation of dietary polyphenols (Clavel et al., 2014). *Erysipelotrichales* phylotypes that has been previously associated with lipid metabolism and inflammation (Kaakoush, 2015) decreased in abundance in BSFO 100 compared to the group in which only corn oil was included. Accordingly, replacing corn oil with 25% of *H. illucens* oil

would be the best suggestion since it increased the prevalence of the genera Lactobacillus, *Eubacterium halii* group and *Fusicatenibacter*.

The positive outcome in the current study is that potentially pathogenic bacteria such as *Campylobacter* and *Corynebacterium* were little to not found. Only *Escherichia-Shigella* was detected in a very low amount (< 0.6%). These genera have been found in several studies done on the microbiome of chicken (Costa et al., 2017; Hou et al., 2016; Lu, Idris, et al., 2003; Zhu et al., 2002). The occurrence of opportunistic bacteria may be associated with the type of diet, indeed, the inclusion of BSF larvae fat in broiler chicken's diet induced a positive reduction in *Clostridium* and *Corynebacterium* which may normally cause infections in poultry.

The bacterial community has an important role in the overall nutritional and health status of the animal. It is strongly affected by several factors such as diet. In the present study, quails were fed different diets and as a result, their caecal microbiota composition and diversity resulted in some differences.

6. CONCLUSION

In the present thesis we examined the effect of partial and total replacement of the commonly used corn oil by Black soldier fly larvae fat on the growth performance, the slaughter performance as well as the bacterial diversity and composition in the caecal content of quails using high-throughput sequencing. The subsequent conclusions were derived from the results.

The inclusion of BSFL oil did not affect the growth performance parameters and the slaughter traits. No variation of the carcass and selected internal organs weights and the gastrointestinal segments lengths were found.

Many other effects of BSFL oil inclusion in the quails' diet are still to be studied (microscopic, biochemical aspects of the different intestinal segments and meat quality parameters).

Regarding the microbial composition, the bacterial community in the caecal samples was dominated by Firmicutes in all groups. The core bacterial genera were *Subdoligranulum, Unclassified genus within family Lachnospiraceae, Blautia, CHKCI001* and *Streptococcus*.

Alpha diversity showed a lower bacterial richness (Shannon entropy), in the BSFO 100 group compared to the control animals and to the BSFO 25 group. Also, the Pielou evenness index, resulted in a significantly lower species evenness in the BSFO 100 group compared to the control animals and to the BSFO 50 group. However, the bacterial phylogenetic diversity was not affected by the animal's diet.

Beta diversity showed the spatial separation of the samples using the The Principal Coordinate Analysis (PCoA). Even though the clusters were not very well separated, statistical analysis revealed significant differences between the groups, mainly the BSFO 100 group compared to the control animals.

The LEfSE analysis identified taxa with significantly different abundances in each animal group. Mainly *Subdoligranulum*, which was the dominant genus among the four groups, was significantly enriched in the BSFO 100 group compared to the control group. As well, the common features

between the three analyses performed were the increased relative abundance of both *Anaerotruncus* (family *Ruminococcaceae*) in the control group and *Fusicatenibacter* (family *Lachnospiraceae*) in the groups in which the *H. illucens* fat was included.

These results suggest that the inclusion of BSFL oil does not negatively affect the growth and slaughter performances, suggesting that *H. illucens* larvae fat can be a suitable fat source in broiler Japanese quail diets and a substituant of the conventionally used corn oil. However, some changes in the bacterial structure diversity and composition is observed. The modification is mainly caused by a different relative abundance of several bacterial species.

7. References

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