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Assessment of the impact of different feeding strategies on chicken gastrointestinal tract by shotgun metagenomic sequencing to fight colonization by potential foodborne pathogens

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To my parents,

for believing in me and for always supporting me in my journey even when it did not seem right,

for always telling me that everything happens for a reason, and that I must face every situation with courage and without fear.

Thank to them I learnt how to befriend strangers, to be always honest and kind with everyone.

Thank you for sharing your love of life and sense of humour with me and for showing me how hard work looks like.

Thank you for being such caring and dedicated parents always willing to help and protect me and my beloved sister.

Without you, I would not be nowhere near the person I would like to became and this thesis would certainly have not existed.

Thank you… I owe you everything.

1. INTRODUCTION

1.1 The chicken gut microbiota: composition and development

Domestic chickens are the most common avian species and valuable sources of proteins for humans. The chicken gastrointestinal tract is densely populated by microorganisms, which closely and intensively interact with the host, the diet and the ingested feed. A better understanding of these interactions, impacting on animal nutrition and health, is required to further enhance poultry productions, safety of poultry products and host growth performance (Rinttilä and Apajalahti, 2013; Pan and Yu, 2014). The total DNA that can be extracted from the chicken gut is the metagenome and is the aggregate of the DNA of the host and the microbiota. Microbiota is the collective microbial community inhabiting the chicken gut. Metagenome and microbiome are often used interchangeably but the microbiome is the collective genomic content of a microbiota and indicates the total genetic capacity of the community (Tremaroli and Bäckhed, 2012).

Between all body sites the gastrointestinal tract (GIT) is the most densely colonized organ by different microbial cells representing the GIT microbiota (Scott et al., 2013; Doré and Blottière, 2015; Jandhyala et al., 2015; Yoon et al. 2015). The microbiota consists of trillions of symbiotic microbial cells harbored by each host, primarily bacteria in the gut; furthermore, the microbiome consists of the genes these cells harbor. In fact, the microbiome is defined as the combined genetic material of the microorganisms in a particular environment (Ursell et al., 2013). All animals coexist with their microbiota establishing a symbiotic equilibrium that confers them a variety of physiologic benefits. The composition of the microbiota is host specific, evolve during the animal's lifetime and is susceptible to both exogenous and endogenous modifications (Neish, 2009; Sekirov et al., 2010). Rawls et al. (2006) showed that the transplantation of microbial communities between different host species results in the transplanted community transforming to resemble the native microbiota of the recipient host. The symbiotic equilibrium between host and microbiota is established soon after the animal birth. Later on, changes in its composition are influenced by the exposure to the microorganisms present in the surrounding environment by diet (Pan and Yu, 2014; Blottière Doré, 2015; Wang et al., 2016). The loss of balance settled between the various populations that compose each microbiota may lead to serious repercussions on the host, such as the onset of a large number of metabolic, immune-mediated, allergic and inflammatory pathologies (Cogen et al., 2008; Yoon et al. 2015).

Compared with mammals, chicken and other poultry, as turkey and duck, have a shorter gastrointestinal tract causing a faster digesta transit, a relatively short retention time for ingested food and consequently selecting a deeply different intestinal microbiota compared to other food animals.

The average transit time through the whole chicken gastrointestinal tract is less than 3.5 h (Hughes, 2008; Pan and Yu, 2014). The chicken gastrointestinal apparatus is divided into histologically and anatomically distinct structure, named esophagus, stomach, small intestine (duodenum, jejunum and ileum) and large intestine (caecum, colon and rectum). The ingested food is initially stored in the crop, an extension of the esophagus, which inner surface is covered with non-secretory stratified squamous epithelium, then it passes to the stomach (Van de Graaff, 1986; Grist, 2004). The food begins the digestion process in the crop where it is fermented by bacteria, especially *Lactobacillus* genus, since crop is the election site for bacteria colonization. From the crop, food passes into the proventriculus and successively into the gizzard, that constitute the glandular and muscular parts of the avian stomach. From the gizzard, the digesta passes into the small intestine which is comprised of the duodenum, jejunum and ileum, where it is mixed with bile salts and proteinases, amylases and lipases secreted in enzyme secretions from the pancreas and others digestive enzyme produced by the secretory mucosa of the small intestine. The small intestine is the major site of chemical digestion and nutrient absorption due, other than presence of pancreatic and small intestine-produced enzymes, to villi and microvilli. The digesta then passes into the large intestine where two caeca branch out forming two separate blind ended pouches (Fuller and Brooker, 1974; Barnes et al., 1980; McLelland, 1989; Mead, 1997) that are filled trough retrograde peristalsis from the colon. The caeca are thought to be involved in the breakdown of plant material indigestible for the host and the absorption of water, glucose and volatile fatty acids. From the ileo-caecal junction the digesta enters the colon where there is very little absorption or digestion processes and then finally the feces pass into the cloaca and expelled mixed with uric acid (McLelland, 1989).

The short digesta retention time, during chicken digestion process, selects bacteria that can adhere to the mucosal layer and/or grow fast. The regions which have less tolerable conditions and faster passage of contents have lower numbers of bacteria. However, abundance and diversity of microbiota are different along the gastrointestinal tract and the ceca. When young chicks are delivered from the hatchery to a chicken house (typically at the age of 1–2 days), their initial gastrointestinal microbiota is very simple, containing a very small number of bacteria belonging to a few species but, after housing, chicks are exposed to several sources of bacteria that can enter in the immature gut. Since the chicks in this stage of life have little colonization resistance, the bacteria coming from litter materials, feed, water, and ambient air readily colonize their gastrointestinal tract. As young chicks grow, their gastrointestinal microbiota undergoes through a series of changes becoming increasingly diverse and complex (Wang et al.,2016; Wei et al., 2013b).

Several studies have been conducted trying to characterize chicken gastrointestinal microbiota and microbiome composition, beginning with early cultivation-based studies that revealed low abundances of Lactobacilli (>10^4/g CFUs) and Clostridia (10^2–10^4/g CFUs) in the small intestines and high abundance (10^{10}–10^{11}/g microscope counts) of anaerobic bacteria in the cecum. Peptostreptococcus, Propionibacterium, Eubacterium, Bacteroides, and Clostridium were the major genera recovered from cecum by cultivation but it was possible to cultivate jusy between 20–60% of the total cecal bacteria (Pan and Yu, 2014). The technologies used for 16S rRNA gene based metataxonomic analyses make it possible to comprehensively characterize the chicken intestinal microbiota and to obtain sequence information, expanding our knowledge on bacterial diversity present in the intestinal tract, particularly the cecum, of chickens and turkeys (Wei et al., 2013b). Wei et al., (2013), identified in chicken gastrointestinal microbiota 117 established genera of bacteria, represented by the sequence collection, with most genera belonging to the phyla Firmicutes, Proteobacteria, and Bacteroidetes. Within phylum Firmicutes, genera Clostridium, Ruminococcus, Lactobacillus, Eubacterium, Fecalibacterium, Butyrivibrio, Ethanoligenens, Alkaliphillus, Butyricicoccus, Blautia, Hespellia, Roseburia, and Megamonas were represented by more than 1% of the total bacterial sequences. Within phylum Proteobacteria, genus Desulfohalobium was represented by the most sequences, and within phylum Bacteroidetes most of the sequences were classified into order Bacteroidales, and only genera Bacteroides, Prevotella, Parabacteroides, and Alistipes were each represented by >1% of the bacterial sequences. Of the minor phyla, Actinobacteria was the most predominant, but only the genus Bifidobacterium was represented by >1% of sequences within this phylum. The most predominant phyla in cecal microbiota included Firmicutes and Bacteroidetes, accounting for approximately 78 and 11% of the total cecal sequences, respectively. Firmicutes alone contained 31 genera, but only Ruminococcus, Clostridium, and Eubacterium each represented ≥5%, of the sequences classified to this phylum. Other genera that contained more than 1% of the total cecal bacterial sequences included Fecalibacterium, Blautia, Butyrivibrio, Lactobacillus, Megamonas Roseburia, Ethanoligenes, Hespellia, Veillonella, and Anaerostipes. Bacteroides was the most predominant genus in the phylum Bacteroidetes, accounting for 40% of the cecal sequences in this phylum. Other relatively predominant genera in this phylum included Prevotella and Paraprevotella, Tannerella and Riemerella. Within phylum Proteobacteria, Desulfohalobium, Escherichia/Shigella, and Neissenia were the most predominant genera.

Comparison of data obtained with different methods shows discrepancy that might reflect the bias of individual studies that could hinder a comprehensive knowledge of composition of the intestinal microbiome (Wei et al., 2013b). Furthermore, the composition of the gut microbiota is strongly

influenced by a large range of factors that include the microbial species acquired at birth, host genetics, immunological factors, antibiotic usage and dietary effects (Scott et al., 2013).

1.2 Interactions between populations belonging to the chicken gut microbiota

It has to be taken into account that, aside from the characterization of the composition of the microbiota is essential to understand the interactions between the different bacterial populations that compose this complex ecosystem. Other than between the microbiota and its host, there are extensive interactions among avian gut microbes. In fact, the avian gastrointestinal tract is an ideal habitat for microorganisms but it does not support unlimited microbial proliferation due to the limited availability of nutrient and niches. Therefore, different bacterial populations have different interactions, such as competition, cooperation, and antagonism. Competition for attachment sites and nutrients among bacteria is a common phenomenon in intestinal ecosystem (Soler et al., 2010). The lack of available attachment sites and niches, due to competition could represent a valid strategy to inhibit the pathogen colonization and proliferation in the host gastrointestinal tract. In fact, in order to cause infections in birds, enteric pathogens need to first attach to the intestinal mucosa and then to break through the epithelial barrier, but the commensal bacterial populations of healthy birds colonize intestinal mucosa forming a protecting layer that cover the mucosal surface. This layer of dense and complex microbial communities occupying the adhering niches can prevent the attachment and the subsequent colonization of most enteric pathogens trough the phenomenon so called “competitive exclusion” (Lan et al., 2005; Gabriel et al., 2006; Lawley and Walker, 2013). For this reason, a critical stage for pathogen colonization could be the post hatching period, when the gastrointestinal tract of chicks is still not colonized by the microbiota and consequently more susceptible to pathogens. Newly hatched chick’s gastrointestinal tract is sterile, but is immediately colonized by microorganisms present in the surrounding environment. In nature, these microorganisms would belong to the mother’s feces microbiota, but in poultry productions, the chicks are hatched in incubators, away from the hens. Since the incubators are relatively clean, there is a delay in normal colonization and succession of intestinal microbiota. The prolonged absence of a normal gut microbiota offers to enteric pathogens in the environment a greater opportunity to colonize gastrointestinal tract and to cause infection in new hatchlings, making them more susceptible to enteric infections, in particular to necrotic enteritis and to the colonization of potential human pathogens, such as *Salmonella enteritidis* (Lan et al., 2005; Dahiya et al., 2006; Lutful Kabir, 2009). Varmuzova et al. confirmed this theory testing whether microbiota from donor hens of different age will protect chicks against *Salmonella Enteritidis* infection. They inoculated groups of newly hatched chicks with cecal extracts of 35-week-old hens either on day 1 of life followed by *S. Enteritidis* infection on day 2 or were

infected with *S. Enteritidis* infection on day 1 followed by therapeutic administration of the cecal extract on day 2 or were inoculated on day 1 of life with a mixture of the cecal extract and *S. Enteritidis*. In this experiment, terminated when the chickens were 5 days old, both *Salmonella* culture and chicken gene expression confirmed that inoculation of microbiota from 35-week-old hens protected chickens even 24 h after while simultaneous administration or therapeutic microbiota administration failed to protect chickens against *S. Enteritidis* infection.

Other than the competitive exclusion, another strategy used by some bacterial populations to gain competitive advantages is to produce bacteriostatic or bactericidal substances hostile to competitors. In fact, the term competitive exclusion generally does not refer only to the mechanism of the site's physical occupation but it includes even other mechanisms as the direct physical or chemical insult to the potential colonist and the resource competition in a physical or chemical niche (Oakley et al., 2014). For example, lactic acid and some short chain fat acids produced by various commensal bacteria have an inhibitory action against certain pathogens. Furthermore, a great number of Gram positive and Gram negative bacteria produces, during their growth, substances of protein structure (either proteins or polypeptides) possessing antimicrobial activities, called bacteriocins. Lactic acid bacteria can inhibit the pathogens growth using both mechanisms. In fact, lactic acid bacteria fermenting the carbohydrates present in chickens' feed produce lactic acid, which, lowering the pH in the surrounding environment, inhibits the growth of certain pathogens such as *Escherichia coli*, *Salmonella Typhimurium* and *Clostridium perfringens* through the production of bacteriocins as a natural barrier against pathogens.

Other than lactic acid that was proven to be effective against *Escherichia coli*, *Salmonella Typhimurium* and *Clostridium perfringens*, in an *in vitro* study conducted on chicken, Van der Wielen et al. (2000) showed that in ceca there is a negative correlation between some short chain fat acids concentrations (acetate, propionate, and butyrate) and Enterobacteriaceae abundance. This could happen because short chain fat acids in un-dissociated form, other than lowering extracellular pH, can diffuse across the bacterial cell membrane. Once into the cell their dissociation causes a lowering of the intracellular pH causing the inhibition of some essential enzymes or metabolism (Van der Wielen et al., 2000; Van Immerseel et al., 2004; Van Immerseel et al., 2006).

The resource competition in a physical or chemical niche mostly refers to the nutrient competition. A good example is the competition for zinc among microbiota's microbes, since zinc is an essential element involved in several cellular functions, such as enzymatic reactions and gene expression. Under low-zinc conditions some pathogen bacteria as *Campylobacter jejuni*, *Salmonella Typhimurium* and *Escherichia coli* use the high affinity ZnuABC transporter mechanism to bring zinc

into cell (Patzer and Hantke, 2000; Campoy et al., 2002; Davis et al., 2009; Gielda and DiRita, 2012). Gielda and DiRita 2012, showed that both a wild-type *C. jejuni* strain and a znuABC- mutant strain of *C. jejuni* were able to colonize limited-mirobiota chicks at similar efficiencies, but only the wild-type *C. jejuni* strain was able to colonize conventional chicks. However, since the zinc level in cecal content was significantly lower in the conventional chicks than in the limited-microbiota chicks, they suggested that under low zinc conditions, *C. jejuni* lacking the high-affinity zinc uptake system was outcompeted by other bacteria present in the GI tract.

Another important interaction between microbes in the horizontal gene transfer that is 'the non-genealogical transmission of genetic material from one organism to another (Goldenfeld and Woese, 2007). The commensal bacteria present in the gastrointestinal microbiota usually possess some characteristics which allow them to survive in the gastrointestinal tract and more importantly, to outcompete other adverse bacteria and the pathogens. However, the horizontal gene transfer make it possible for the pathogens to acquire these characteristic traits and became more competitive, and for commensal bacteria to acquire virulence factors from pathogens becoming pathogenic for chickens. Finally, poultry enteric pathogens can directly exchange the virulence traits increasing their pathogenicity (Johnson et al., 2010; Van Reenen and Dicks, 2011). The inappropriate or prolonged use of antibiotics can lead to the horizontal transfer of resistance genes and may contribute to spread of antimicrobial resistance among adverse and pathogenic bacteria. In particular, the litter, if used for multiple growth cycles, can represent the main source of antibiotic resistant bacteria in poultry allowing their recycle between litter and gastrointestinal tract of animals (Dhanarani et al., 2009).

1.3 Microbiota's role in host physiology

In the past decades, most of the research on the impact of bacteria in the intestinal environment has focused on gastrointestinal pathogens and the way they cause disease, while there has recently been a considerable shift towards the study of the effect that commensal microbes exert on the host gut. In fact, the intestinal microbiota is an extremely dense and complex ecosystem, which plays a relevant role in the maintenance of the animal's well-being through the production of biologically relevant metabolites and the prevention of pathogenic microorganisms' colonization, acting as an intestinal barrier. The microbiota is intimately involved in numerous aspects of normal host physiology. It can influence the usage of nutrients by the host and host's stress and immune response. Furthermore, it contributes to the optimal development of its intestinal mucosa and immune system (Scott et al., 2013; Doré and Blottière, 2015; Jandhyala et al., 2015; Yoon et al. 2015). The mechanisms through which microbiota exerts its beneficial or detrimental influences remain largely undefined, but it includes

elaboration of signaling molecules and recognition of bacterial epitopes by both intestinal epithelial and mucosal immune cells (Sekirov et al., 2010).

1.3.1 Immunostimulation, immunomediation and mucosal development

The gastrointestinal microbiota contributes to gut immunomodulation in tandem with both the innate and adaptive immune systems and maintain gut homeostasis by protecting the host from infections stimulating the gut enteric system and keeping it always active inducing a base level of inflammation. The cells of the host immune system that cooperate with gut microbiota in the immunomodulatory process are the gut associated lymphoid tissues (GALT), effector and regulatory T cells, IgA producing B (plasma) cells, Group 3 innate lymphoid cells, and, resident macrophages and dendritic cells in the lamina propria (Cebra, 1999; Chung et al., 2012; Jandhyala et al., 2015). The implication of gut microbiota in shaping a normal GALT is implied by the reduced development of the Peyer's patches and isolated lymphoid follicles that are marked by the abundance of IgE+ B cells instead of the normal IgA+ B cells, documented by Durkin et al. (1981), in germ free mice compared to conventionally raised mice. Gut microbiota is also associated to the normal development and function of Foxp3+, a protein regulator of regulatory T cells (Tregs) even if the mechanism by which this is mediated is still not clear. Furthermore, short chain fat acids (CFAs), especially butyrate, has also been implicated in the development and function of Tregs. In fact, they activate G-protein coupled receptors expressed by the IECs and regulate Treg by epigenetic regulation of the Foxp3 (Smith et al., 2013; Arpaia et al., 2013). Other roles played by the microbiota during the immunostimulation process regards the My-D88 signaling, the differentiation of innate lymphoid cells and the support of IL1β in response to pathogen. The production of IgA is induced by DCs. MyD88 signaling is the mediator of this function and its signaling process can be activated by the gut microbiota. Furthermore, the microbiota can stimulate directly DCs in the Peyer's patches to secrete TGF-β, CXCL13, and B-cell activating protein leading to IgA production and class switching (Suzuki et al., 2010).

The microbiota's composition can also affect the diverse differentiation in the innate lymphoid cells or in T helper Th17 cells of a common lymphoid precursor. Commensal flora induces MyD-88 dependent mechanisms, which are essential during the rapid production of the mature IL1β, from the pro-IL1β, in response to pathogen invasion (Spits and Cupedo, 2012). Other than an involvement in the immunostimolation, the gut microbiota plays a relevant role in maintaining the structure and function of the gastrointestinal tract. This theory is supported by the observation that germ free mice shows a lower intestinal surface area, a significant reduction of villus capillary network and a decreased nutrient digestion and absorption (Gordon and Bruckner-Kardoss, 1981). In fact, the gut

microbiota induces the transcription factor angiogenin-3, implied in the development of intestinal microvasculature. It can prevent cytokine induced apoptosis of the intestinal epithelial cells through the production by *Lactobacillus rhamnosus* GG strain of two soluble proteins (p40 and p75) and can increase the levels of endocannabinoids, trough *Akkermansia muciniphilia* strain action, that control gut barrier functions by decreasing metabolic endotoxemia (Jandhyala et al., 2015). Furthermore, the microbial cell wall peptidoglycan stimulates signaling path of TLR2, a mechanism that is necessary for the maintenance of the tight junctions (Cario et al., 2007).

Other than stimulating the immuneresponse, some bacteria populations produce butyrate, a short chain fatty acid, that it is the preferred energy source for the enterocytes in the lower intestinal tract and is known to regulate cellular differentiation and proliferation within the intestinal mucosa, thereby increasing intestinal tissue weight. So, it should be pointed out that, the contribution of butyrate, other than feeding the enterocytes, since the epithelium act as a highly selective barrier preventing the passage of toxic and proinflammatory molecules into the submucosa and systemic circulation, is indirectly essential in the maintenance of normal intestinal barrier functions (Waite and Taylor, 2014).

1.3. 2 Synthesis of dietary compounds

Microbe-host interactions are mutualistic. In fact, commensal intestinal bacteria help during the digestion and synthesis of dietary compounds, some of which could not be otherwise available, and energy metabolism. In return the host provides to the microbes a secure growth conditions and a constant stream of nutrients (Waite and Taylor, 2014). However, the amounts and types of compound produced trough bacterial fermentation depend on relative amounts of each substrate available and fermentation strategy of bacteria involved in the fermentation process (Waite and Taylor, 2014). Some of the principal end products of intestinal microbial fermentation are short-chain fatty acids, vitamins and protein degradation products.

Vitamins are critically involved in regular energy metabolism and enzymatic functions important for gene expression. Deficiency of one or more water-soluble vitamins can contributes to various diseases and dysfunctions. An adequate supply of vitamins obtained by dietary intake seems necessary to ensure sufficient vitamin status, but microbiota can also act as an important supplier of vitamins. (Biesalski, 2016). Some bifidobacterial species are claimed to convert a number of dietary compounds into health-promoting bioactive molecules, such as conjugated linoleic acid and certain vitamins. Such findings have been confirmed by *in vivo* studies: administration of high-producing folate strains was shown to cause an increased faecal level of folate in rats (Pompei et al., 2007a, Pompei et al., 2007b). Folate biosynthetic properties of bifidobacteria, though folate de-novo

biosynthesis, appear to be restricted only to certain species/strains, while other species are capable of folate biosynthesis just in the presence of para-aminobenzoic acid (pABA) (LeBlanc et al., 2013). In fact, *Bifidobacterium adolescentis* ATCC15703 and *Bifidobacterium dentium* Bd1 are the only strains in which genome possesses the genetical determinants for entire de novo-pathway for pABA biosynthesis. No complete pathways for the biosynthesis of biotin, panthothenate, pyridoxine, cobalamin and menaquinone are present in any of the so far sequenced bifidobacterial genomes. Lactobacilli do not appear to harbour the genetic determinants for *de novo* pABA synthesis, except for *Lactobacillus plantarum* WCFS1, suggesting that the vast majority of Lactobacilli are unable to synthesize folate in the absence of pABA (LeBlanc et al., 2013; Ventura et a., 2009).

Regarding instead riboflavin, the enzymes needed for the biosynthesis of this vitamin seem to be partially or completely absent from most of the currently available bifidobacterial genomes (Ventura et al., 2009). Cobalamin is the only vitamin that is exclusively produced by microorganisms, particularly by anaerobes, in fact the commercial vitamin B12 is bacterial produced. *Lactobacillus reuteri* CRL1098 is able to produce a cobalamin-like compound with an absorption spectrum closely resembling that of standard cobalamin, but with a different elution time. The asset of 30 genes, involved in the B12 vitamin biosynthesis of *Lactobacillus reuteri* CRL1098 is similar to those found in *Salmonella enterica* and *Listeria innocua* genome, with the exception of hem genes location on their genome that appear to be different. Propionibacteria and *L. reuteri* are normally present in the intestine and may thus (partially) fulfil the vitamin B12 requirement of the host (Santos et al., 2007; LeBlanc et al., 2013). Gut microbiome of poultry may also serve as a vitamin (especially B vitamins) supplier to its host. Similar as bacterial protein, most of the vitamins synthesized by gut bacteria are excreted with feces because they cannot be absorbed in the cecum. However, coprophagic birds may benefit from bacterial vitamin synthesis. This is evidenced by a greater vitamin requirement by chickens housed in wire cages, where coprophagy is prevented, than by chickens raised on hard floors (Pan and Yu, 2014).

Aside from vitamins, short-chain fatty acids (acetate, butyrate, propionate, succinate, and lactate) represent relevant end products of intestinal microbial carbohydrate fermentation that benefits the host. Short-chain fatty acids are mainly used by the host as source of energy but they can bring even other benefits as reducing pH of the intestinal environment in chicken cecum, potentially inhibiting acid-sensitive pathogenic bacteria, such as members of the family Enterobacteriaceae, by dissipating the proton motive force across the bacterial cell membrane (van Der Wielen et al., 2000). Butyrate is used from the host enterocytes as energy source. Furthermore, it contributes to the regulation of cellular differentiation and proliferation within the intestinal mucosa, consequently increasing

intestinal tissue weight. The contribution of butyrate and other SCFA to epithelial development is essential in the maintenance of normal intestinal barrier functions and, therefore, indirectly in the protection of the host from the pathogens (Rinttilä and Apajalahti, 2013). Although butyrate production is distributed across many Clostridial clusters like the IV and the XIV, belonging to phylum Firmicutes, it is mainly produced by members of *Roseburia* spp. and *Eubacterium rectale*, both members of the family Lachnospiraceae, and especially from *Faecalibacterium prausnitzii* of family Ruminococcaceae (Rinttilä and Apajalahti, 2013; Miquel et al., 2014).

The production of short chain fatty acids by bacteria's fermentation can be observed in most part of the avian gut, but primarily takes place in the cecum, which is the most densely populated and diversified ecosystem in the gastrointestinal tract. However, fermentation increases as young birds grow. In fact, cecal acetate, propionate and butyrate are almost undetectable in 1-d-old broilers. As the cecal microbiota becomes established, these short chain fat acids reach high concentrations in 15-d-old broilers and remain stable afterwards. Chicken microbiome produce greater concentrations of short chain fatty acids than human microbiome (Rehman et al., 2007; Pan and Yu, 2014).

Lactic acid is another compound produced by gastrointestinal bacteria and particularly from lactic acids bacteria. This compound would tend to reduce residual pH more than other short chain fatty acids but it is normally absorbed from the intestine or used as a substrate for lactate-utilizing bacteria, such Eubacterium, Anaerostipes, Veillonella, and Megasphaera genera, quickly enough to not being able to pathologically acidify the gut environment (Harmsen et al., 2002; Belengueret al., 2007; Rinttilä and Apajalahti, 2013).

Another noteworthy microbiota's metabolism is the protein and amino acid fermentation in the lower intestine. The real role of this metabolism lies, more than in the energy or compound production, in the fermentation, by putrefactive bacteria, of potential systemic toxins and carcinogens resulted from the protein catabolism. In fact, in broiler chicken cecum once carbohydrate sources are exhausted, sources of protein material are fermented and metabolized to salvage energy but at that gastrointestinal level, proteins and amino acids provide a less significant energy source. Common examples of undesired metabolic end products include phenols and indoles (as a result of anaerobic fermentation of the aromatic amino acids, tyrosine and tryptophan, by intestinal bacteria), ammonia (as a result of oxidative or reductive deamination of amino acids), and amines (as a result of amino acid decarboxylation in the gut). These compounds other than be toxic could result in an increase the pH of intestinal contents, but a low pH is beneficial for the suppression of the growth of the acid-sensitive pathogenic microorganisms. As the bacteria generally favor fermentable sources of carbohydrates, protective measures against excessive putrefactive activity and the undesired

metabolites in the hindgut can be achieved by adding dietary fiber in the diet or limiting the intake of poorly digested proteins (Apajalahti, 2005; Rinttilä and Apajalahti, 2013).

Gut bacteria also contribute to host nitrogen metabolism. Since in birds the intestinal and urogenital tracts meet in the cloaca where urine mixes with feces, some urine, may travel to the ceca due to the retrograde peristalsis in the rectum. Cecal bacteria can then catabolize uric acid to ammonia, which can be absorbed by the host and used to synthesize a few amino acids such as glutamine (Pan and Yu, 2014). Some of the dietary nitrogen is incorporated into bacterial cellular proteins. Therefore, gut bacteria themselves can be a source of amino acids. However, the majority of these bacterial proteins are lost by the host with the excretion of feces, because most of the intestinal bacteria in birds reside in the cecum which does not have the ability to digest and absorb proteins. Utilization of bacterial proteins is possible when chickens are housed on hard floors, where coprophagy (ingestion of feces) can occur and bacterial proteins can be digested and absorbed in proximal gut (Pan and Yu, 2014).

1.4 Factors affecting and modulating the chicken GI microbiota

The intestinal track of poultry harbors a complex and dynamic microbiota that has a symbiotic relationship with its host. The interaction between host and microbiota affects the physiological, immunological, and nutritional status of the host and consequently his growth performance and health status. The evidence for metabolic interactions is particularly strong, as many data support the conclusion that gut microbiota influences the energy harvest from dietary components, particularly complex carbohydrates, and that metabolites, such as the short-chain fatty acids produced by gut bacteria, can perturb metabolic traits. The gut microbiota communities are assembled each generation, since their composition is influenced by environmental factors, age and diet (Wei et al., 2013b; Org et al., 2015). In particular, the symbiotic equilibrium between host and microbiota is established soon after hatching and later on bacteria present in microbiota can be affected by a range of factors, such as host genetics and age, litter management, diet, and feed additives. Since the gastrointestinal microbiota is strongly related to several host functions, numerous efforts have been attempted on manipulation and control of the exogenous factors, especially dietary intervention and litter management, in order to modulate its composition to enhance feed conversion and gut health (Wei et al., 2013b; Pan and Yu, 2014; Blottière Doré, 2015; Wang et al., 2016).

1.4.1 Environment, age and diet

For commercial chickens, the environment is represented by the litter. Litter can have a significant effect on the initial composition and structure of the microbiota gastrointestinal tract of chickens, while later on the main role in the microbiota shaping process is played by the diet. In fact, when young chicks are delivered from the hatchery their gastrointestinal tract contains very small number

of bacteria belonging to a few species, and just when they begin pecking at and consuming litter materials they inoculate their young gastrointestinal tract from the litter (Wang et al., 2016). If hatching takes place in an environment where the microbial load has been minimized, individual chicks may pick up a random inoculum from their surroundings, which may lead to differences in the intestinal physiology of individual birds in a flock. It is worth noting that intentional inoculation of the chicks with a competitive exclusion culture at hatch might render a flock microbiota composition more uniform (Rinttilä and Apajalahti, 2013). Poultry litter is a mixture of bedding materials and in the USA, where the litter is changed every 6 cycles, even of chicken excreta that contain chicken GI bacteria, undigested feed, uric acid, and other substances of host origin. Several studies have documented that poultry litter contains a complex and dynamic microbiota, composed primarily of environmental bacteria and its composition can be affected by the bedding materials used (Lu et al., 2003; Torok et al., 2009). Repeated use of poultry litter and poor litter management can result in considerable changes in microbiological conditions leading to an increase of density and diversity of microbes. In addition, reused litter can serve as a driving force that shapes the chicken GI microbiota because exposing young chicks to different bacterial inocula can profoundly affect GI microbiota development (Cressman et al., 2010). It was shown that reused litter harbors less *Salmonella* and *Clostridium perfringens* but enables *Campylobacter jejuni* and *C. coli* to survive longer compared to fresh litter (Kassem et al., 2010; Roll et al., 2011; Wei et al., 2013a). Moreover, two recent studies have shown that reused litter can affect the immune system of chickens, which suggests that litter conditions can also affect the GI microbiota of chickens indirectly through their immune system (Lee et al., 2011; Shanmugasundaram et al., 2012).

Cressman et al., (2010) examined the microbiota both in the GI tract and in the poultry litter and their interaction revealing that the litter microbiota and the GI microbiota affected each other in a reciprocal manner. In fact, fresh litter resulted in increased diversity and predominance of environmental bacteria in the GI microbiota of young chicks, while reused litter increased the bacteria of gut origin. Another study conducted by Wang et al., (2016), investigating the effect of fresh and reused litter on chicken gastrointestinal microbiota at different ages, showed that the ileal mucosa and the cecal contents were affected by both litter management regimen and age of birds. At days 10 and 35, in the cecal luminal microbiota eight and three genera, respectively, differed significantly in relative abundance between the two litter management regimens. Compared to the fresh litter, reused litter increased predominance of halotolerant/alkaliphilic bacteria and *Faecalibacterium prausnitzii*, a gut butyrate-producing bacterium. This study suggests that litter management regimens affect the chicken GI microbiota, which may impact the host nutritional status and intestinal health.

Torok et al., (2009) investigated linkages between litter material, gut microbiota and chicken growth performance. Cecal microbial populations were investigated at 14 and 28 d of age and at both ages. The caeca microbiota of chickens raised on reused litter was significantly different from that of chickens raised on any of the other litter materials, except for softwood shavings at d 28. However, age had a significant influence on ceca microbiota composition regardless of litter material. We can conclude that the environment and consequently the type of litter material and management, can influence colonization and development of cecal microbiota in chickens. Litter-induced changes in the gut microbiota may be partially responsible for some of the significant differences observed in early rates of growth; therefore, litter choice may have an important role in poultry gut health particularly in the absence of in-feed antibiotics (Torok et al., 2009).

Gong et al. (2008) examined the effect of dietary bacitracin, bird age and access to range on the richness and microbiota community structure concluding that age had the most profound effect on microbiota composition. This is demonstrated clearly since most birds of the same age were grouped together and, regardless of access to range or dietary treatment, the richness of microbiota increased as the birds grew older. Furthermore, they showed that chickens at 42 days of age had a well-developed bacterial microbiota in both ileum and caeca and at 14 days the development of caecal bacterial microbiota was close to that of 42-day-old chickens, while the ileal microbiota did not appear to be fully developed, a stage which could be more sensitive to dietary treatments and other environmental factors. It is worth pointing out that, the abundance of Lactobacilli in the caecal microbiota was greatly higher in 3-day-old chickens than in 42-day-old chickens, suggesting the importance of Lactobacilli in the early development of caecal microbiota while, on the contrary, the abundance of Bifidobacteria population in the ileum and caeca were hardly detected in 3-day-old chicks and high in 42-day-old chickens.

Regarding the immature chicken microbiota, Lu et al. (2003b) reported that 3-day-old broiler chickens had a similar community structure of bacterial microbiota in ileum and caeca and the caecal microbiota was a subset of the ileal microbiota during the first 14 days of age based on the metataxonomic analysis of random clone libraries of partial 16S rRNA genes. On the contrary, Gong et al., 2008 in their study, reported that PCR–DGGE profiles of bacterial microbiota from the ileum and caeca of 3-day-old chicks were significantly different, suggesting two different bacterial communities in these two intestinal regions at that age.

The correlation between the advancing of age and the increase of microbiota composition richness were reported even by other authors, like Van der Wielen et al. (2002), Knarreborg et al. (2002), Van Wielen et al. (2002) and Hume et al. (2003). Van der Wielen et al., 2002 found that the PCR–DGGE

profiles of the microbiota were similar in crops, duodenum and ileum in 4-day-old chicks, but observed an increased diversity of bacterial microbiota in crops, duodenum, distal ileum and caeca when broiler chickens aged. All these studies highlight that, regardless the influence of other factors, microbiota changes and matures on time during the chicken's life.

Another factor that can influence the microbiota composition is host's genotype. The role of host genetics in shaping microbial communities' composition is not clear but it could be speculated that the host may affect its microbiota composition either directly, through secretions into the gut, control of gut motility and modification of epithelial cell surfaces, or indirectly, through food and lifestyle preferences (Zhao et al., 2013). Zhao et al. (2013) conducted an experiment using next generation sequencing technology to investigate the effect of genetic on the gut microbiota's population structure in two different lines (56-day high or low body weight) of adult chickens. The pattern of host genetic influence was different in adult males and females, demonstrating gender as a factor that impacts the composition of gut microbiota. Of 190 species, 68 were affected by genotype (line), gender and by genotype and gender interactions, where 15 of the 68-species belonged to *Lactobacillus*. In fact, of host-microbe interactions, *Lactobacillus* was the major influenced genus showing different abundances between low body weight males and high body weight females. Beside environment, age and genotype the chicken gut microbiota composition is mainly shaped by diet and feed additives.

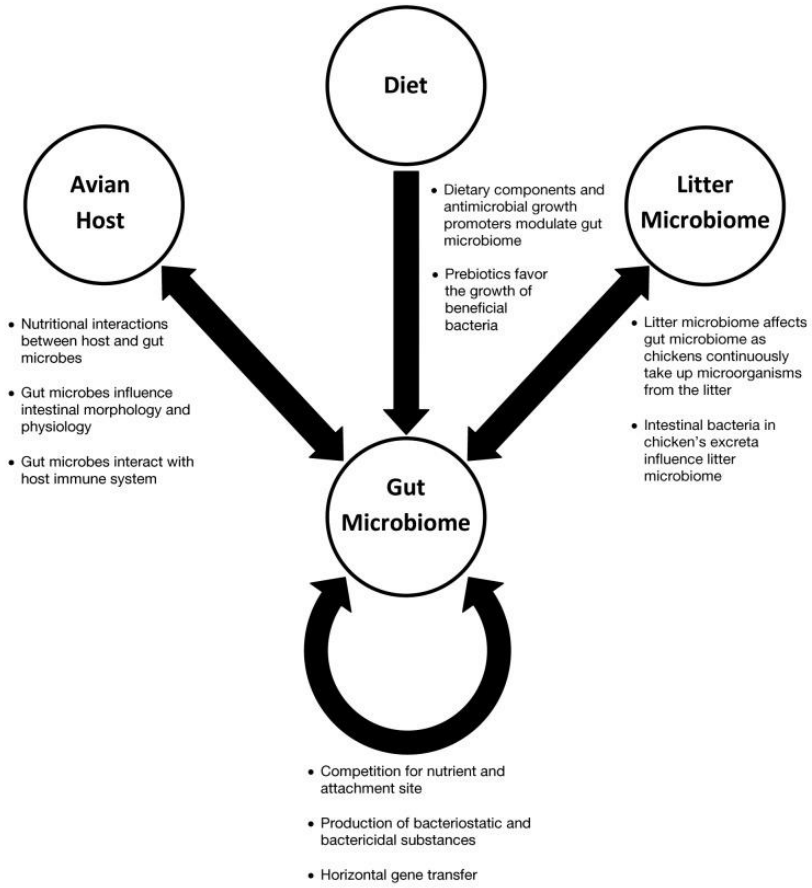


Figure 1. Interactions among gut microbiome, avian host, diet, and litter microbiome (Pan and Yu, 2014)

1.4.2 Diet and feed additives

Diet is the most relevant factor impacting on intestinal microbiome in poultry as dietary components that escape host digestion and absorption serve as substrates for the growth of intestinal bacteria (Pan and Yu, 2014). In fact, several studies already demonstrated its potential to impact the chicken GI microbiota with respect to diversity, composition, and structure. It was also showed that the same action can be performed by feed additives (Amerah et al., 2011; Danzeisen et al., 2011; Rodriguez et al., 2012; Wang et al., 2016).The first studies focused on how feed and feed additives affect the prevalence of enteric pathogens, such as *Salmonella* (Santos et al., 2008), *Clostridium perfringens* (Si et al., 2009; Wei et al., 2013a), and *Campylobacter jejuni* (Ridley et al., 2011), while the prevalence of these pathogens could be decreased by the effect of a healthy GI microbiota. In fact, microbiota can perform colonization resistance and competitive exclusion to inhibit the pathogen growth (Wagner, 2006; Kerr et al., 2013). Furthermore, other than creating this positive barrier, the commensal bacteria can positively affect the efficiency of feed utilization by the chicken host. That's why, now the interest of the researchers shifted towards the understanding of how diet and feed

additives, could modulate the GI microbiota of chickens instead of just focusing on their effect on pathogens bacteria (Gong et al., 2008; Santos et al., 2008; Danzeisen et al., 2011).

One of the most remarkable example of how diet can modulate the microbiota, is represented by the use of diets containing high levels of indigestible, water-soluble, non-starch polysaccharides as wheat-, barley-, or rye-based diets, that favor the proliferation of *Clostridium perfringens* and predispose young chicks to necrotic enteritis, while diets poor in non-starch polysaccharides, such as corn-based diets, do not. In fact, this proliferation can be due to increase of digesta viscosity, decrease of digesta passage rate and a decline in nutrient digestibility caused by high level of non-starch polysaccharides, supporting the growth of *Clostidium perfringens*. When compared with corn-based diet, wheat-based diets also affect a number of other bacteria (Annett et al., 2002; Pan and Yu, 2014).

In a study conducted in 2010, Hammons et al. showed that even a small variation in dietary cereal grain composition can potentially affect the intestinal bacteria at species and strain levels. In fact, they showed that a standard corn-soybean ration supported *Lactobacillus agilis* type R5, whereas a ration high in wheat favored *L. agilis* type R1. In order to see the effect of grain base on the microbial community profile Apajalahti et al., (2004), analyzed 256 caecal samples of broilers being fed either wheat or corn based diet from all around the world. The % G+C profiling method used to reveal the most significant sources of variation showed that the two grain bases favored different bacterial groups in the caecum. This analysis did not reveal the identity of the bacteria, but it was possible to establish that corn favors low G+C Clostridia, Enterococci and/or Lactobacilli and wheat improves higher %G+C Bifidobacteria.

Another dietary nutrient category that can affect gut microbiota can be the protein. In fact, the source and level of dietary protein have been demonstrated to stimulate the proliferation of different bacteria populations. Sun et al. (2013) noticed that the use of fermented cottonseed meal as protein source, instead of soybean meal which is widely used as a source of protein in poultry production, increases the population of Lactobacilli and decreases the number of coliforms in cecum of broiler chickens. Furthermore, other than water-soluble and non-starch polysaccharides, even diets containing high percentages of animal protein support the growth of *Clostridium perfringens* in the chicken gut and are considered as one of the predisposing factors of necrotic enteritis. In addition, it has been reported that *C. perfringens* proliferation can be improved even by dietary fat source, as it was more abundant in the ileum of broiler chickens fed diet with animal fat than chickens fed diet with soy oil (Pan and Yu, 2014).

However, not only the nutrients inside the diet affect the microbiota composition, even processing significantly affects the characteristics of the feed as a substrate for the bacterial community. If the

bacterial shifts following different set of feed processing conditions were understood, the manufacturing process itself could be used to partly control and manage the gastrointestinal microflora trough the identification of signature species. Apajalahti et al. (2004) investigated the changes caused by inclusion of whole wheat in the feed on the bacterial community structure in chicken caecum, giving an overview of the effect of processing procedure on microbial community compositions. They showed that grinding and processing of wheat affect wheat characteristics and act as a microbial modulator. In particular, they investigated the changes caused by of feed amendment with the addition of whole wheat, compared to the commercial feed with no amendments, on microbiota using the G+C% profiling. Effect of whole-wheat addition on microbial community structure was statistically significant and in particular bacteria with %G+C ranging from 35 to 54 were stimulated by whole wheat, while those with %G+C between 60 and 69 were suppressed (e.g., Bifidobacteria).

Another way to influence gut microbiota in poultry, reducing enteric pathogens and increasing growth performances, is using feed additives. In the past growth-promoting antibiotic (AGP), as feed additives, were added in the feed to gain effects on gastrointestinal microflora and performance. These effects were obtained because growth-promoting antibiotic reduced competition for nutrients in the small intestine, reduced local inflammation due to control of pathogens, and reduced intestinal thickening and length, as a result of improved digestibility and reduced pathogen loading (Thomke and Elwinger, 1998b; Thomke and Elwinger, 1998a). The latter two mechanisms result in a more efficient digestion and reduced maintenance energy requirement (Apajalahti et al., 1999; Apajalahti et al., 2004). However, it was hypothesized by Niewold (2007) that a different mechanism is behind the AGP positive effect on animals' growth performances. According to the author, the different microbial compositions when using AGP are a consequence of an altered immune status of the host rather than of a direct effect on the microbiota. The changes in microflora are most likely the consequence of an altered condition of the intestinal wall due to the anti-inflammatory effect on intestine cells. Growth promoter's antibiotic have long been supplemented to poultry feed to stabilize the intestinal microbial flora, improving the general performances and prevent some specific intestinal pathology. However, due to the growing concern about microbes resistant to antibiotics used to treat human and animal infections, the European Commission (EC) decided to phase out, and ultimately ban (1 January 2006), the marketing and use of antibiotics as growth promoters in feed (EC Regulation No. 1831/2003; http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2003:268:0029:0043:EN:PDF).

Since the ban of AGP as feed additives for livestock production, the research of new molecules able to selectively promote the growth of microbial populations related with positive effect on animals' growth performances and health has always be continuous (Butaye et al., 2003; Huyghebaert et al., 2011). Whatever the mechanism of action of AGPs, the main characteristic of a good alternative, from a practical point of view, should be that it must positively modulate microbiota composition improving good microbial populations and reducing pathogens. Good alternatives to AGPs to influence the intestinal microbiota population is using probiotic and feed enzymes.

Modulation of the intestinal bacteria by feeding probiotics is currently under active research (Garriga et al., 1998; Jin et al., 1998; Gusils et al., 1999; Samli et al., 2007; Gérard et al., 2008; Nakphaichit et al., 2011; Babot et al., 2014; Pedroso et al., 2016; Hu et al., 2017). The target of such nutraceutical products is to improve gastrointestinal health by selecting for beneficial microflora and suppressing known intestinal and food-borne pathogens. Direct-fed microbials (probiotics) are products which are targeted to improve the health of the gastrointestinal tract, but these are likely to be effective only if the requirements for their growth are fulfilled. Many of these benefits apply also to the use of feed enzymes. Dietary enzymes, such as xylanase and β-glucanase, has already been showed to increase intestinal lactic acid bacteria and decrease the population of adverse and pathogenic bacteria, such as *E. coli* (Rodríguez et al., 2012). Dietary supplementation with xylanase and β-glucanase can also offer chickens some protection against necrotic enteritis as the enzymes breakdown the non-starch polysaccharides in the diet and reduce the digesta viscosity. Furthermore, feed enzymes have the ability to remove fermentable substrate from the small intestine, that could be an optimal substratum for some pathogens growth requirements (Apajalahti et al.,2004; McDevitt et al., 2006; Owens et al., 2008).

The role of feed enzymes in improving the productive value of diets for monogastric animals has received extensive reviews, and several modes of action have been proposed. They include hydrolysis of specific chemical bonds in feedstuffs that are not sufficiently degraded or indeed not at all by the animal's own enzymes (for example, mixed salts of phytic acid); the elimination of the nutrient-encapsulating effect of the cell wall polysaccharides and therefore increased availability of starches, amino acids and minerals; the breakdown of anti-nutritional factors that are present in many feed ingredients (for example, soluble NSP and phytic acid) and the complementation of the enzymes (for example, amylase, protease, lipase) produced by young animals where, because of the immaturity of their own digestive system, endogenous enzyme production may be inadequate. For example, the indigestibility of some protein contents could limit the inclusion of these nutrients into pig feed. However, the supplementation of proteases might allow high inclusion of such feedstuffs. However,

it must be taken into consideration that the supplementation of any feed enzyme does not just impact directly animal nutrition but, since the gastrointestinal tract is densely populated, it will impact also the microbiota. Since microbiota plays a critical role for animal nutrition, performance and safety of animal products, there is a clear need to understand the role of feed enzymes in influencing gut health through its modulation (Kiarie and Nyachoti, 2009; Kiarie et al., 2013). The feed additives whose modulating action on chicken gut microbiota will be analysed in this study are a probiotic (i.e., *Lactobacillus acidophilus*) and two feed enzymes, a commercial protease and a commercial phytase, alone and combined with inositol.

1.5 Analysis of chicken gut microbiota: from traditional techniques to metagenomic analysis

One of the most remarkable events in the field of microbial ecology in the past decade has been the advent and development of metagenomics. Metagenomics is the study of the metagenome (microbiome). Metagenomics can either be targeted (usually 16S ribosomal RNA) or untargeted (shotgun sequencing) (Tremaroli and Bäckhed, 2012). Metagenomics provides both access to the functional gene and the composition of microbial communities, within an environmental sample (Thomas et al., 2012). Metagenomic analysis has recently developed. Previously microbial communities were characterized culturing them on selective growth media and subsequently carrying out a range of biochemical tests to identify the bacteria that survived under the specific culture conditions employed. Such methods, other than being laborious and time consuming, were not suitable for extensive monitoring of the unknown microflora, because only a small fraction of bacteria composing the community could be found. In fact, up to 99% of the bacteria in many environments fail to grow under artificial conditions. This disadvantage is due to the growth requirements of most bacteria that are still unknown or cannot be mimicked under laboratory conditions, leading to an incomplete data recovery regarding the whole community. Since microbial communities have individual bacterial members specialized on different functions and providing elements to other bacterial members, conducting a metagenomic analysis investigating these communities' dynamic and interactions with the omission of unculturable populations would have been impossible (Apajalahti et al., 1999; Apajalahti et al., 2001; Apajalahti et al., 2004).

That is why, in the late 1970s, Woese and Fox proposed the use of ribosomal RNA genes as molecular markers, revolutionizing the classification of microorganisms. Some decades later, advances in molecular techniques were applied to microbial diversity description and granted access to a "new uncultured world" of microbial communities. DNA-based culture-independent methods' basic principle was the analysis of the bacterial DNA without harvesting it from *in vitro* isolated pure cultures. Total bacterial DNA is directly recovered from a sample derived from the site of interest,

extracted and then analysed. Some of these techniques were the polymerase chain reaction (PCR), fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE and TGGE), restriction-fragment length polymorphism, and terminal restriction-fragment length polymorphism (T-RFLP) (Woese and Fox, 1977; Apajalahti et al., 2001; Apajalahti et al., 1999; Apajalahti et al., 2004; Hiergeist et al., 2015; Escobar-Zepeda, 2015).

The target for many of the molecular profiling techniques is the 16S ribosome and its encoding gene. In bacteria, the three rRNA molecules are genetically organized in a ribosome operon and primarily transcribed as a single 30S rRNA precursor that is subsequently cleaved by RNase III into 16S, 23S, and 5S rRNA subunits. Operon size, sequences, and secondary structures of these three rRNA genes are conserved within a bacterial species. The 16S rRNA gene contains both variable regions and conserved regions, allowing the design of PCR primers which target all or specific bacterial DNA. The 16S rRNA gene is constituted of nine variable regions (V1-V9), where the V1 region was found to be the most variable, followed by V9 and then by V3 (Yu and Morrison, 2015; Hedgiest., 2015). For more than 30 years, culture-independent microbial profiling has been based on the 16S ribosomal rRNA gene (Olsen et al. 1986). By doing this, researchers received a key tool to species and phylogenetic trees identification by comparing these relatively stable parts of the genome. However, these 16S ribosomal rRNA gene based technologies, other than being low-throughput technologies, could not deliver exhaustive insight into microbial diversity and metabolic and ecological functions, making impossible to deduce the potential biological tasks carried out by a community as a whole (Woese and Fox, 1977; Escobar-Zepeda, 2015; Hiergeist., 2015). In fact, metagenomics provides access to the functional gene composition, e.g. metagenome, of microbial communities and therefore gives genetic information on potentially novel biocatalysts or enzymes, genomic linkages between function and phylogeny for uncultured organisms, and evolutionary profiles of community function and structure, a much broader description than phylogenetic surveys based on 16S rRNA gene (Thomas et al., 2012).

In 1990, for the first time, clone libraries of 16S rRNA genes from environmental bacteria were directly amplified and sequenced by the Sanger method (Giovannoni et al., 1990). This procedure represented a breakthrough that permanently changed the way prokaryotes in the environment were analyzed, leading to the advent of the metagenomic analysis era trough sequencing techniques (Hiergeist., 2015). Sanger and others introduced the concept of DNA sequencing called the chain-terminator method. This first-generation sequencing technology is based on incorporation of fluorescently labelled deoxynucleoside triphosphate and primers into a PCR that set the stage for automated high-throughput DNA sequencing. With the information obtained from the last terminator

base in the four individual base reaction tubes after size separation, the original sequence could be determined (Sanger and Coulson, 1975; Sanger et al., 1977). However, Sanger sequencing method presented a high number of limitations and disadvantages, mainly associated with the low throughput of DNA sequences obtained and the high cost. That's why others sequencing technologies were developed during the following years (Schloss, 2008; Metzker, 2010).

Particularly relevant was the development of pyrosequencing technique, also called sequencing by synthesis, that permits the detection of pyrophosphate released when a nucleotide is incorporated in the chain resulting in detectable light in a real-time format. Improvements and development of this technology resulted in advances in the next-generation devices based on the same principle, leading to so-called next generation HT-NGS platforms produced by Roche, Illumina-Solexa, Life Technologies, Helicos, and other companies.

Illumina sequencing technology is based on reversible dye-terminators principle and can perform shotgun High throughout Whole Genome sequencing. In fact, in shotgun sequencing random DNA fragments are immobilizes on a surface and then a solid-surface PCR amplification is performed with the result of clusters of identical DNA fragments. These are then sequenced with four types of reversible terminator bases in a sequencing-by-synthesis process. After the incorporation of reversibly terminating nucleotides, a camera capture images of the fluorescence and the dye along with the terminal 3′ blocker is chemically removed from the DNA allowing the next cycle. Clustered fragments can be sequenced from both ends (pair-end mode) and the cluster density is enormous, with hundreds of millions of reads per surface channel. The read length can be different in relation to the Illumina instrument and the sequencing mode chosen but, in any case, it is relatively short compared to other sequencing technologies read length. Yields of ~60 Gbp can therefore be typically expected in a single channel. In fact, this technology can sequence the equivalent of one-third of the entire human genome in a single run (approx. 10 days), while the sequencing of the entire human genome with the sanger method lasted 10 years and costs 2 billion dollars. The lower costs of this technology and recent success in application to metagenomics, are currently making the Illumina technology an increasingly popular choice. The only limitation of Illumina technology is the read length. In fact, a limited read length means that a greater proportion of unassembled reads might be, after the quality clipping of the first bad quality sequences of the reads, too short for functional annotation. However, some current software packages (e.g. MG-RAST and Mg-Mapper) are designed to analyze unassembled Illumina reads of 75 bp and longer, bypassing this limit (Thomas et al., 2012; Diaz-Sanchez et al., 2015).

Next-generation sequencing platforms (NGS) have allowed the substantial researches into the diversity and functions of microbiota from the guts of various livestock animals. High-throughput NGS generates large volumes sequence data containing genetic information and this allows hypothesis-driven researches on chicken GIT microbiota, thereby highlighting the roles of previously unknown and rare microbial GIT species. Furthermore, metagenomic data have raised new questions such as how microbiota stability and ecological shifts in species diversity are influenced by nutrients and hosts (Metzker, 2010; Medinger et al., 2010; Choi et al., 2015).

The application of HT-NGS sequencing is emerging and moving toward the development and the improvement of the poultry industry raising the food safety measures and avoiding foodborne pathogens. Relative few studies have been conducted on chicken gut microbiota to determine any changes that affect health and disease and a detailed assessment of probiotics or/and other feed additive to control pathogenic growth and or shaping the gut microflora, trying to lead to the development of novel alternatives to antibiotic growth promoters. Frequently, the sequencing methods used to gain data regarding the microbiota composition, aside from the platforms, are the small-subunit ribosomal RNA (16S rRNA gene) amplicons or shotgun metagenomic sequencing (Diaz-Sanchez et al., 2013).

1.5.1 16S rRNA gene based metataxonomic analysis

Amplicon sequencing is the most widely used method for characterizing the diversity of microbiota, even in chicken. For bacteria and archaea classification, the small-subunit ribosomal RNA (16S rRNA gene) locus is targeted and amplified by PCR. The obtained amplicons are sequenced and characterized to determine microbial community composition and population relative abundance (Pace et al., 1986; Hugenholtz and Pace, 1996). Comparing 16S rRNA gene based metataxonomic analyses profiles across samples clarifies how microbial diversity is associated with environmental conditions generating insight into host–microbe interactions and yields hypotheses about microbiota-based disease mechanisms (Muegge et al., 2011; Sharpton, 2014). However, amplicon sequencing presents several limitations. In fact, it may fail to resolve a substantial fraction of diversity in a community because of various biases associated with PCR and can produce widely estimates of diversity (Hong et al., 2009; Sharpton et al., 2011; Sharpton, 2014; Jumpstart Consortium Human Microbiome Project Data Generation Working Group, 2012). Furthermore, amplicon sequencing only provides insight into the taxonomic composition of the microbial community, with taxa for which taxonomically informative genetic markers are known, and sometime result in an overestimation of community diversity, since the 16S rRNA gene locus can be transferred between distantly related taxa. Another limitation is the impossibility to provide information on the biological

functions associated with the single population. In some cases, phylogenetic reconstruction can be used to infer those biological functions that are encoded in a genome containing a particular 16S rRNA gene sequence (Langille et al., 2013) but the accuracy depends on how well the genomic diversity of the community is represented by the genomes available in sequence databases.

1.5.2 Shotgun whole genome metagenomic sequencing

Shotgun whole genome sequencing is an alternative approach that can avoid all 16S rRNA gene metataxonomic amplicon sequencing limitations. In fact, instead of targeting a specific genomic locus, all DNA is subsequently sheared into tiny fragments that are independently sequenced making possible to sequence numberless genomes present in the sample, including non-microbes or microbes with unknown taxonomically informative genetic markers. The DNA sequences are sampled from taxonomically informative genomic loci (e.g., 16S rRNA gene) and from coding sequences that provide insight into the biological functions encoded in the genome. However, even if using shotgun whole genome sequencing the metagenomic data do not present the previously described limitations, there are anyways several challenges. Metagenomic analysis tends to require a large volume of data to identify meaningful results because of the vast amount of genomic information being sampled. Furthermore, the data, other than representing a big amount, are relatively complex and large, sometimes making it difficult to determine the genome from which a read belongs. That's because such rich diversity of genomes can result in a not complete representation of all communities and two reads from the same gene may not overlap and are thus impossible to directly compare through sequence alignment. However, if two reads do overlap, it is not always evident if they are from the same or distinct genomes, which can challenge sequence assembly (Mavromatis et al., 2007; Schloss and Handelsman, 2008; Sharpton et al., 2011; Mende et al., 2012; Sharpton, 2014). Another challenge is represented from eventual contamination, since, once the genetic material is sequenced, is particularly difficult to discriminate the contaminant's reads. This contamination could mislead analyses of community genetic diversity if the contaminant's genome is enriched of genes that are uncommon in the community and is highly abundant or has a large genome. However, other than limiting the contamination applying good sampling and DNA extraction practices, bioinformatic software allows identification and filter of contaminant and host sequences in the metagenomic data (Schmieder and Edwards, 2011). Shotgun whole genome sequencing, for the advantages regarding the large amount of data generated, has become the most used methods for most laboratories, even because the limits are lately being overcome by the development in bioinformatics. In recent years, metagenomic sequencing has been used to identify new viruses, characterize the genomic diversity

and function of uncultured bacteria, reveal novel and ecologically important proteins, and identify taxa and metabolic pathways that differentiate gut microbiota (Sharpton, 2014).

1.5.3 Sequences' data analysis and MG-RAST platform

When performing shotgun metagenomics, the complete sequences of protein coding genes (previously characterized or novel) as well as full operons in the sequenced genomes can offer invaluable functional knowledge about the community. The first approach to analyse the output data of sequencing is the assembly of shorter reads into genomic contigs and orientation of these into scaffolds to provide a more compact and concise view of the sequenced community under investigation. The process of assembling shorter reads into contigs can be performed using reference-based assembly or *de novo* assembly. Reference-based assembly refers to the use of one or more reference genomes as a "map" in order to create contigs, which can represent genomes or parts of genomes belonging to a specific species or genus. The tools commonly used in metagenomics for performing referenced-based assemblies are not computationally intensive and perform well when metagenomic samples are derived from extensively studied areas. In that case the sequences from closely related organisms would have already been deposited in online databases, allowing them to be used as references for the assembly process.

De novo assembly refers to generation of assembled contigs using no prior reference to know genome(s). This task is computationally expensive and relies heavily on sophisticated graph theory algorithms, such as de-Bruijn graphs tools. These tools initially were built for assembling a single genome and often underperform when used for metagenome assemblies. This limitation led to the development of the next generation of assembly tools, such as MetaVelvet and Meta-IDBA. MetaVelvet and Meta-IDBA employ a combined binning and assembly approach to create more accurate assemblies from datasets containing a mixture of multiple genomes. They make use of k-mer frequencies to detect kinks in the de-Bruijn graph and then use these k-mer thresholds to decompose the graph into subgraphs. These tools further assemble contigs and scaffolds based on the decomposed sub-graphs, and thus perform a more efficient grouping/assembly of contigs, effectively separating those belonging to different species (Zerbino et al., 2008; Miller et al., 2010; Peng et al., 2010; Thomas et al., 2012; Oulas et al., 2015).

The annotation of metagenomes is the alternative, specifically designed to work with mixtures of genomes but requires some pre-processing step to prepare the reads for annotation. Briefly, the first step is the trimming of low-quality reads based on the Phred or Q quality scores, the thresholds of which depend on the sequencing technology, followed by the masking of low-complexity reads process and a final de-replication step that removes sequences that are more than 95% identical. The

last step is the screening of the sequences performed by tools like MG-RAST. MG-RAST provides an option to remove reads that matches to the genomes of model organisms, including human and chicken, allowing to remove of host associated sequences and eventual human contaminations. The next main stage of the annotation pipeline is the identification of genes within the reads/assembled contig, a process often denoted as "gene calling" (Glass et al., 2010, Thomas et al., 2012; Oulas et al., 2015) Genes are labelled as coding DNA sequences and noncoding RNA genes, and certain annotation pipelines also predict for regulatory elements, such as clustered regularly interspaced short palindromic repeats.

Coding DNA sequences are identified using a number of tools including MetaGeneMark, Metagene, Prodigal, Orphelia, and FragGeneScan, all of which utilize ab initio gene prediction algorithms. Often, annotation pipelines use an intersection of these tools to obtain a more informative prediction of the protein coding genes. Gene prediction tools utilize codon information (ie, start codon – AUG) to identify potential open reading frames and hence label sequences as coding or non-coding. Most tools can be trained by using the desired training sets (Thomas et al., 2012; Oulas et al., 2015). For example, FragGeneScan is trained for prokaryotic genomes only, and is used by IMG/MER and MG-RAST as well as EBI Metagenomics. It is believed to be one of the most accurate gene-prediction tools currently available. Noncoding RNAs, such as tRNAs are predicted using programs like tRNAscan. Ribosomal RNA (rRNA) genes (5s, 16s, and 23s) are predicted using internally developed rRNA models for IMG/MER, and MG-RAST uses similarity to compare three known databases (SILVA, Greengenes, and the Ribosomal Database Project-RDP95) to predict rRNA genes (Rho et al., 2010; Glass et al., 2010). The next stage of the annotation pipeline involves functional assignment to the predicted protein coding genes. This is currently achieved by homology-based searches of query sequences against databases containing known functional and/or taxonomic information. BLAST or other sequence-similarity-based algorithms often run on high-performance computer clusters. Some widely-used data repositories to obtain annotation for metagenomic datasets include functional annotation databases, such as KEGG, SEED, eggNOG, COG/KOG, as well as protein domain databases, such as PFAM and TIGRFAM (Tatusov et al., 2000; Du et al., 2014; Powell et al., 2014; Oulas et al., 2015).

MG-RAST utilizes many of the databases for annotation mapping, as well as the NCBI taxonomy. The primary data product displayed to the user by MG-RAST is in the form of abundance profiles, and taxonomic information is projected against this data. Both IMG/MER and MG-RAST are widely used data management repositories and comparative genomics environments. They are fully automated pipelines that provide quality control, gene prediction, and functional annotation. Both tools support user download of data products generated, as well as optional sharing and publishing

within the respective portals. MG-RAST predicts all genes in the metagenome, and then identifies the best homologs of those genes in the isolate genomes using a tool called BLAT (BLAST-like alignment tool). BLAT misses similarities below 70% identity, so many strong hits to other genes are missed. After the best hits to genes from an isolated genome are identified, all subsequent analysis is done using the genes of the isolate genomes, not the genes of the metagenome at hand. This creates a lot of limitations due to the fact that the analysis is not performed on the original genes of the metagenome, but on the "proxy" genes to the isolated genomes instead. The advantage of this method is its speed; the only computationally intensive step is to find the best hits of the metagenomes against the isolates. Once this is done, all other comparisons are already pre-existing. (Kent et al., 2002; Oulas et al., 2015).

2. OBJECTIVES

The symbiotic equilibrium between a host and its microbiota is established immediately after the animal birth, then during the early stages of life its development is influenced by exposure to microorganisms present in the environment. However, the main role in the microbiota shaping process is played by the diet (Pan and Yu, 2014; Doré and Blottière, 2015). The intestinal microbiota is an extremely dense and complex ecosystem which plays a relevant role in the maintenance of the animal's well-being through the production of biologically relevant metabolites. The microbiota can influence the usage of nutrients by the host and furthermore it contributes to the optimal development of his intestinal mucosa (Scott et al., 2013; Doré and Blottière, 2015). In fact, the loss of balance between the various populations composing the microbiota may lead to the onset of pathologies and to a prevalence of pathogens (Yoon et al. 2015).

Since the diet is considered the main way to influence intestinal bacterial populations, maintaining their homeostasis status, it is already common to introduce feed additives in animal diets in order to positively modulate gut microbiota (Angelakis and Raoult, 2010; Huyghebaert et al., 2011; Kiarie et al., 2013; Pan and Yu, 2014). In livestock production, the research of new molecules able to selectively promote the growth of microbial populations related with positive effect on animals' growth performances has always be continuous, especially after the ban of antibiotic growth promoters within the European Union. Metagenomic analysis is an effective strategy to characterize the effect of feed additives on modulation of chicken gut microbiome, which is the collection of all genomes from all members of a microbial community from a specific environment (Handelsman et al., 1998). Several studies regarding the characterization of chicken gut microbiome are available but there is surprisingly little literature regarding the characterization of the chicken skin microbiota through metagenomic analysis. In fact, in the majority of the studies, even if they focus on understanding the structure of the population's flora inhabiting the skin and especially on how a subset of these microbes can become human pathogens, metagenomic analysis is not used and little attention is paid to the analysis of the microbiota and how it can be modulated (Cogen et al., 2008, Yoon et al. 2015).

The objectives of this Ph.D. project have been:

1. to characterize the impact of the administration of different feed additives on gut microbiota as well as the functional genes of commercial chicken;
2. to provide new information regarding the chicken skin microbiota and the effect of the diet on its modulation.

Both these objectvises will allow to highlight the connection between skin and gut microbiota in order to find nutritional strategies able to fight foodborne pathogen colonization in the chichen, improving the safety of poultry meat.

The tested feed additives were a probiotic (i.e., *Lactobacillus acidophilus* D2/CSL), a serine protease and phytase, administered alone or in combination with inositol. The feed additives were tested in separate trials involving commercial chickens. The animals investigated in this Ph.D. project were farmed and slaughtered under commercial conditions, according to Italian and European law for broiler chicken production. Whenever possible, the animal gastrointestinal tract was collected at the slaughterhouse at the end of animal rearing period. At the slaughterhouse, the chickens were electrically stunned in agreement with the Council Regulation (EC) No. 1099/2009 on protection of animals at the time of killing. Moreover, all slaughter procedures were monitored by the veterinary team appointed by the Italian Ministry of Health. The few chickens sacrificed along the trials (i.e., 1, 14 and 42 days) were humanely euthanized by cervical dislocation according to the principles stated in EU Directive 63/2010 (European Union, 2010) regarding the protection of animals used for experimental and other scientific purposes and according to the guidelines of the Animal Ethic Committee of the University of Bologna.

For all feeding trials, described below, the work flow included (1) rearing of the chickens fed with a control diet and the diet supplemented with the additive; (2) chicken gastrointestinal tract collection and total DNA extraction; (3) library preparation and shotgun metagenomic sequencing; (4) sequences analysis and statistical analysis; (5) results interpretation and discussion.

3. TRIALS PERFORMED

3.1 Trial on metagenomic investigation of caeca of chickens fed with *Lactobacillus acidophilus* D2/CSL

3.1.1 Background

Probiotics, or direct-fed microbials, have been defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Pineiro and Stanton, 2007). Probiotics supplement is used in livestock and poultry production to protect animals from enteric pathogen infection and improve animal health (Dahiya et al., 2006; Kabir, 2009). The mode of action of probiotics can vary depending on the traits of the specific probiotic strains/species used. Ideally, researchers select the promising probiotic strains from the autochthonous intestinal microbiota by supposing that these microorganisms have a symbiotic relationship with the host, so they could colonize the gastrointestinal tract. The mechanisms through which the probiotic, in particular Lactic Acid Bacteria (LAB), benefits the host include: competitive exclusion toward harmful bacteria, through competition for nutrient and attachment site, production of bacteriostatic and bactericidal substances, alteration of microbial and host metabolism, neutralization of enterotoxins, enhance of gut barrier functions and stimulation of host immunity (Van der Wielen et al., 2000; Haghighi et al., 2005; Lan et al., 2005; Huyghebaert et al., 2011; Lawley and Walker, 2013).

To archive these beneficial effects, the probiotic species/strains can be administrated to the host individually or combined. The effect of multispecies probiotics has also been investigated by many authors as Mountzouris et al., 2007 and Ghareeb et al., 2012. Mountzouris et al., 2007 investigated the effect of a multispecies probiotic containing *Enterococcus faecium*, *Bifidobacterium animalis*, *Pediococcus acidilactici*, *Lactobacillus salivarius* and *Lactobacillus reuteri* isolated from chicken gut, showing that its administration decreased cecal coliform population and that probiotic inclusion level had a significant effect on broiler chicken growth responses, nutrient apparent digestibility coefficients, apparent metabolizable energy, and cecal microflora composition. Another study conducted by Ghareeb et al. (2012) demonstrated that multispecies probiotics containing *Enterococcus faecium*, *Pedococcus acidilactici*, *Lactobacillus salivarius* and *Lactobacillus reuteri* significantly reduced cecal colonization by *Campylobacter jejuni*. This result suggested that probiotic can also be used to higher the food safety levels of poultry products by reducing the population of human pathogens, such as *Campylobacter jejuni*.

However, the most common choice is the administration of a single species/strain probiotic because its effect is more easily defined and more predictable. In fact, probiotic effects are strain-specific

(FAO/WHO, 2002) and selection criteria must consider the safety and efficacy on the target species. However, not only the bacteria strains, but even the dosage (i.e., colony forming unit (cfu)/bird/day) and the duration of the treatment are critical factors that should be considered since they can influence probiotic efficacy. Nevertheless, the health status of the flock and/or the farm hygienic conditions may suggest modulating the dose. Since it is necessary to preserve the bacteria vitality until their ingestion and assist their intestinal colonization, other important variables that must be considered are probiotic conservation and distribution technology, feed composition, antimicrobial agents in the feed or in the drinking water used as probiotic carrier (Gallazzi et al., 2008).

Lactobacillus strains have been described to be beneficial additives because of their effects in promoting poultry production performance and stimulating immune responses, improving digestive health in chickens. It has even been shown that *Lactobacillus* probiotic strains can decrease the population of *Salmonella*, *Campylobacter*, *Clostridium* and some other non-beneficial bacterial groups in chickens, improving the safety of poultry meat (Kim et al., 2012; Brisbin et al., 2011; Ghareeb et al., 2012; Neal-McKinney et al., 2012; Askelson et al., 2014; Spivey et al., 2014; La Ragione et al., 2004; Chen et al., 2012.

Other than reducing some pathogen proliferation and producing bacteriostatic or bactericidal molecules, as bacteriocins, several *Lactobacillus* species have been shown to even stimulate the immune-response of the host. Some studies suggest that various strains of *Lactobacilli* have a stimulating effect on antibody-mediated response in chickens. Such effect is dependent on the strain of *Lactobacillus* administrated, genotype and age of the chickens. *Lactobacillus acidophilus*, *Lactobacillus reuteri* and *Lactobacillus salivarius* are members of the chicken intestinal microbiota and have been shown to induce different cytokine profiles in mononuclear cells *in vitro* (Robredo and Torres, 2000; Brisbin et al. 2011). Brisbin et al. (2011) examined the effects of these bacteria individually or in combination on the induction of antibody- and cell-mediated immune responses *in vivo* proving the effectiveness of *Lactobacilli* oral treatment in the immune response modulation. However, these bacteria may vary in their ability to modulate the immune response. In particular, the birds received *Lactobacilli* weekly via oral gavage starting on day of hatch and subsequently, at 14 and 21 days, were immunized with sheep red blood cells (SRBC), keyhole limpet hemocyanin (KLH), Newcastle disease virus vaccine, and infectious bursal disease virus vaccine. *Lactobacillus salivarius*-treated birds had significantly more serum antibody to SRBC and KLH than birds that were not treated with probiotics. *Lactobacillus salivarius*-treated birds also had decreased cell-mediated immune responses to recall antigen stimulation while *Lactobacillus acidophilus* treatment increased the antibody response to KLH. Even if the mechanism through which the probiotics

enhance the antibody-mediated immune response is still not fully understood, probiotics could stimulate the production of Th2 cytokines, which may subsequently increase the antibody immune-mediated response (Robredo and Torres, 2000; Koenen et al., 2004; Haghighi et al., 2005; Brisbin et al., 2011). Brisbin et al. (2012) evaluated even the effects of *Lactobacillus acidophilus* bacteria as a probiotic on chicken T cell subset populations in peripheral blood and lymphoid tissues. The findings indicate that probiotics may alter the distribution of T cells in the blood and lymphoid tissues in young chickens; however, transient changes in lymphoid tissues indicate that probiotics likely do not permanently affect mucosal immunity. In fact, in the probiotic-fed group after 21 days of treatment the percentage of blood CD4+, CD8+, and TCR1+ cells was significantly higher than in the control group. After 14 days and 21 days of treatment, a significantly greater number of CD4+ T cells were found in the ileum of probiotic-fed chickens than in chickens from the other two groups.

In the present project, the effect of the addition of a probiotic to the diet (i.e., *Lactobacillus acidophilus*) on chicken gut microbiota populations and composition of functional genes will be investigated.

3.1.2 Methodology

3.1.2.1 Animals and diet groups

The trial in which the chickens were fed with *L. acidophilus* D2/CSL experiment was approved by the Ethical Committee of the University of Bologna on 17/3/2014 (ID: 10/79/2014). A total of 1,100-day-old male Ross 308 chicks, obtained from the same breeder flock and hatching session, were used. Birds were vaccinated against infectious bronchitis virus, Marek's disease virus, Newcastle and Gumboro diseases and coccidiosis at the hatchery. Before housing, chicks were individually weighed and divided according to their live weight in 5 classes: >42 g, 42-44 g, 45-47 g, 48-50 g, >50 g. The first and the last groups (i.e. >42 g and >50 g) were discarded, while the remaining ones were distributed in 32 pens (2.5 m^2 each) at the stocking density of 10 chicks/m^2 (25 birds/pen), while maintaining the same class distribution of live-weight of the population placing in each pen an equal number of chicks belonging to the three classes. Pens were equipped with pan feeders to assure at least 2cm/bird of front space and an independent drinking system with 1 nipple/5 birds. Feeders were of identical manufacture, type, size, colour, and other notable physical features. Each pen was equipped with an individual bin clearly labelled as reservoir for the experimental feed.

On a daily basis, the experimental feed was manually transferred from the bin to the feeder. Any change in the diet was made uniformly for all animals. Feed and water were provided for *ad libitum* consumption. At each diet switch, feeders were emptied, orts were weighed back and the feeders were filled with the diets described below. Twice daily observations were recorded for general flock

condition, temperature, lighting, water, feed, litter condition and mortality. The experiment lasted 41 days when birds reached the slaughter weight of about 2.8 kg of live weight. Photoperiod and temperature programs were set up according to the European welfare regulation 43/2007 (European Commission, 2007). The chicks were divided into 2 groups of 16 replicates each, fed with the basal diet (*control group, CON*) (Table 1) or the basal diet supplemented with *Lactobacillus acidophilus* D2/CSL (bacterial concentration of 5.0 x 1010 CFU g–1) at the dosage of 20 g ton-1 feed (*treated group, LA group*). The probiotic strain *L. acidophilus* D2/CSL has been isolated from the GI tract of a healthy adult chicken (Bianchi Salvadori et al., 1985) and supplied by Centro Sperimentale del Latte S.r.l. (Lodi, Italy). The experimental diets were weekly produced by adding the LA to the common basal diet. The feed was supplied *ad libitum* in mash form throughout the experiment. The feeding program included three feeding phases: Starter (0-14 d), Grower (15-28 d) and Finisher (29-41 d). The basal diet composition is given in Table 1.

Table 1. Basal diets composition (%) of the feed administered to the chicks belonging to the trial with *Lactobacillus acidophilus* D2/CSL (%)

	Starter (0-14 d)	Grower (15-28 d)	Finisher (29-41 d)
Corn	42.17	34.96	12.73
White corn	0.00	0.00	15.00
Wheat	10.00	20.00	25.01
Sorghum	0.00	0.00	5.00
Soybean meal	23.11	20.63	17.60
Expanded soybean	10.00	10.00	13.00
Sunflower	3.00	3.00	3.00
Corn gluten meal	4.00	3.00	0.00
Soybean oil	3.08	4.43	5.48
Dicalcium phosphate	1.52	1.20	0.57
Calcium carbonate	0.91	0.65	0.52
Sodium bicarbonate	0.15	0.10	0.15
Salt	0.27	0.27	0.25
Choline chloride	0.10	0.10	0.10
Lysine sulphate	0.59	0.55	0.46
Dl-methionine	0.27	0.29	0.30
Threonine	0.15	0.14	0.14
Xylanase	0.08	0.08	0.08
Phytase	0.10	0.10	0.10
Vitamin-mineral premix[1]	0.50	0.50	0.50
Dry matter	88.57	88.65	88.64
Protein	22.70	21.49	19.74
Lipid	7.06	8.24	9.74
Fiber	3.08	3.04	3.07
Ash	5.85	5.17	4.49
ME (kcal/kg)	3,076	3,168	3,264

[1] Provided the following per kg of diet: vitamin A (retinyl acetate), 13,000 IU; vitamin D3 (cholecalciferol), 4,000 IU; vitamin E (DL-α_tocopheryl acetate), 80 IU; vitamin K (menadione sodium bisulfite), 3 mg; riboflavin, 6.0 mg; pantothenic acid, 6.0 mg; niacin, 20 mg; pyridoxine, 2 mg; folic acid, 0.5 mg; biotin, 0.10 mg; thiamine, 2.5 mg; vitamin B_{12} 20 μg; Mn, 100 mg; Zn, 85 mg; Fe, 30 mg; Cu, 10 mg; I, 1.5 mg; Se, 0.2 mg; ethoxyquin, 100 mg.

3.1.2.2 Sample collection

In the trial with *Lactobacillus acidophilus* D2/CSL, four chickens were randomly selected and humanely euthanized at day 1, before starting the dietary treatment. Moreover, five chickens were randomly selected from both control and treated group at the end of the rearing period (i.e., 41 days). From each of the 14 selected birds, the caeca were dissected out and a small sample of cecum content (i.e., 0.5 to 2 g) was collected into 10-ml sterile plastic tubes. The samples collected were then stored at -80°C until further testing.

3.1.2.3 DNA extraction from chicken caecum contents

The DNA was extracted from each sample of caecum content using a bead-beating procedure (Danzeisen et al., 2011). Briefly, 0.25 g of cecal content were suspended in 1 ml lysis buffer (500 mM NaCl, 50 mM Tris-Cl, pH 8.0, 50 mM EDTA, 4 % SDS) with MagNA Lyser Green Beads (Roche, Milan, Italy) and homogenized on the MagNA Lyser (Roche) for 25 secs at 6500 rpm. The samples were then heated at 70°C for 15 min, followed by centrifugation to separate the DNA from the bacterial cellular debris. This process was repeated with a second 300 µl aliquot of lysis buffer. The samples were then subjected to 10 M v/v ammonium acetate (Sigma, Milan, Italy) precipitation, followed by isopropanol (Sigma) precipitation and a 70% ethanol (Carlo Erba, Milan, Italy) wash and re-suspended in 100 ul 1X Tris-EDTA (Sigma). The samples were treated with DNase-free RNase (Roche) and incubated overnight at 4°C, before being processed through the QIAmp® DNA Stool Mini Kit (Qiagen, Milan, Italy) according to manufacturer's directions with some modifications. Samples were measured on a BioSpectrometer® (Eppendorf, Milan, Italy) to assess DNA quantity and quality.

3.1.2.4 Library preparation and metagenomic sequencing

The DNA extracted from each sample were quantified on a BioSpectrometer® (Eppendorf, Milan, Italy) to assess DNA yield, in terms of quantity and quality. Moreover, DNA purity was assessed in terms of absence of contaminants according to value of the A260 / A280 nm ratio.

The DNA extracted and assessed for quality and quantity was submitted to the library preparation procedure with the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA). Nextera technology provides an input DNA fragmentation and transposase mediated ligation of oligo-adapters, essentials to anchor the amplified DNA fragment (around 500 bp) to the sequencer flow cell and to amplify the insert DNA by PCR. Illumina's preparation procedure was chosen because it improves traditional protocols by combining DNA fragmentation, end-repair, and adaptor-ligation into a single step using an engineered enzyme (Head et al., 2014). The PCR reaction also adds index (barcode) sequences. However, the use of an engineered enzyme makes this protocol very sensitive

to the amount of DNA input compared with other fragmentation methods (Head et al., 2014). Since the ratio of transposase complexes to sample DNA is critical and the subsequent fragment size is also dependent on the reaction efficiency DNA concentration was evaluated other than by BioSpectrometer (Eppendorf) even using fluorimetric analysis by Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen).

In particular, according to the first quantification obtained through BioSpectrometer (Eppendorf), the DNA was brought to the concentration required (0.2 ng/µl) through others intermediate dilutions (i.e., 25, 10 and 2 ng/µl) all quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) on the Infinite 200 PRO (Tecan) instrument. All libraries have been validated accordingly to Illumina's protocol. To determinate the nanomolarity of each library, the concentration and average length of the DNA fragments have been checked through fluorimetric analysis by Quant-iT™ PicoGreen® dsDNA Assay Kit and through Chip DNA Hi Sensitivity analysis on Bioanalyzer 2100 (Agilent Technologies). Each library pool of 24 samples was adjusted to a micro molarity between 1.3 to 2 (depending on the pool's library with the lower molarity) to be sequenced. A total of 5 µl of each library (1.3-2 nM) were pooled together. Each pool of 24 libraries was load into a flow cell of glass slide. Each fragment of DNA library was anchored on complementary oligo-adapters placed on the flow cell and clonally amplified through a solid-phase amplification called bridge amplification and then sequenced by synthesis. Whole genome sequencing was performed using the HiScanSQ sequencer (Illumina) at 100 bp in paired-end mode. Metagenomic sequencing yielded an average of 6.841 million mapped reads/sample, with a Phread quality score always higher than 30.

3.1.2.5 Sequences analysis

The metagenomic sequences belonging to the *Lactobacillus acidophilus D2/CSL* were analysed using the MG-RAST pipeline. The MG-RAST pipeline (Meyer et al., 2008) (metagenomics.anl.gov) was used to identify the relative abundances of bacterial taxa performing a BLAST similarity search for the longest cluster representative against the M5rna database, integrating SILVA (Pruesse et al., 2007), Greengenes (De Santis et al., 2006) and RDP (Cole et al., 2003). Moreover, the sequenced reads were assigned to functional groups using the Kyoto Encyclopedia of Genes and Genome (KEGG) database (www.genome.jp/kegg/) (Kanehisa, 2002) and the percentage of abundance was calculated. For both taxonomic and functional classification the following parameters were set: maximum e-value 1e-5, minimum identity 60%, and minimum alignment length 15 bp.

The metagenomic sequences belonging to the *Lactobacillus acidophilus D2/CSL* trial were deposited on MG-RAST at the following link: http://metagenomics.anl.gov/linkin.cgi?project=13081. The metagenome ID mgm 4624898.3, 4625263.3, 4625261.3 and 4625265.3 refer to samples collected at

day 1. The mgm 4625297.3, 4625262.3, 4625269.3, 4625304.3 and 4625316.3 refer to samples collected from the control group. The mgm 4625288.3, 4625285.3, 4625287.3, 4625273.3 and 4625272.3 refer to samples collected from the treated group.

3.1.2.6 Statistical analysis

The results regarding the relative abundances of bacterial taxa and functional groups were compared through the White's non-parametric t-test, using Statistical Analysis of Metagenomic profile Software v 2.0.9 (STAMP) (Parks et al., 2014).

3.1.3 Results

3.1.3.1 Sequences obtained

All samples with a A260 / A280 nm ratio value out of the range between 1.7 and 1.9 were excluded from the analysis and the DNA was extracted again from the original sample. The quantity and quality parameters of the DNA samples sequenced in this project along with corresponding library parameters abd reads achieved for each individual sample are described in Table 2.

Table 2. Parameters of the samples and libraries sequenced in the project.

Sample			Library			
ID	Conc (ng/ml)	Ratio 260/280 nm	Conc (ng/µl)	Fragment lenght	mM	Reads (n)
***Lactobacillus acidophilus* trial**						
XT_45	136	1.78	6.63	696	14.67	6,489,130
XT_120	771.7	1.78	6.72	998	10.36	4,757,509
XT_121	129.9	1.85	8.66	1327	10.05	4,342,708
XT_122	113.1	1.84	6.13	1075	8.78	6,825,047
XT_74	851.1	1.77	7.04	1224	8.85	7,899,518
XT_75	979.2	1.8	6.18	1003	9.48	6,875,605
XT_76	1342.8	1.81	5.18	1370	5.83	7,089,936
XT_77	1161.5	1.85	3.74	896	6.42	6,189,645
XT_78	819.1	1.84	4.66	1232	5.82	5,348,518
XT_79	1200.9	1.87	6.29	930	10.40	5,364,250
XT_80	1192.3	1.83	1.55	1158	2.06	5,891,661
XT_81	907.4	1.83	2.49	1310	2.93	5,408,894
XT_82	1003	1.84	3.741	876	6.57	5,104,388
XT_83	1286	1.76	1.18	980	1.86	5,225,063

3.1.3.2 Caeca microbiota composition

The microbiota composition of the chicks fed with *Lactobacillus acidophilus* D2/CSL (bacterial concentration of 5.0 x 1010 cfu g–1) at the dosage of 20 g ton-1 feed was compared with that of chicks fed with a control diet at 41 days of rearing. Moreover, both microbiota were compared with the microbiota of day old chicks tested before starting the feeding trial. The results obtained in day old chicks (i.e., 1 d), in chicks fed with the basal diet (control group, CON) and in chicks fed with the basal diet supplemented with *Lactobacillus acidophilus* (treated group, LA group) are detailed in Table 3. In the microbiota composition of day-old chicks more than 95% of bacterial population was represented by Firmicutes (85.5%) and Proteobacteria (9.61%). Both these Phyla were largely represented also in the caeca of CON and LA birds at 41 days (Table 3). The relative frequency of abundance of Firmicutes in day-old chicks was significantly lower than that observed in both groups at 41 days (P=0.01), whereas Proteobacteria were significantly higher (P=0.0067).

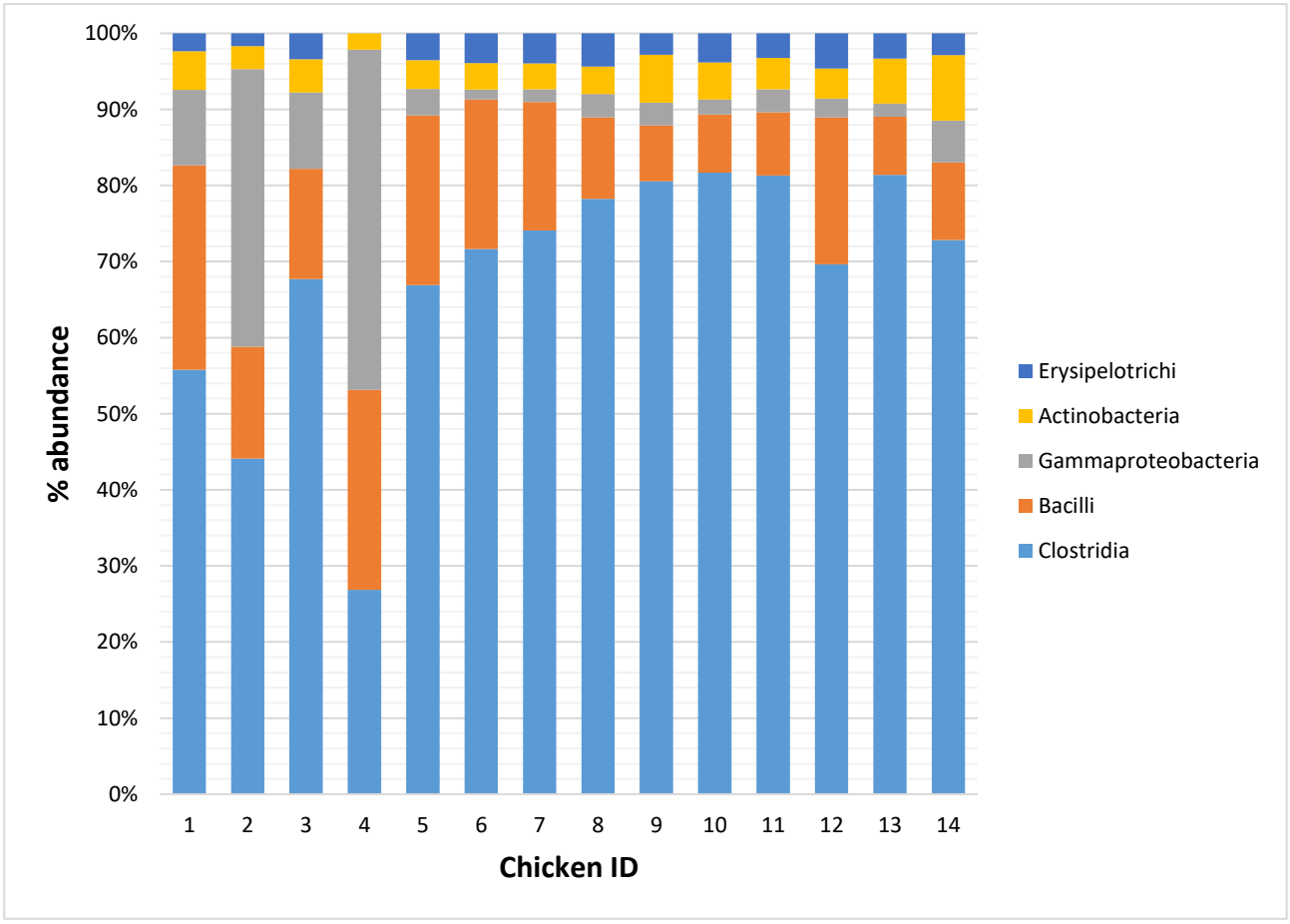
Within the phylum of Firmicutes, in day-old chicks Bacilli was the most abundant class, followed by Clostridia. On the contrary, at 41 days Clostridia represented the most abundant class in both LA and CON groups (70.8 and 70.5% respectively) followed by Bacilli, presenting a relative frequency of abundance of 20.7 and 18.9%, respectively. The mean relative abundances of Clostridia and Bacilli in the birds at the end of the rearing period were significantly higher (P=0.0086) and lower (P=0.0094) in comparison to those of one day-old chicks (Table 3). In day-old chicks, as well as LA and CON groups, Gammaproteobacteria was the most representative class of the Proteobacteria phylum. This class was the only one significantly higher in day-old chicks in comparison to CON and LA group at 41 days of age (P=0.015 and P=0.017 respectively). Moreover, in day-old chicks Enterobacteriaceae was the most represented family (7.63%) in comparison to the birds at 41 days, where the relative abundances of the same family were as low as 0.77 and 0.84% in the CON and LA groups, respectively (Table 3). Within the Bacilli class, the most represented family in day-old chicks was objeceae (33.5%), followed by Enterococcaceae (3.72%), Streptococcaceae (1.79%) and Bacillaceae (1.17%) (Table 3). This distribution was similar in CON and LA birds at 41 days, except for Bacillaceae and Paenibacillaceae, representing the second most abundant families in CON and LA birds at 41 days, respectively.

Table 3. Mean relative frequency of abundance (%) of Phyla, Classes and Families of caecum bacteria in day-old and 41 day-old chickens untreated (CON) and treated with *L. acidophilus* (LA).

Phylum	Class	Family	1 d	CON 41 d	LA 41 d
Firmicutes			85.85	93.93	92.14
	Bacilli		43.55	20.72	18.91
		Bacillaceae	1.17	1.12	0.81
		Paenibacillaceae	0.18	0.49	0.95
		Staphylococcaceae	0.93	0.22	0.18
		Enterococcaceae	3.72	0.50	0.37
		Lactobacillaceae	33.45	17.22	15.62
		Streptococcaceae	1.79	0.44	0.32
	Clostridia		41.92	70.51	70.79
		Clostridiaceae	12.28	11.89	14.30
		Eubacteriaceae	3.47	3.87	3.73
		Lachnospiraceae	13.25	14.39	17.07
		Peptococcaceae	0.18	0.60	0.43
		Peptostreptococcaceae	1.18	3.89	3.39
		Ruminococcaceae	6.8	29.53	26.27
	Erysipelotrichi		0.37	2.21	1.83
	Negativicutes		0	0.47	0.60
Proteobacteria			9.61	1.74	2.10
	Alphaproteobacteria		0.49	0.57	0.66
	Betaproteobacteria		0.18	0.10	0.10
	Deltaproteobacteria		0.18	0.27	0.48
	Gammaproteobacteria		8.74	0.77	0.84
		Enterobacteriaceae	7.63	0.62	0.70
Actinobacteria			1.6	0.92	1.43
Bacteroidetes			0.18	0.35	0.29
Tenericutes			1.42	1.18	1.47

Comparing the abundance of the top five classes between chickens belonging to the same group chicken ID 1 and 3 showed a percentage of abundance of Gammaproteobacteria lower in comparison to the other day one chicks (i.e., 9.91 and 10.03 vs 36.47 and 44.70%) (Figure 2). The decrease in Gammaproteobacteria corresponded to a higher abundance of Clostridia (55.81 and 67.70% respectively). On the contrary, Chicken ID 4 showed the highest abundance of Gammaproteobacteria (44.70%) and the lowest abundance of Clostridia (26.88%) in comparison with the other day old chicks. At 41 days, the percentage of abundance of the top five classes was quite similar between both groups of chickens belonging to the CON and LA group. However, chicken ID 12 showed a higher percentage of abundance of Bacilli and a lower abundance of Clostridia in comparison to the other LA chickens (Figure 2).

Figure 2. Mean relative frequency of abundance (% abundance) of most represented bacterial classes in each of the 14 chickens tested (Day 1: chicken ID 1-4; Control 41 days: chicken ID 5-9; Treated 41 days: chicken ID 10-14).



In comparison to day-old chicks, Lactobacillaceae, Enterococcaceae and Streptococcaceae decreased significantly in both CON (i.e., P=0.023, P=0.042 and P=0.01, respectively) and LA groups (i.e. P=0.031, P=0.013, P=0.002, respectively) at 41 d. Lachanospiraceae was the most represented family identified within the Clostridia class in one-day old chicks (13.25%). On the contrary, in CON and LA at 41 days Ruminococcaceae was the most represented family (29.53 and 26.27%, respectively) and showed a relative frequency of abundance significantly higher than in day-old chicks (P=0.00044 and P=0.0107 for CON and LA, respectively). At the end of the rearing period Lachanospiraceae were significantly higher in LA birds in comparison to CON (17.07 vs 14.39; P=0.036) and the same trend was observed for Clostridiaceae (14.30 vs 11.89%; P=0.074) (Table 3).

Overall, among the first 30 bacterial species identified in day-old chicks the most represented species were *Lactobacillus johnsonii, Lactobacillus crispatus, Escherichia coli, Ruminococcus torques, Lactobacillus helveticus, Lactobacillus gasseri, Ruminococcus obeum, Ruminococcaceae bacterium* D16, *Clostrifium hylemonae, Eubacterium limosum* (Table 4). At 41 days, the most represented species in CON group were *Faecalibacterium prausnitzii, Lactobacillus crispatus, Ruminococcus torques, Subdoligranulum variabile, Ruminococcaceae bacterium* D16, *Lactobacillus johnsonii, Pseudoflavonifractor capillosus, Ruminococcus obeum, Clostridium difficile* and *Blautia hydrogenotrophica,* whereas in LA group they were *Faecalibacterium prausnitzii, Lactobacillus johnsonii, Ruminococcus obeum, Subdoligranulum variabile, Ruminococcus torques, Ruminococcaceae bacterium* D16, *Lactobacillus reuteri, Lactobacillus crispatus, Blautia hydrogenotrophica* and *Clostrium leptum* (Table 4).

Table 4. Mean relative frequency of abundance (%) of the 30 most representative species (MRS) of caecum bacteria in day-old and 41 day-old chickens untreated (CON) and treated with *L. acidophilus* (LA).

MRS	Species 1 d	Mean	Species CON 41 d	Mean	Species LA 41 d	Mean
1	*Lactobacillus johnsonii*	11.36	*Faecalibacterium prausnitzii*	17.35	*Faecalibacterium prausnitzii*	14.00
2	*Lactobacillus crispatus*	6.14	*Lactobacillus crispatus*	5.62	*Lactobacillus johnsonii*	4.17
3	*Escherichia coli*	4.80	*Ruminococcus torques*	4.41	*Ruminococcus obeum*	3.76
4	*Ruminococcus torques*	3.80	*Subdoligranulum variabile*	3.26	*Subdoligranulum variabile*	2.99
5	*Lactobacillus helveticus*	2.94	*Ruminococcaceae bacterium D16*	3.10	*Ruminococcus torques*	2.86
6	*Lactobacillus gasseri*	2.73	*Lactobacillus johnsonii*	2.44	*Ruminococcaceae bacterium D16*	2.73
7	*Ruminococcus obeum*	1.98	*Pseudoflavonifractor capillosus*	2.05	*Lactobacillus reuteri*	2.44
8	*Ruminococcaceae bacterium D16*	1.98	*Ruminococcus obeum*	1.68	*Lactobacillus crispatus*	2.12
9	*Clostridium hylemonae*	1.85	*Clostridium difficile*	1.59	*Blautia hydrogenotrophica*	1.62
10	*Eubacterium limosum*	1.80	*Blautia hydrogenotrophica*	1.38	*Clostridium leptum*	1.62
11	*Clostridium bolteae*	1.74	*butyrate-producing bacterium SM4/1*	1.30	*Pseudoflavonifractor capillosus*	1.54
12	*Lactobacillus vaginalis*	1.69	*Clostridium leptum*	1.27	*Blautia sp. Ser8*	1.31
13	*Lactobacillus reuteri*	1.68	*Lactobacillus reuteri*	1.23	*Ruminococcus bromii*	1.29
14	*Enterococcus faecalis*	1.48	*Lactobacillus acidophilus*	1.18	*Clostridium difficile*	1.29
15	*Shigella boydii*	1.43	*Ruminococcus bromii*	1.11	*Clostridium clostridioforme*	1.29
16	*Enterococcus faecium*	1.32	*Ruminococcus albus*	1.06	*Clostridium bolteae*	1.16
17	*Clostridium asparagiforme*	1.30	*Lactobacillus vaginalis*	1.05	*butyrate-producing bacterium SM4/1*	1.03
18	*Lactobacillus delbrueckii*	1.30	*Lactobacillus helveticus*	1.02	*Ruminococcus flavefaciens*	0.94
19	*Ruminococcus lactaris*	1.18	*Ruminococcus flavefaciens*	0.90	*Lactobacillus helveticus*	0.92
20	*butyrate-producing bacterium SL7/1*	1.11	*Lactobacillus agilis*	0.87	*Lactobacillus agilis*	0.90
21	*Granulicatella adiacens*	0.93	*butyrate-producing bacterium SL7/1*	0.84	*Ruminococcus gnavus*	0.90
22	*Blautia hydrogenotrophica*	0.93	*Clostridium bartlettii*	0.84	*Clostridium scindens*	0.89
23	*Clostridium sphenoides*	0.93	*Ruminococcus gnavus*	0.83	*butyrate-producing bacterium SL7/1*	0.88
24	*Enterococcus pseudoavium*	0.92	*Anaerotruncus colihominis*	0.81	*Ruminococcus albus*	0.86
25	*Lactobacillus acidophilus*	0.87	*Butyricicoccus pullicaecorum*	0.81	*Clostridium saccharolyticum*	0.84
26	*Faecalibacterium prausnitzii*	0.87	*Clostridium bolteae*	0.80	*Lactobacillus vaginalis*	0.82
27	*Pseudoflavonifractor capillosus*	0.87	*Eubacterium hallii*	0.76	*Lactobacillus acidophilus*	0.78
28	*Lactobacillus plantarum*	0.87	*Blautia sp. Ser8*	0.74	*Roseburia intestinalis*	0.77
29	*Clostridium scindens*	0.80	*Dorea formicigenerans*	0.68	*Anaerotruncus colihominis*	0.70
30	*butyrate-producing bacterium SM4/1*	0.80	*Clostridium clostridioforme*	0.66	*Blautia hansenii*	0.65

[1]MRS: most represented species

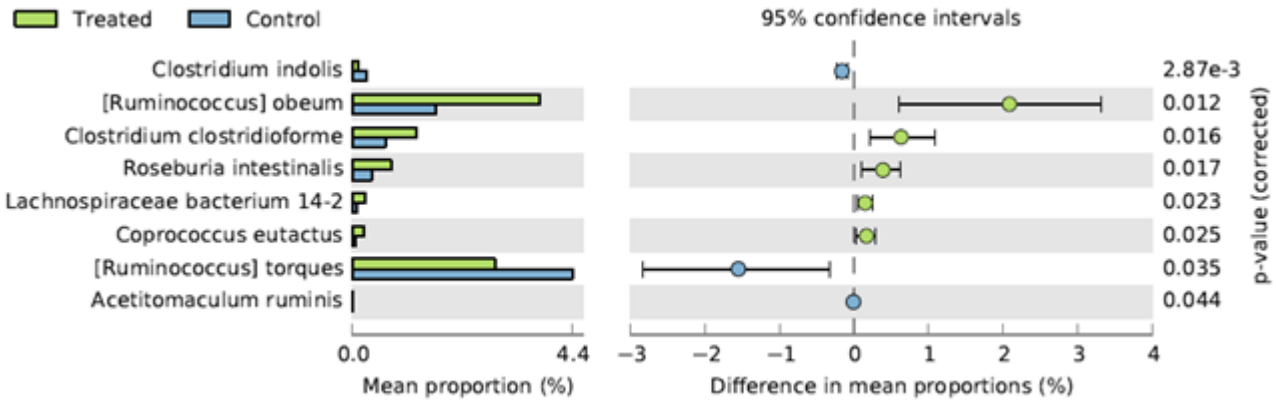
In relation to the bacterial species significantly different among the tested groups, the relative frequency of abundance of *Lactobacillus crispatus* was significantly higher in day-old chicks in comparison to LA, whereas *Ruminococcus lactaris* was significantly higher in day-old chicks in comparison to CON at 41 days. The species *Faecalibacterium prausnitzii* and *Subdoligranulum variabile* showed a significantly higher relative frequency of abundance in LA and CON birds in comparison to day-old chicks. On the contrary, all the other species were significantly higher in day-old chicks in comparison to both LA and CON groups (Table 5).

Table 5. Statistically significant differences between means of relative frequency of abundance (%) of caecum bacterial species in day-old and 41 day-old chickens untreated (CON) and treated with *L. acidophilus* (LA).

		CON	LA	1 d vs	1 d vs
	1 d	41 d	41 d	CON 41 d	LA 41 d
Species		Mean		P-values	
Lactobacillus johnsonii	11.36	2.44	4.17	0.0088	0.0045
Lactobacillus crispatus	6.14	-	2.12	0.005	0.0049
Escherichia coli	4.80	0.40	-	0.0165	0.022
Lactobacillus gasseri	2.73	0.37	0.45	0.0058	0.0079
Clostridium bolteae	1.74	0.80	1.16	0.0032	0.039
Shigella boydii	1.43	0.03	0.03	0.0006	0.0009
Lactobacillus delbrueckii	1.30	0.23	0.28	0.0191	0.028
Ruminococcus lactaris	1.18	0.17	-	0.0458	0.106
Faecalibacterium prausnitzii	0.87	17.35	14.00	3.57E-05	0.0005
Subdoligranulum variabile	0.62	3.26	2.99	0.0025	0.0034

Overall, among all the bacterial species identified in CON and LA groups at 41 days those showing a significantly higher relative frequency of abundance in LA birds were *Ruminococcus obeum*, *Clostridium clostridioforme, Roseburia intestinalis, Lachnospiraceae bacterium* 14-2T and *Coprococcus eutactus*. On the contrary, *Clostridium indolis* and *Ruminococcus torques* were significantly higher in CON at 41 days (Figure 3).

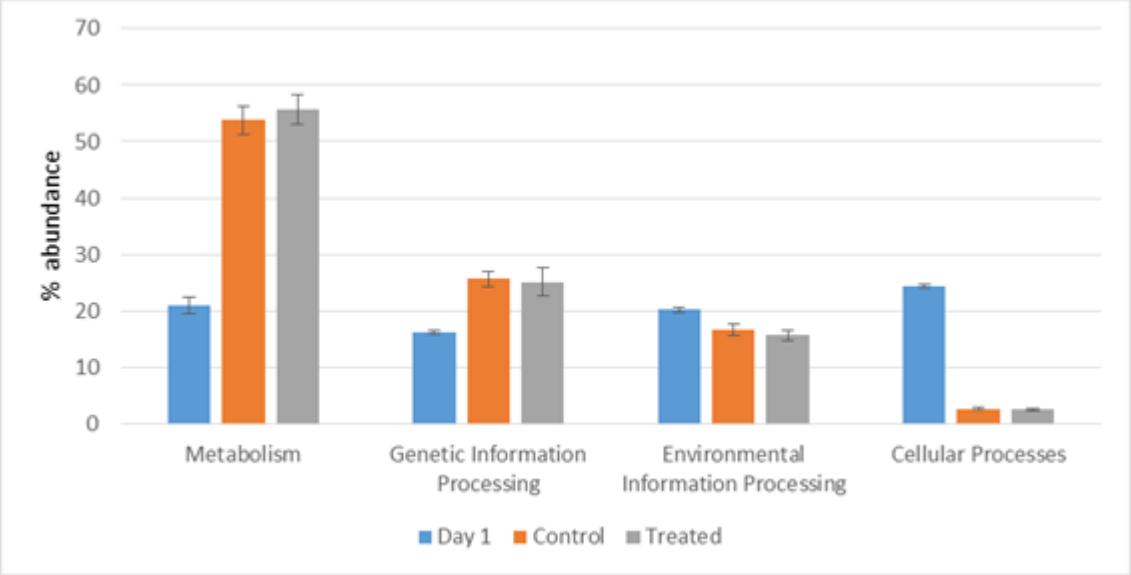
Figure 3. Bacterial species resulting significantly different in chickens treated with *L. acidophilus* (Treated) in comparison to the untreated birds (Control) at 41 days.



3.1.3.3 Caeca metabolic genes composition

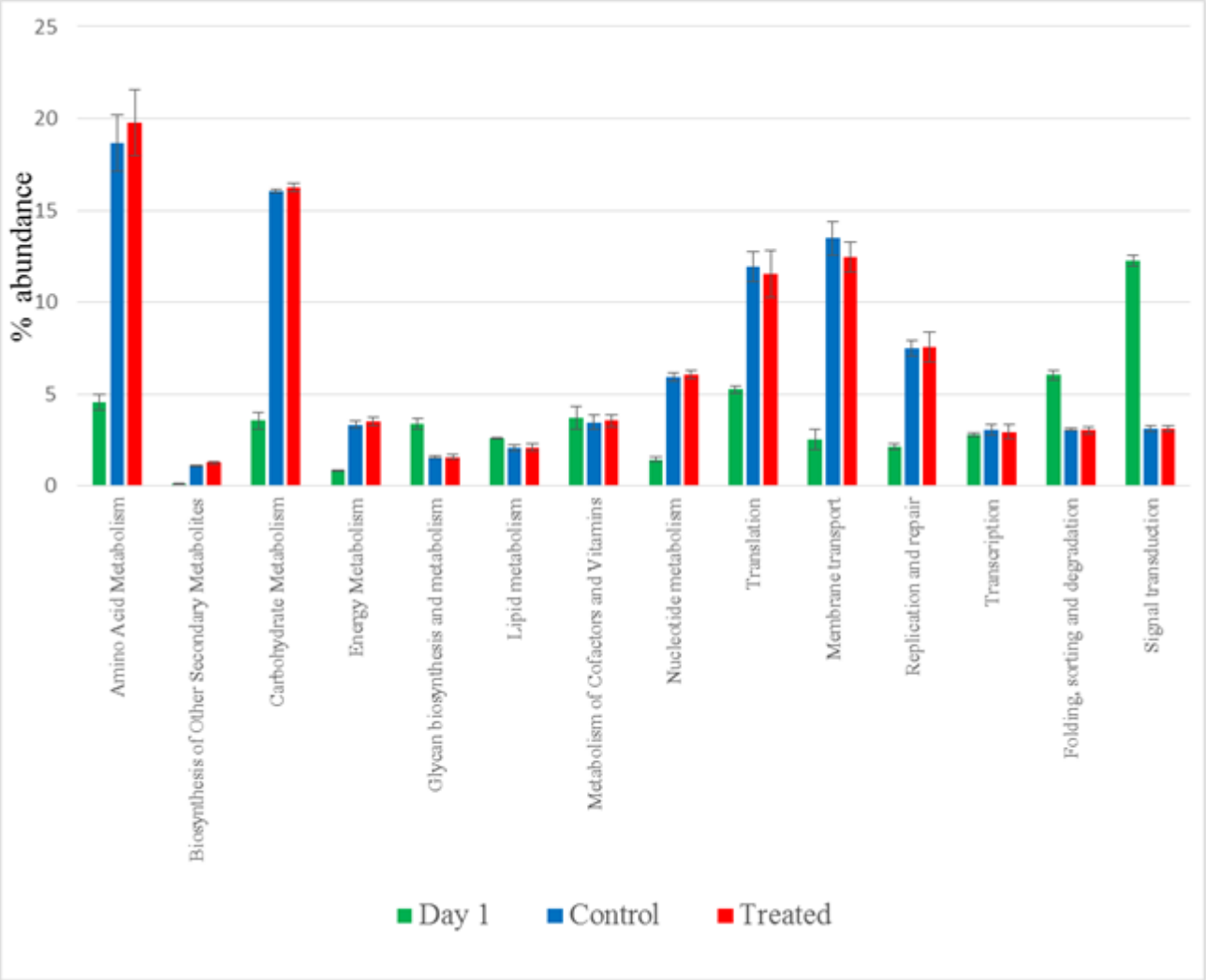
The mean relative abundance of the KEGG pathways related to metabolism and genetic information processing in day-old chicks corresponded to 20.9 and 16.2%, respectively. These values were significantly lower than those detected at 41 days in both CON and LA groups (53.8 vs 55.8% and 25.6 vs 25.0%, respectively) (Figure 4). On the contrary, the environmental information processing and cellular processes pathways were significantly higher in day-old chicks.

Figure 4. Mean relative frequency of abundance (% abundance) of the KEGG pathways in caeca of Day 1 chickens (Day 1) and in ceca of chickens treated with *L. acidophilus* (Treated) in comparison to the untreated birds (Control) at 41 days.



In relation to the specific metabolism pathway, day-old chickens showed relative frequencies of the aminoacid and carbohydrate metabolisms significantly lower (4.54 and 3.55%, $P<0.001$) of those detected in both LA and CON groups (19.8 and 18.6%; 16.2 and 16.1%, respectively) (Figure 5). The biosynthesis of other secondary metabolites was the only metabolism pathway significantly higher in LA than CON birds (1.27 vs 1.12% respectively; $P<0.012$) at 41 days (Figure 5).

Figure 5. Mean relative frequency of abundance (% abundance) of the KEGG in caeca of day 1 chickens (Day 1) and in ceca of chickens treated with *L. acidophilus* (Treated) in comparison to the untreated birds (Control) at 41 days.



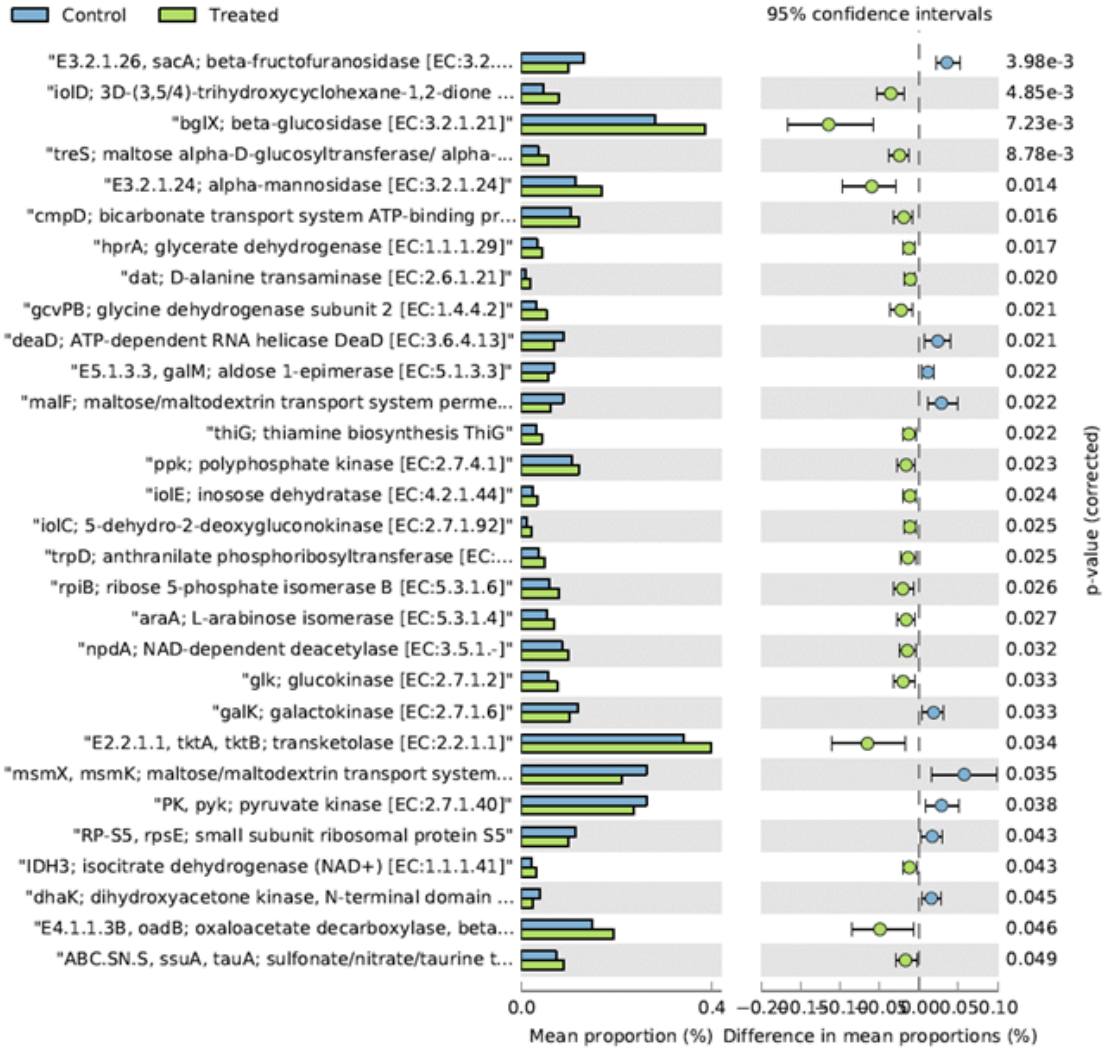
Overall, in terms of mean relative frequency of abundance, the top 20 metabolic functions identified using the KEGG database are reported in Table 6.

Table 6. Mean relative frequency of abundance (%) of the top 20 metabolic functions identified in caeca of day-old and 41 day-old chickens untreated (CON) and treated with *L. acidophilus* (LA) by using KEGG database.

Metabolic functions	1 d		CON 41 d		LA 41 d	
	Mean	SD	Mean	SD	Mean	SD
uvrA; excinuclease ABC subunit A	0.06	0.020	0.95	0.09	1.06	0.08
gpmA. PGAM; 2.3-bisphosphoglycerate-dependent phosphoglycerate mutase [EC:5.4.2.1]	0.04	0.010	0.49	0.03	0.48	0.06
LARS. leuS; leucyl-tRNA synthetase [EC:6.1.1.4]	0.10	0.020	0.48	0.05	0.48	0.09
ABC-2.AB.A; antibiotic transport system ATP-binding protein	0.03	0.010	0.54	0.08	0.49	0.06
ppdK; pyruvate.orthophosphate dikinase [EC:2.7.9.1]	0.03	0.007	0.5	0.04	0.49	0.06
E2.3.1.54. pflD; formate C-acetyltransferase [EC:2.3.1.54]	0.03	0.007	0.47	0.05	0.50	0.08
glnA; glutamine synthetase [EC:6.3.1.2]	0.06	0.020	0.51	0.03	0.53	0.06
uvrB; excinuclease ABC subunit B	0.02	0.010	0.52	0.03	0.53	0.06
DPO3A1. dnaE; DNA polymerase III subunit alpha [EC:2.7.7.7]	0.03	0.020	0.53	0.02	0.56	0.06
VARS. valS; valyl-tRNA synthetase [EC:6.1.1.9]	0.05	0.009	0.58	0.04	0.58	0.06
DPO3A2. polC; DNA polymerase III subunit alpha. Gram-positive type [EC:2.7.7.7]	0.03	0.010	0.6	0.07	0.58	0.13
dnaK; molecular chaperone DnaK	0.06	0.020	0.62	0.04	0.64	0.05
secA; preprotein translocase subunit SecA	0.03	0.010	0.66	0.05	0.64	0.06
cbiO; cobalt/nickel transport system ATP-binding protein	0.07	0.030	0.7	0.09	0.65	0.08
IARS. ileS; isoleucyl-tRNA synthetase [EC:6.1.1.5]	0.07	0.010	0.66	0.02	0.66	0.11
E6.3.5.3. purL; phosphoribosylformylglycinamidine synthase [EC:6.3.5.3]	0.03	0.010	0.58	0.18	0.69	0.15
nrdD; ribonucleoside-triphosphate reductase [EC:1.17.4.2]	0.04	0.020	0.74	0.10	0.74	0.12
carB. CPA2; carbamoyl-phosphate synthase large subunit [EC:6.3.5.5]	0.04	0.010	0.78	0.03	0.82	0.09
rpoC; DNA-directed RNA polymerase subunit beta' [EC:2.7.7.6]	0.05	0.002	1.03	0.13	0.95	0.14
rpoB; DNA-directed RNA polymerase subunit beta [EC:2.7.7.6]	0.07	0.030	1.00	0.09	0.96	0.15

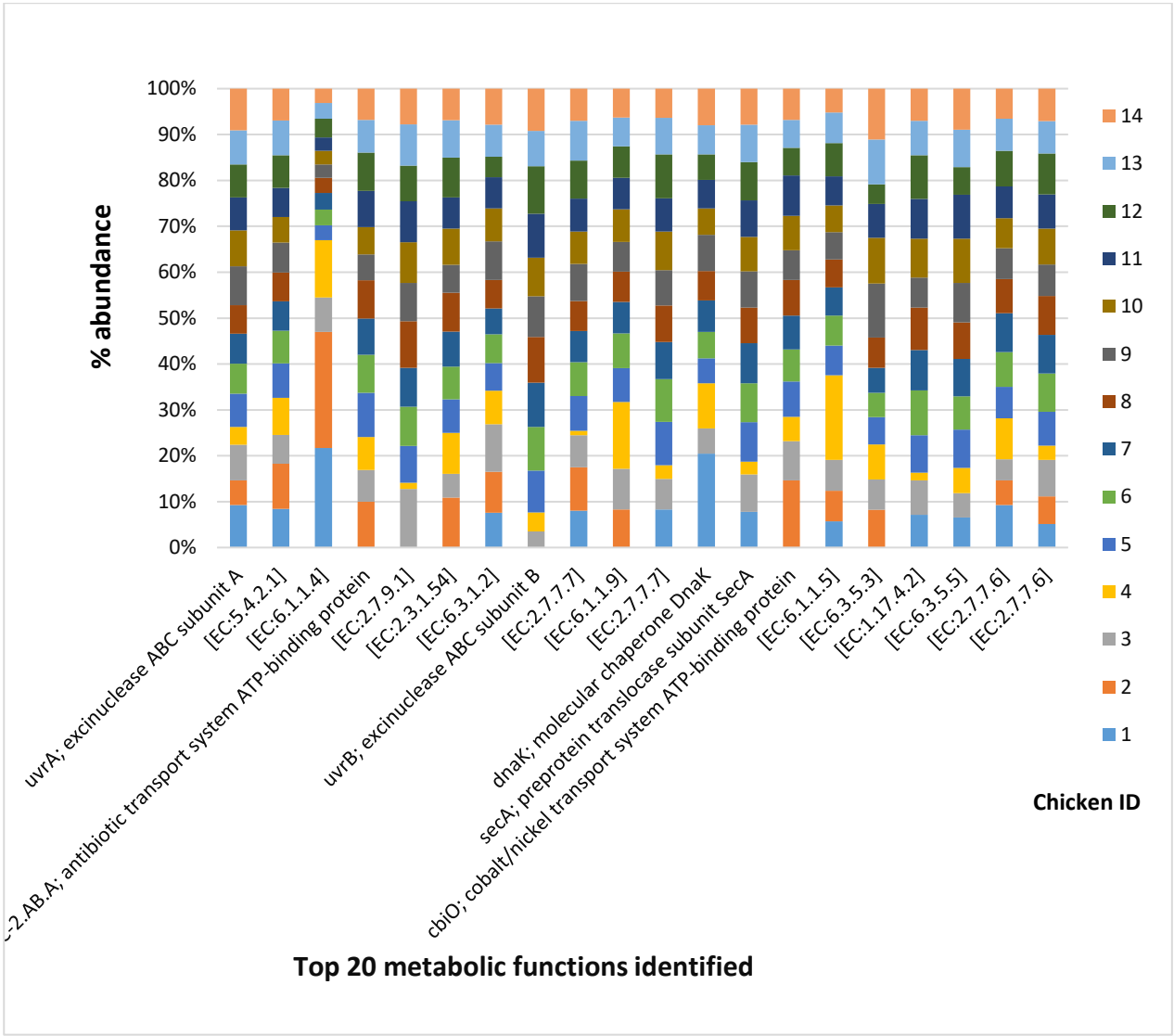
At the end of the rearing period the following functions resulted significantly higher in LA vs CON birds: bglX beta-glucosidase (EC:3.2.1.21), tkatkb transketolase EC:2.2.1.1, alpha-mannosidase (EC:3.2.1.24), ppk polyphosphate kinase (EC:2.7.4.1), oadB oxaloacetate decarboxylase beta subunit (EC: 4.1.1.3), glk glucokinase (EC:2.7.1.2), rpiB ribose 5-phosphate isomerase B (EC: 5.3.1.6), araA L.arabinose isomerase (EC:5.3.1.4) and npdA NAD-dependent deacetylase (EC:3.5.1.-). On the contrary sacA beta-fructofuranosidase (EC:3.3.1.26), malF:maltose/maltodextrin transport system permease, msmX, msmK; maltose/maltodextrine transport system ATP-binding protein, pyk; pyruvate kinase (EC:2.7.1.40) were higher in CON vs LA (Figure 6).

Figure 6. Mean relative frequency of abundance (% abundance) of the KEGG functions showing P < 0.05 between chickens treated with *L. acidophilus* (Treated) in comparison to the untreated birds (Control) at 41 days.



The distribution of the 20 metabolic functions among the 14 tested chickens showed slight differences only among the four chickens collected from the day-old chicks (Figure 7).

Figure 7. Mean relative frequency of abundance (% abundance) of the top 20 KEGG functions in each of the 14 chickens tested (Day 1: chicken ID 1-4; Control 41 days: chicken ID 5-9; Treated 41 days: chicken ID 6-10).



3.2 Trial on metagenomic investigation of caeca of chickens fed with serine protease

3.2.1 Background

Proteins consist of polymers of amino acids. All amino acids commonly consist of an amino and carboxylic group, which interconnect the amino acids with peptide bonds that comprise the backbone of protein. Each amino acid has in addition a side-group, which has different chemical properties and is the basis for grouping the amino acids into hydrophobic, hydrophilic and aromatic groups. The specific composition and order of the amino acids in the protein, together with the three-dimensional structure, determines the properties of the final protein. The enzymes that degrade proteins, named proteases, are characterized by their ability to hydrolyse bonds before and after specific amino acids.

The use of exogenous proteases in poultry diets has significantly improved during the last decade. The first commercial protease was introduced into the poultry feed market in the 1990s in combination with other enzymes, with the aim to increase the ileal digestibility of grain and oilseed meal based diets (Simbaya et al., 1996; Olukosi et al., 2015). Recently serine proteases were introduced into the market for broilers. This feed proteases are claimed to act through solubilisation and hydrolysis of dietary proteins, and to have an unspecific mode of action on a broad range of dietary proteins (Fru-Nji et al., 2011).

Several proteases are now commercially available and their use has significantly increased because of the significant pressure on the price of soybean meal, which has motivated nutritionists to further evaluate proteases for their ability to improve protein and amino acid digestibility of diets (Olukosi et al.,2015). Romero et al (2013, 2014) in their studies proved this theory describing the possibility to increment in the ileal digestibility of protein and amino acids feeding the chickens with an exogenous protease supplementation in the feed. They fed the chickens with two different diets, a simple maize–soya diet and the same diet fortified with dried distiller's grains with soluble and rapeseed meal. The results showed that a combination of xylanase, amylase and protease improved ileal digestibility of starch, fat and protein significantly, improving the birds' ability to extract energy from the different diets. The accelerated intestinal digestion and the removal by feed enzymes of what would otherwise be apparently indigested however, could lead to a limitation of nutrients' availability for the microbiota populations.

In fact, proteases don't just interact with dietary proteins hydrolysing them, they even have interaction with the digestions of other nutrients in the feed matrix and with the intestinal mucosa increasing the mucus layer's thickness, but most importantly they interact with the microbes composing the

microbiota. Protease's effect of increasing the availability of easily accessible proteins in different parts of intestinal lumen can lead to changes in the microbial communities' composition (Olukosi et al.,2015; Scott et al., 2013). Even though the effect of exogenous proteases on ileal digestibility, on total tract retention of energy and protein and on growth performance of the chicken has been extensively studied, there are few information regarding their effect on chicken's microbiota modulation.

One of few studies that examined not only the correlation between the addition of protease to the feed and the growth performance but even the effect of protease on chicken gut microbiota's composition was conducted by Torok et al. (2008). This study implied that the changes in the species composition may have contributed to improve the performances of chickens fed with the addition of feed enzymes in the diet. The authors used the terminal restriction fragment length polymorphism method to examine the changes in gut microbial communities in response to the addition of a product containing b-glucanase, xylanase and protease enzymes in a barley-based diet with the intent of improving the degradation of starch polysaccharides. Other than improvement of growth performance and energy utilisation related to the use of the product, the authors described a correlation between the dietary apparent metabolisable energy and microbial community composition within the ileum and caeca of the chickens, revealing distinct composition between the control and enzyme-supplemented birds. It was noticed that for both cereals, enzyme supplementation tended to decrease specific groups of bacteria and in particular, Coliforms and Enterobacteriaceae. This must be regarded as a beneficial property of using feed enzymes since the Enterobacteriaceae family, besides commensal coliform bacteria, also includes pathogens and zoonotic bacteria, like *Salmonella*.

The main factors that drive the fitness and colonisation efficiency of the microbes in the vary intestinal compartments of the gastrointestinal tracts are the availability of suitable growth substrates. Ingested feed has a high concentration of readily available substrates which could potentially be utilised by a wide variety of bacteria and its availability decreases moving down the gastrointestinal tract. That is why bacteria in the lower intestine are often specialists in utilising feed components that are resistant to the endogenous digestive system of the host as non-starch polysaccharides, resistant starch or resistant proteins, while the small intestine is dominated by lactic acid bacteria which have complex nutrient requirements resembling those of the chicken host itself. For example, *Lactobacilli* are unable to synthesise amino acids for their anabolism and are highly dependent on amino acid availability in the growth environment (Apajalahti and Vienola, 2016). This need lead to a competition for amino acids between the chicken and host the microbiota, that is estimated to utilize from 3 to 6% of the protein. Exogenous protease can provide a competitive advantage to the chicken, offering less growth

potential for amino acid-dependent bacteria. So, it can be concluded that some positive populations of bacteria populations dependent on amino acid availability could be affected from the addition of protease enzymes in the chicken diet. However, on the other hand, some others non-beneficial populations, as some members of the Enterobacteriaceae family or putrefactive bacteria, could be reduced. In fact, ilea bypass protein is subject to fermentation by putrefactive bacteria in the caecum with the production of many harmful and toxic compounds, which in high concentrations may have adverse effects on chicken growth and performance. These compounds include amines, indoles, phenols, cresol and ammonia, which can all negatively affect host or cell health. All actions to reduce the amount of ilea bypass protein potentially also reduce production of toxic protein fermentation metabolites in the caecum. Enzymes which facilitate protein digestion in the upper intestine and soluble carbohydrates resistant to ilea digestion both reduce caeca putrefaction.

Since the effects of the protease on chicken gut microbiota are still not clearly and completely defined due to the few numbers of study available, the present project aims to characterize the effect of a commercial protease, in particular a serine protease, on chicken gut microbiota's composition and modulation.

3.2.2 Methodology

3.2.2.1 Animals and diet groups

In the trials with serine protease, a total of 1755 one-day-old male chicks (Ross 308), obtained in September 2012 from the same breeder flock and hatching session, were used. The chicks were housed in a poultry house containing 27 pens of 6 m^2 each. Before housing, birds were individually weighed and divided according to their live weight in 3 classes: 42-44 g, 45-47 g, 48-50 g. The groups were distributed in 27 pens at the stocking density of about 10 chicks/m2 (i.e., 65 birds/pen), while maintaining the same class distribution of live-weight of the population placing in each pen an equal number of chicks belonging to the three classes. The 27 pens were divided in three diet groups of 9 replicates each. Pens labelled as *group A* hosted birds fed with the basal diet (i.e., diet A) (Table 7); those of *group B*, birds fed with basal diet - 7 % protein (i.e., diet B); those of *group C*, birds fed with basal diet -7 % protein and supplementation of 15000 units of serine protease (i.e., diet C). Feeds, formulated according to the three different diets, were supplied *ad libitum* in mash form throughout the experiment. The feeding program included four phases: starter (0-10 day), grower first period (11-21 day), grower second period (22-35 day) and finisher (36-42 day). The experiment lasted at 42 days, when birds reached the slaughter weight of about 2.8 kg of live weight. Photoperiod and temperature programs were set up according to the European welfare regulation 43/2007 (Europian Union, 2007).

Table 7. Basal diets composition (%) of the feed administered to the chicks belonging to the trial with serine protease

	0-10 days			11-21 days			22-35 days			36-42 days		
	Diet A	Diet B	Diet C	Diet A	Diet B	Diet C	Diet A	Diet B	Diet C	Diet A	Diet B	Diet C
Corn	35.206	46.804	46.804	41.229	52.449	52.449	42.841	53.969	53.969	43.676	54.835	54.835
Soybean meal	28.880	19.140	19.140	23.960	14.413	14.413	23.747	14.307	14.307	23.107	13.600	13.600
Wheat	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
Expanded soybean	10.000	10.000	10.000	10.000	10.000	10.000	10.000	10.000	10.000	10.000	10.000	10.000
Vegetable oils	3.260	1.500	1.500	3.793	2.100	2.100	4.940	3.247	3.247	5.207	3.513	3.513
Corn gluten meal	3.000	3.000	3.000	2.000	2.000	2.000	0.000	0.000	0.000	0.000	0.000	0.000
Dicalcium phosphate	1.480	1.600	1.600	1.200	1.320	1.320	0.760	0.893	0.893	0.547	0.667	0.667
Calcium carbonate	0.980	0.960	0.960	0.733	0.707	0.707	0.653	0.627	0.627	0.653	0.640	0.640
Lysine sulphate	0.484	0.434	0.434	0.449	0.445	0.445	0.332	0.349	0.349	0.305	0.337	0.337
Dl-methionine	0.360	0.248	0.248	0.336	0.240	0.240	0.339	0.245	0.245	0.320	0.233	0.233
Salt	0.260	0.260	0.260	0.260	0.160	0.160	0.240	0.193	0.193	0.240	0.187	0.187
Sodium butyrate	0.120	0.120	0.120	0.120	0.120	0.120	0.000	0.000	0.000	0.000	0.000	0.000
Thannins	0.120	0.120	0.120	0.120	0.120	0.120	0.153	0.153	0.153	0.153	0.153	0.153
Threonine	0.110	0.074	0.074	0.099	0.079	0.079	0.103	0.085	0.085	0.081	0.072	0.072
Sodium bicarbonate	0.100	0.100	0.100	0.100	0.247	0.247	0.100	0.160	0.160	0.100	0.173	0.173
Choline chloride	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.060	0.060	0.060
Vitamin-mineral premix[1]	0.440	0.440	0.440	0.400	0.400	0.400	0.340	0.340	0.340	0.200	0.200	0.200
Carotenoids	0.000	0.000	0.000	0.000	0.000	0.000	0.252	0.231	0.231	0.251	0.229	0.229
Phytase	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100
Serine protease	0.000	0.000	0.100	0.000	0.000	0.100	0.000	0.000	0.100	0.000	0.000	0.100
Dry matter	88.415	88.415	88.415	88.374	88.374	88.374	88.228	88.228	88.228	88.232	88.232	88.232
Protein	20.501	20.501	20.501	18.020	18.020	18.020	16.832	18.832	16.832	16.531	16.531	16.531
Lipid	5.507	5.507	5.507	6.212	6.212	6.212	7.358	7.358	7.358	7.643	7.643	7.643
Fiber	2.546	2.546	2.546	2.482	2.482	2.482	2.476	2.476	2.476	2.465	2.465	2.465
Ash	5.500	5.500	5.500	4.761	4.761	4.761	4.211	4.211	4.211	3.960	3.960	3.960
ME (Kcal/kg)	3.070	3.070	3.070	3.160	3.160	3.160	3.240	3.240	3.240	3.275	3.275	3.275

[1]Provided the following per kg of diet: vitamin A (retinyl acetate), 13,000 IU; vitamin D3 (cholecalciferol), 4,000 IU; vitamin E (DL-α_tocopheryl acetate), 80 IU; vitamin K (menadione sodium bisulfite), 3 mg; riboflavin, 6.0 mg; pantothenic acid, 6.0 mg; niacin, 20 mg; pyridoxine, 2 mg; folic acid, 0.5 mg; biotin, 0.10 mg; thiamine, 2.5 mg; vitamin B12 20 μg; Mn, 100 mg; Zn, 85 mg; Fe, 30 mg; Cu, 10 mg; I, 1.5 mg; Se, 0.2 mg; ethoxyquin, 100 mg.

3.2.2.2 Sample collection

In the trial with serine protease five chickens were randomly selected and humanely euthanized at day 14 and 42 from each diet group (i.e., A, B and C). The entire gastrointestinal tract of each individual selected bird was dissected out and a small sample of caecum content was collected into collected from both caeca into 2 ml sterile plastic tubes and then stored at -80°C until DNA extraction.

3.2.2.3 DNA extraction from the chicken caecum contents

The DNA was extracted from each sample of caecum content using a bead-beating procedure (Danzeisen et al., 2011). Briefly, 0.25 g of cecal content were suspended in 1 ml lysis buffer (500 mM NaCl, 50 mM Tris-Cl, pH 8.0, 50 mM EDTA, 4 % SDS) with MagNA Lyser Green Beads (Roche, Milan, Italy) and homogenized on the MagNA Lyser (Roche) for 25 secs at 6500 rpm. The samples were then heated at 70°C for 15 min, followed by centrifugation to separate the DNA from the bacterial cellular debris. This process was repeated with a second 300 µl aliquot of lysis buffer. The samples were then subjected to 10 M v/v ammonium acetate (Sigma, Milan, Italy) precipitation, followed by isopropanol (Sigma) precipitation and a 70% ethanol (Carlo Erba, Milan, Italy) wash and re-suspended in 100 ul 1X Tris-EDTA (Sigma). The samples were treated with DNase-free RNase (Roche) and incubated overnight at 4°C, before being processed through the QIAmp® DNA Stool Mini Kit (Qiagen, Milan, Italy) according to manufacturer's directions with some modifications. Samples were measured on a BioSpectrometer® (Eppendorf, Milan, Italy) to assess DNA quantity and quality.

3.2.2.4 Library preparation and metagenomic sequencing

The DNA extracted from each sample were quantified on a BioSpectrometer® (Eppendorf, Milan, Italy) to assess DNA yield, in terms of quantity and quality. Moreover, DNA purity was assessed in terms of absence of contaminants according to value of the A260 / A280 nm ratio.

The DNA extracted and assessed for quality and quantity was submitted to the library preparation procedure with the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA). Nextera technology provides an input DNA fragmentation and transposase mediated ligation of oligo-adapters, essentials to anchor the amplified DNA fragment (around 500 bp) to the sequencer flow cell and to amplify the insert DNA by PCR. Illumina's preparation procedure was chosen because it improves traditional protocols by combining DNA fragmentation, end-repair, and adaptor-ligation into a single step using an engineered enzyme (Head et al., 2014). The PCR reaction also adds index (barcode) sequences. However, the use of an engineered enzyme makes this protocol very sensitive to the amount of DNA input compared with other fragmentation methods (Head et al., 2014). Since the ratio of transposase complexes to sample DNA is critical and the subsequent fragment size is also

dependent on the reaction efficiency DNA concentration was evaluated other than by BioSpectrometer (Eppendorf) even using fluorimetric analysis by Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen).

In particular, according to the first quantification obtained through BioSpectrometer (Eppendorf), the DNA was brought to the concentration required (0.2 ng/µl) through others intermediate dilutions (i.e., 25, 10 and 2 ng/µl) all quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) on the Infinite 200 PRO (Tecan) instrument. All libraries have been validated accordingly to Illumina's protocol. To determine the nanomolarity of each library, the concentration and average length of the DNA fragments have been checked through fluorimetric analysis by Quant-iT™ PicoGreen® dsDNA Assay Kit and through Chip DNA Hi Sensitivity analysis on Bioanalyzer 2100 (Agilent Technologies). Each library pool of 24 samples was adjusted to a micro molarity between 1.3 to 2 (depending on the pool's library with the lower molarity) to be sequenced. A total of 5 µl of each library (1.3-2 nM) were pooled together. Each pool of 24 libraries was load into a flow cell of glass slide. Each fragment of DNA library was anchored on complementary oligo-adapters placed on the flow cell and clonally amplified through a solid-phase amplification called bridge amplification and then sequenced by synthesis. Whole genome sequencing was performed using the HiScanSQ sequencer (Illumina) at 100 bp in paired-end mode. Metagenomic sequencing yielded an average of 6.841 million mapped reads/sample, with a Phread quality score always higher than 30.

3.2.2.5 Sequences analysis

The metagenomic sequences belonging to the serine protease trial were analysed using the MG-RAST pipeline. The MG-RAST pipeline (Meyer et al., 2008) (metagenomics.anl.gov) was used to identify the relative abundances of bacterial taxa performing a BLAST similarity search for the longest cluster representative against the M5rna database, integrating SILVA (Pruesse et al., 2007), Greengenes (De Santis et al., 2006) and RDP (Cole et al., 2003). Moreover, the sequenced reads were assigned to functional groups using the Kyoto Encyclopedia of Genes and Genome (KEGG) database (www.genome.jp/kegg/) (Kanehisa, 2002) and the percentage of abundance was calculated. For both taxonomic and functional classification, the following parameters were set: maximum e-value 1e-5, minimum identity 60%, and minimum alignment length 15 bp.

The metagenomic sequences belonging to the serine protease trial were deposited in MG-RAST (http://metagenomics.anl.gov/) under project label as "protease" and metagenome ID mgm 4624489.3 to mgm 4624518.3.

3.2.2.6 Statistical analysis

The results regarding the relative abundances of bacterial taxa and functional groups were compared through the White's non-parametric t-test, using Statistical Analysis of Metagenomic profile Software v 2.0.9 (STAMP) (Parks et al., 2014). After removing non-bacterial species, taxa abundances obtained from MG-RAST were normalized so that each sample total abundance resulted 1. The samples heterogeneity was investigated both in terms of alpha and beta diversity.

Alpha diversity was computed according to Pielou's definition

$$\alpha = \frac{-\sum_{i=1}^{S} p_i ln(p_i)}{ln(S)}$$

(Pielou, 1966), where pi is the proportion of the *i*-th species. This is a normalized index related to Shannon entropy, that ranges from 0, when all individuals belong to the same species, corresponding to minimum diversity, to 1, when each individual belongs to a different species, corresponding to maximum diversity. Beta diversity, that refers to the compositional difference between samples, was computed in the form of Bray-Curtis distance with pdist function of scipy module (0.17.0). Principal Coordinate Analysis (PCoA) was computed with python module skbio 0.4.2 to represent the samples in a 2D space that satisfies the Bray-Curtis distances.

In order to assess which species mostly contribute to the samples Bray-Curtis distances, SIMilarity PERcentages analysis (SIMPER) (Clarke et al., 1993) was computed with R package vegan 2.3-5, setting 100000 permutations. Species that contribute at least to 70% of the differences between groups were selected by SIMPER as important in explaining the beta diversities. Student t-test and 2-way Analysis Of Variance (ANOVA) were computed with scipy 0.17.0, after scaling the data, to compare taxas abundances and samples diversity and to evaluate the influence of time, diet and their combination on the microbiota composition. In order to determine a signature that characterizes the four diet groups, for each time point we considered those phyla that included at least 10% of the species that were found to be significantly different in at least one pairwise comparison (p-value < 0.05). Moreover, species with an average abundance in the four groups less than 0.025% were discarded. We propose to use as signature the set of selected species that are also important in explaining the beta diversities, that is species that were found to be important also by SIMPER, as previously defined. All the statistical analyses were performed in python 2.7 and R 3.3.0.

3.2.3 Results

3.2.3.1 Sequences obtained

All samples with a A260 / A280 nm ratio value out of the range between 1.7 and 1.9 were excluded from the analysis and the DNA was extracted again from the original sample. The quantity and quality parameters of the DNA samples sequenced in this project along with corresponding library parameters and reads achieved for each individual sample are described in Table 8.

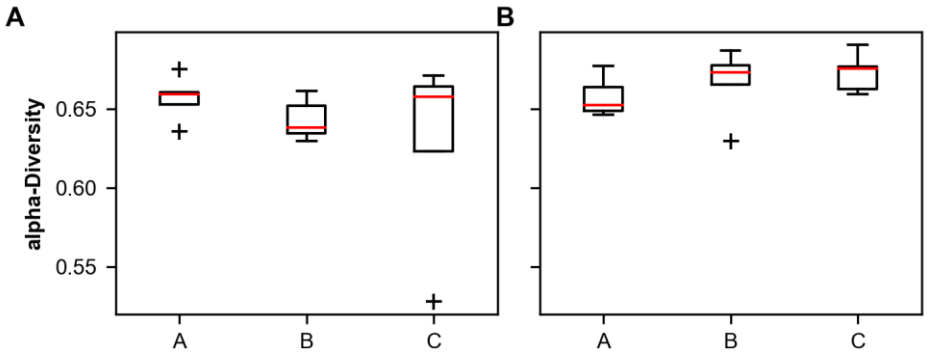
Table 8. Parameters of the samples and libraries sequenced in the project.

Sample			Library			
ID	Conc (ng/ml)	Ratio 260/280 nm	Conc (ng/µl)	Fragment lenght	mM	Reads (n)
XT_10	90.7	1.78	4.21	445	14.56	6188550
XT_12	116.6	1.72	3.99	1072	5.72	6773629
XT_14	20.1	1.78	6.86	854	12.36	6449097
XT_15	97.7	1.7	6.56	943	10.67	7508957
XT_17	107.7	1.82	5.73	929	9.49	6432429
XT_18	74	1.80	5.88	904	10	7050352
XT_20	96	1.89	6.98	962	11.17	7184616
XT_21	25.6	1.89	6.26	612	15.74	6208473
XT_22	52	178	5.39	403	20.56	7088568
XT_23	13.7	2.06	3.84	729	8.10	4804282
XT_24	78.6	1.90	5.65	472	18.42	4813726
XT_25	540	1.83	3.61	631	8.82	5661233
XT_26	406	1.84	7.45	894	12.82	5683528
XT_27	394.8	1.82	4.50	520	13.31	6424469
XT_28	501.2	1.85	2.15	636	5.21	5447944
XT_29	305.6	1.85	2.27	614	5.69	6893134
XT_30	383.3	1.85	4.50	530	13.08	6719001
XT_31	286	1.86	4.61	628	11.31	6389432
XT_32	125.1	1.88	2.45	728	5.19	6267990
XT_33	255.2	1.87	2.32	634	5.63	6219556
XT_34	189.3	1.86	2.55	486	8.07	7544966
XT_3	64.3	1.78	6.07	671	13.90	6913357
XT_41	79.4	1.82	7.40	493	23.09	4280630
XT_42	115.8	1.82	3.82	929	6.32	5398553
XT_43	70	1.80	7.20	608	18.22	4581356
XT_44	113.9	1.81	6.53	831	12.09	5486244
XT_5	120.7	1.75	7.37	477	23.78	5014363
XT_6	47.6	1.78	7.37	408	29.16	4347928
XT_7	83.7	1.80	6.02	487	19.02	4128956
XT_9	136.1	1.8	6.59	853	6.45	5601013

3.2.3.2 Caeca microbiota composition

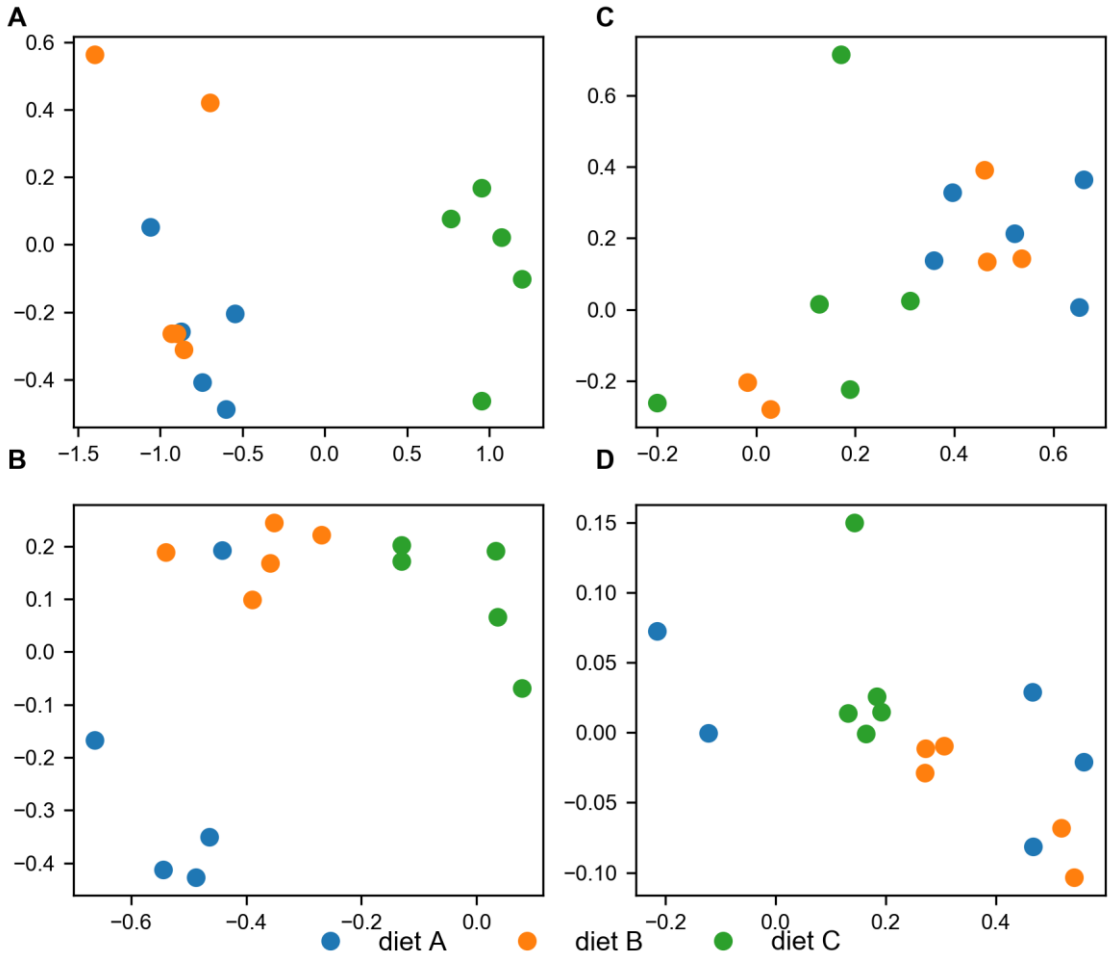
In the trials with serine protease, the microbiota composition of the chicks fed with the basal diet (group A) were compared with those of chicks fed with basal diet - 7 % protein (group B) and those of birds fed with basal diet -7 % protein and supplementation of 15000 units of serine protease (group C). The species diversity observed in microbiota colonizing the caecum contents of broilers fed with the different diets at 14 and 42 days, quantified by the Pielou alpha-diversity, was similar in all tested groups (Figure 8).

Figure 8. Pielou-alpha diversity calculated for the species colonising the caeca of the broilers fed with the different diets (i.e. A, B, C) at (A) 14 days and (B) 42 days.



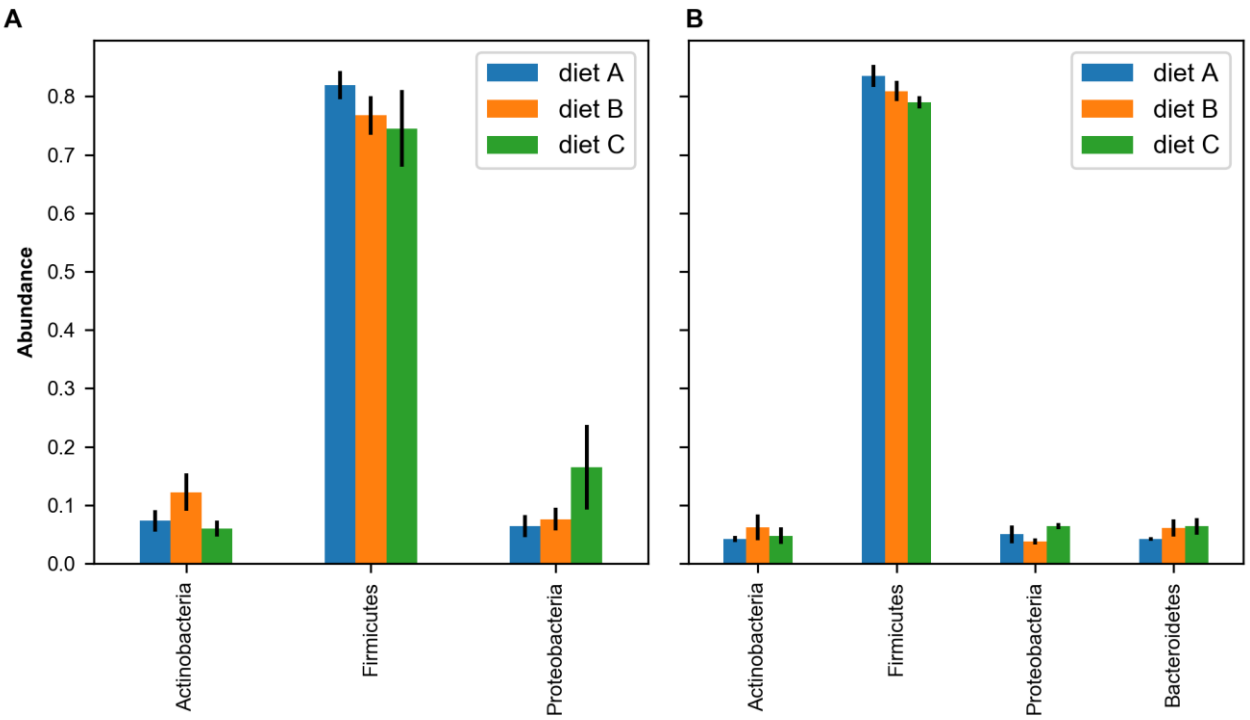
The Principal Coordinate Analysis (PCoA) plots (Figure 9), generated using the Bray-Curtis distance metric, confirmed that, despite the strong relationship between age of the birds and microbiota composition, the microbiota compositional diversity (measured by the Beta diversity parameter) did group samples belonging to diet C at family level, at 14 and 42 days (Figures 9 A, 9 B), but not at species level (Figure 9 C, 8 D).

Figure 9. PCoA plots generated using the Bray-Curtis distance metric showing the impact of age of the birds and diet on microbiota composition in terms families and species. (A) family 14 days; (B) family 42 days; (C) species 14 days; (D) species 42 days.



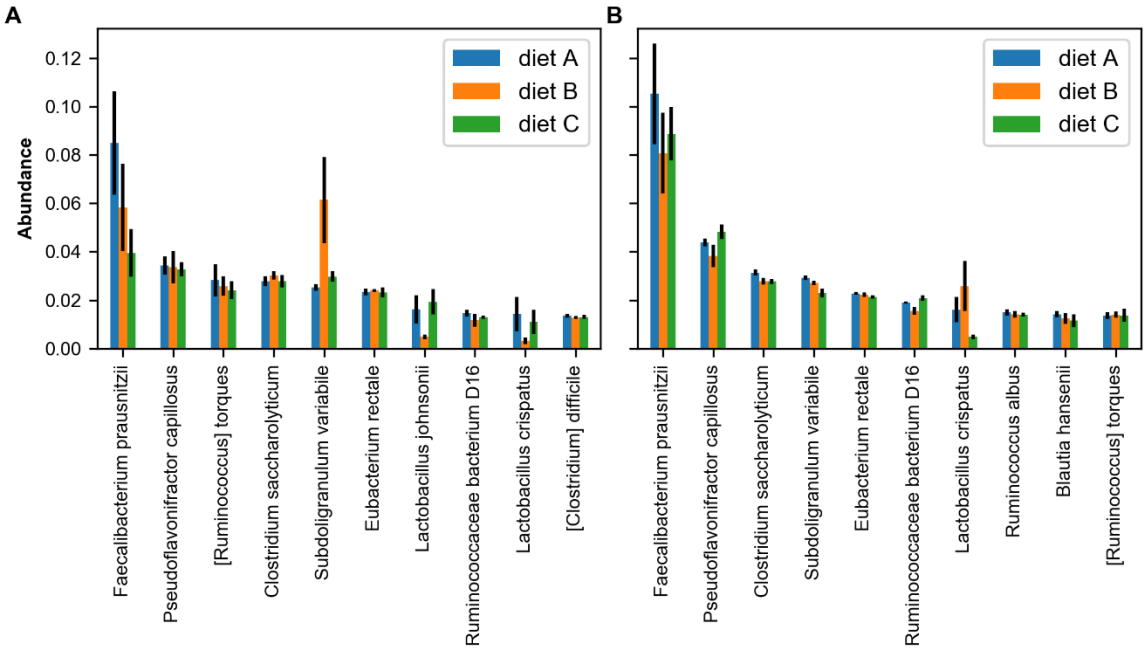
At phylum level, Firmicutes accounted for up to 70% of the differences observed between all pairwise comparisons (i.e., diet *vs* time) at both 14 (Figure 10 A) and 42 (Figure 10 B) days. The additional phyla accounting for such difference were Actinobacteria and Proteobacteria at both sampling time, as well as Bacteroidetes at 42 days only (Figures 10 A and 10 B).

Figure 10. Phyla in charge of 70% of the Bray-Curtis dissimilarities in birds tested at (A) 14 and (B) 42 days.



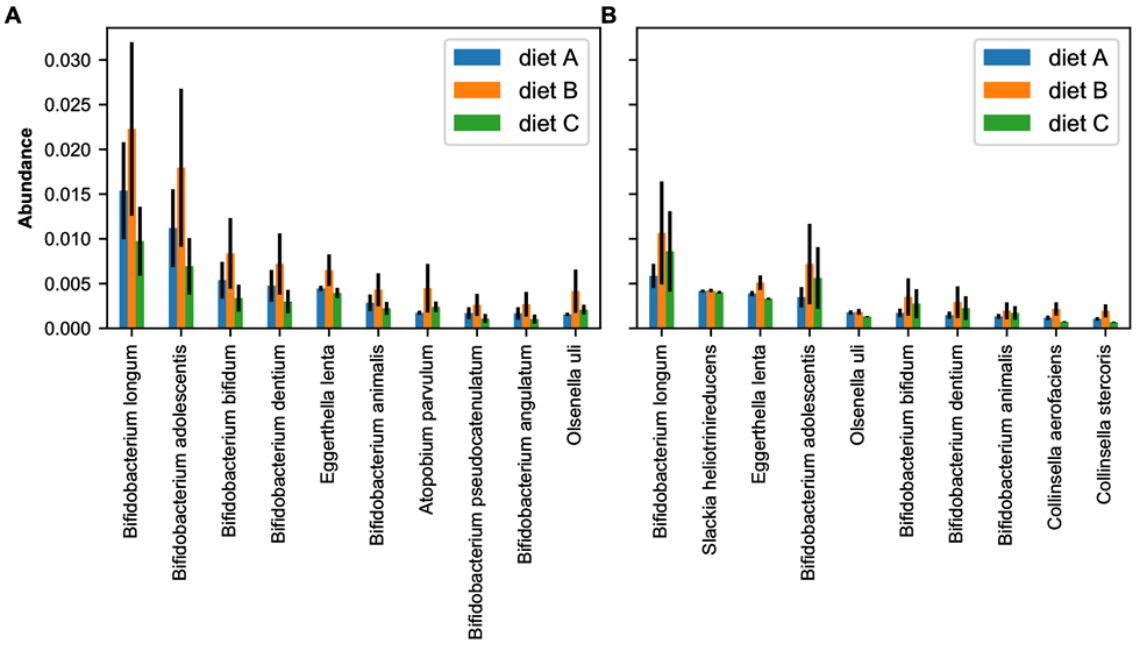
At species level, *Faecalibacterium prausnitzii* was the most abundant species among those accounting for up to 70% of the differences observed at both 14 and 42 days within the phylum Firmicutes, followed by *Subdoligranum variabile* at 14 days (Figure 11 A) and *Pseudoflavonifactor capillosus* at 42 days (Figure 11 B).

Figure 11. Firmicutes species included in the first 70% of the Bray-Curtis dissimilarities in birds tested at (A) 14 and (B) 42 days.



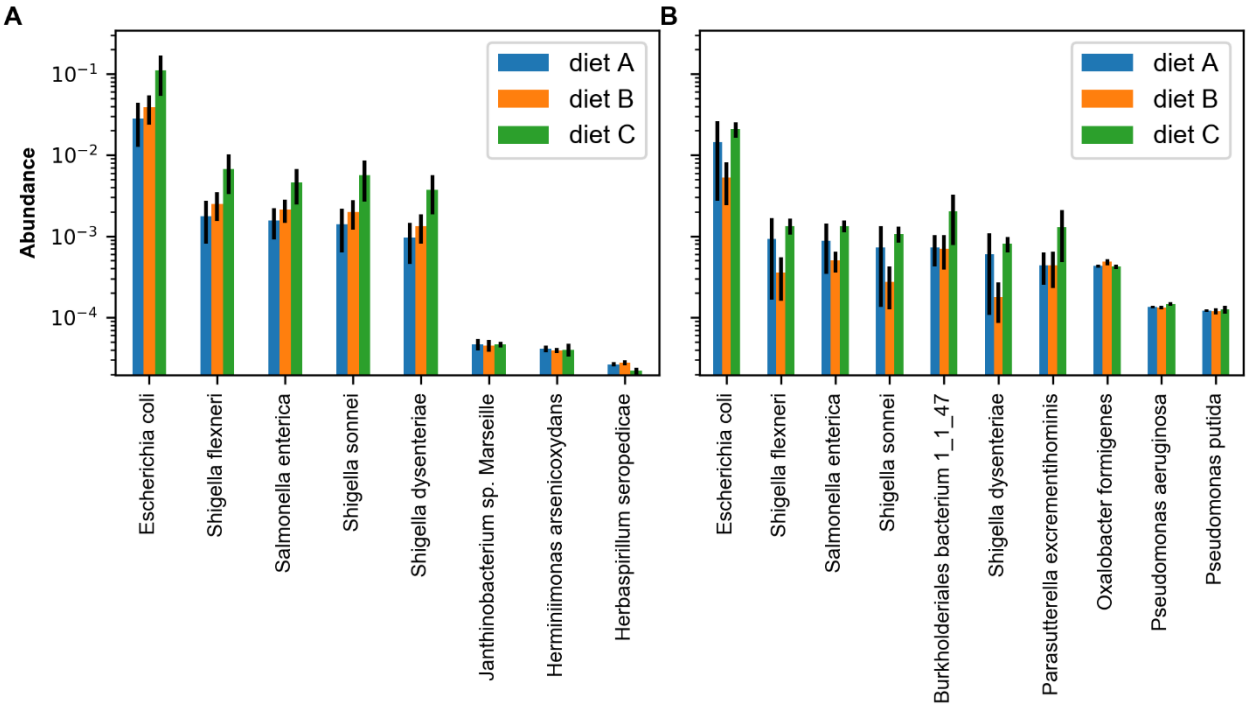
Bifidobacterium longum was the most abundant species among those accounting for up to 70% of the differences observed at both 14 (Figure 12 A) and 42 (Figures 12 B) days within the phylum Actinobacteria, followed by *Bifidobacterium adolescentis* at 14 days (Figure 12 A).

Figure 12. Actinobacteria species included in the first 70% of the Bray-Curtis dissimilarities in birds tested at (A) 14 and (B) 42 days.



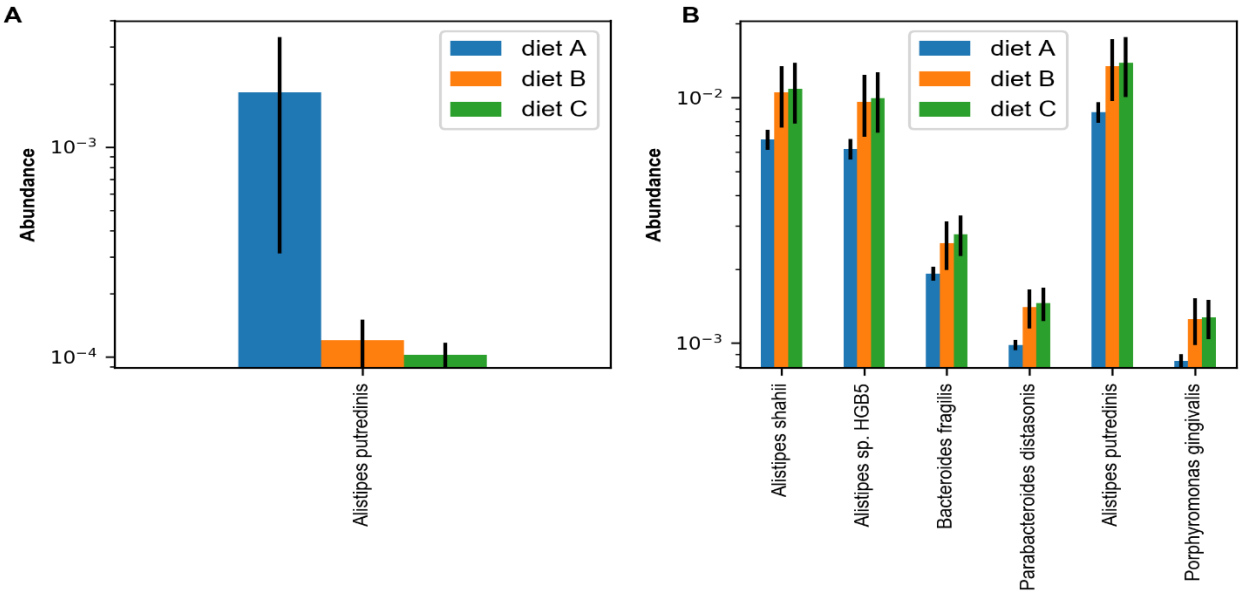
Escherichia coli was the most abundant species causing differences between pairwise comparisons at both sampling time within the phylum Proteobacteria, followed by *Salmonella enterica* and different species belonging to the genus Shigella at 14 (Figure 13 A) and by those species along with *Burkholderiales bacterium* 1_1_47, *Parasitterella excrementihominis* and *Oxalobacter formigenes* at 42 days (Figure 13 B).

Figure 13. Proteobacteria species included in the first 70% of the Bray-Curtis dissimilarities in birds tested at (A) 14 and (B) 42 days.



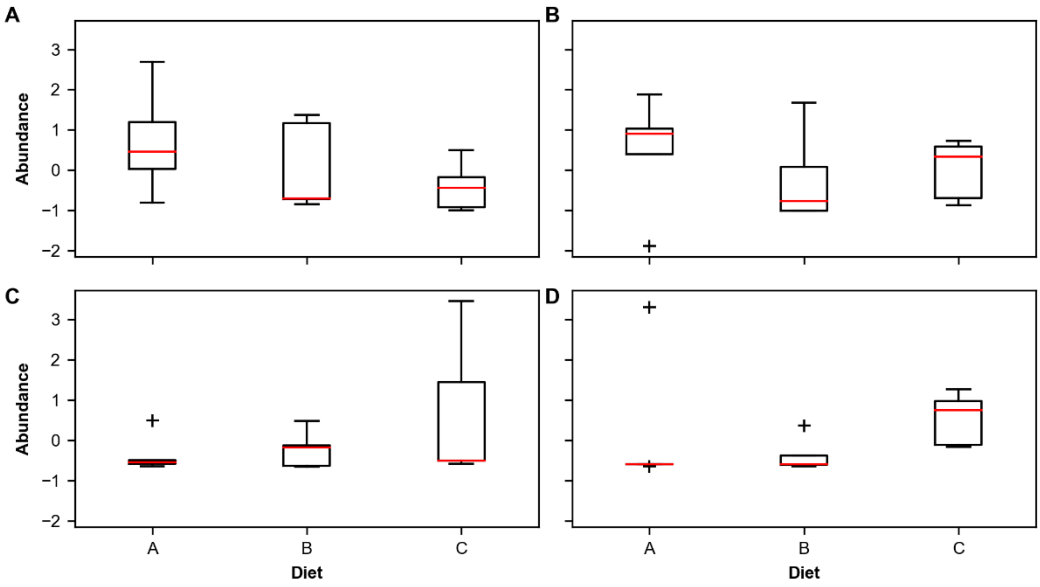
Alistipes putredinis accounted for the majority of the differences observed at species level between pairwise comparisons at both 14 (Figure 14 A) and 42 (Figure 14 B) days within the phylum Bacteroidetes, followed by *Alistipes shahii* and *Alistipes sp. HGB5* at 42 days (Figure 14 B).

Figure 14. Bacteroidetes species included in the first 70% of the Bray-Curtis dissimilarities in birds tested at (A) 14 and (B) 42 days



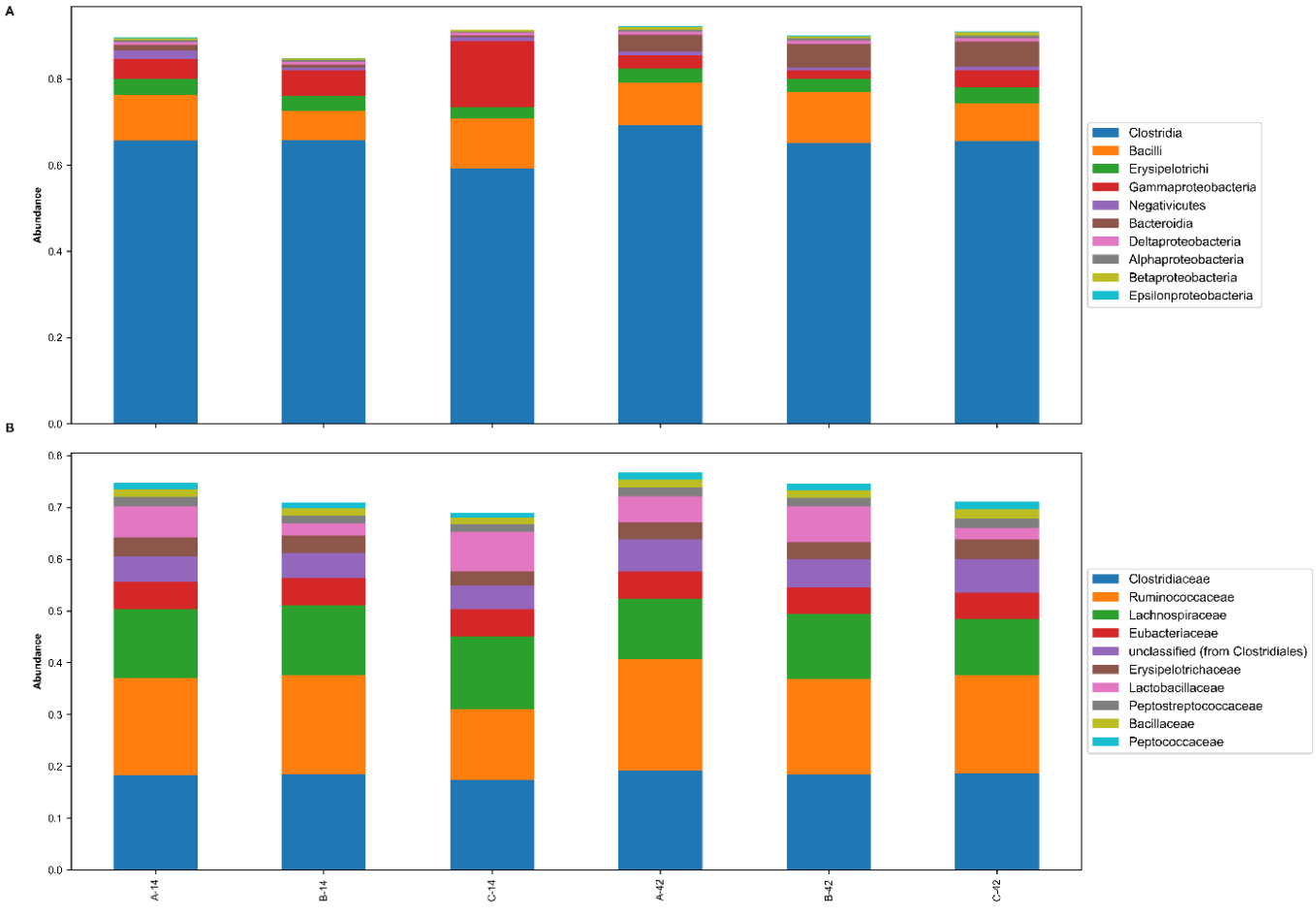
Overall, the abundance of *Faecalibacterium prausnitzii* in chickens fed with the different diets did not significantly change at both 14 and 42 days. The same trend was observed for *Escherichia coli* at 14 days. However, the abundance of *E. coli* in the caeca of birds fed with diet C at 42 days was significantly higher in comparison to groups A and B (Figure 15).

Figure 15. Boxplots showing the distribution of *Faecalibacterium prausnitzii* at 14 (A) and 42 (B) days and the distribution of *Escherichia coli* at 14 (C) and 42 (D) days.



At both sampling time, in all tested diets, the most abundant class was represented by Clostridia, followed by Bacilli showing an abundance ≥ 0.592 in all tested groups (Figure 16 A). The class of Erysipelotrichi was stable at both sampling time in all tested diets (i.e., abundance ranging between 0.026 and 0.037), whereas Gammaproteobacteria decreased and Bacteroidia increased in all tested groups between 14 and 42 days (Figure 16 A). However, at 14 days Gammaproteobacteria were significantly higher in group C fed with serine protease in comparison to the other groups. At family level, the most abundant groups were Clostridiaceae and Ruminococcaceae (Figure 16 B), followed by Lachnospiraceae, Eubacteriaceae and Lactobacillaceae. Clostridiaceae and Eubacteriaceae showed an abundance quite stable at both sampling times in all tested diets (i.e., abundance ranging between 0.173 and 0.192 and 0.050 and 0.053, respectively), whereas Ruminococcaceae increased over time in groups A and C. Lachnospiracea slightly decreased in all tested groups between 14 and 42 days, whereas Lactobacillaceae decreased in groups A and C and increased in group B over time (Figure 16 B).

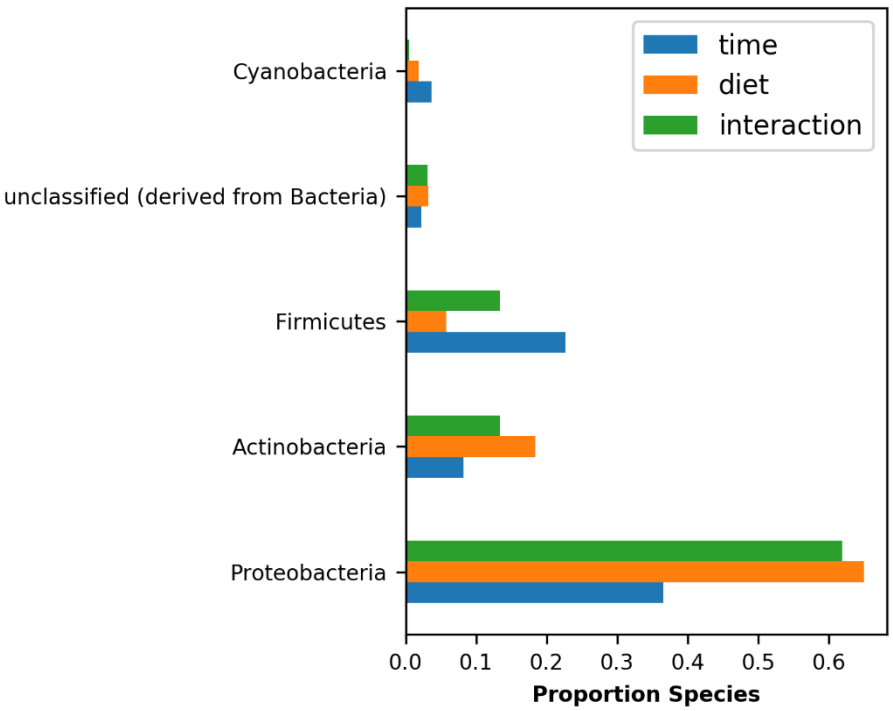
Figure 16. (A) Classes and (B) families belonging to the 10 most abundant phyla colonising the birds fed with each diet at each sampling time.



3.2.3.3 Impact of diet, age and their interactions on microbiota composition

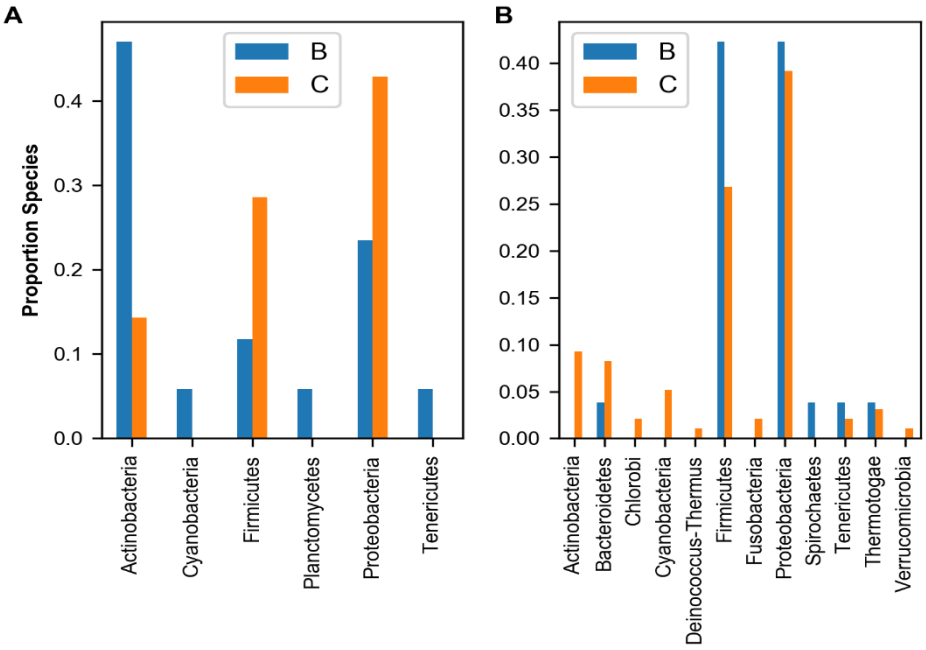
The ANOVA two-way analysis, assessing the impact of diet, time and their interaction on abundance of phyla colonising the caeca, showed that abundances of Actinobacteria and Proteobacteria were mainly affected by the diet, as well as interaction between diet and time. On the contrary, abundances of Firmicutes and Cyanobacteria were mainly affected by age of the birds (Figure 17).

Figure 17. Phyla showing an abundance significantly changed according to sampling time, diet or their interaction assessed using the ANOVA two way. The x-axis shows the proportion of significantly changed species belonging to the phyla shown in the y-axis.



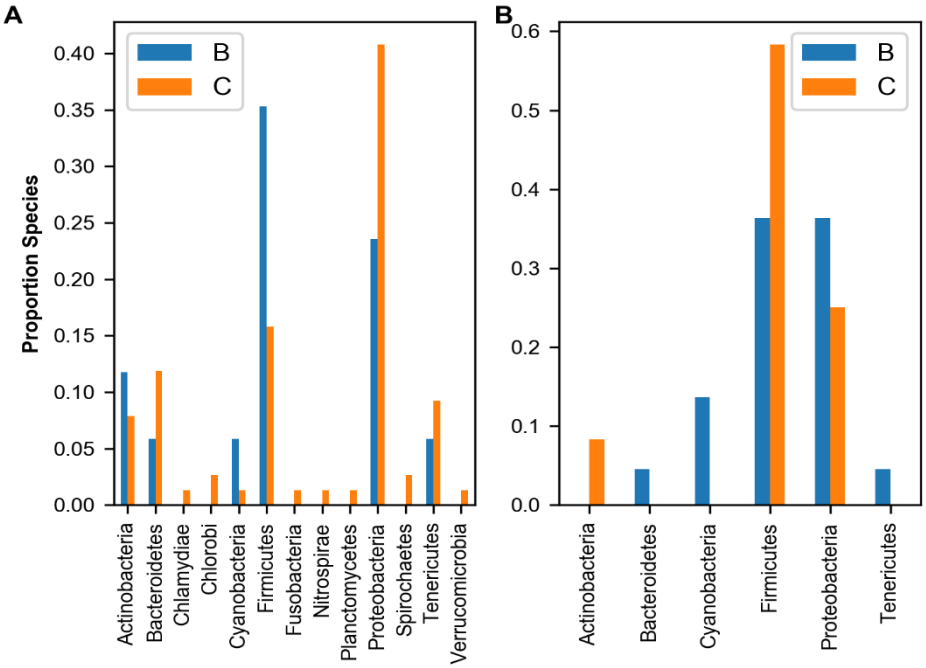
Overall, the number of species with significantly changed abundances in ceca of broilers fed with diet B in comparison to the control diet was similar at both sampling time (i.e., 43 and 49 species at 14 and 42 days, respectively). At 14 days, most of the species with increased abundances in comparison to the control diet belonged to the phyla Actinobacteria, Cyanobacteria and Planctomycetes (Figure 18 A), whereas most of the species with decreased abundances belonged to the phyla Bacteroidetes, Spirochetes and Thermotogae (Figure 18 B).

Figure 18. Phylum classification of the species with significantly (A) increased and (B) decreased abundance levels in the comparisons between diets B and C with the control diet (T-test) at 14 days.



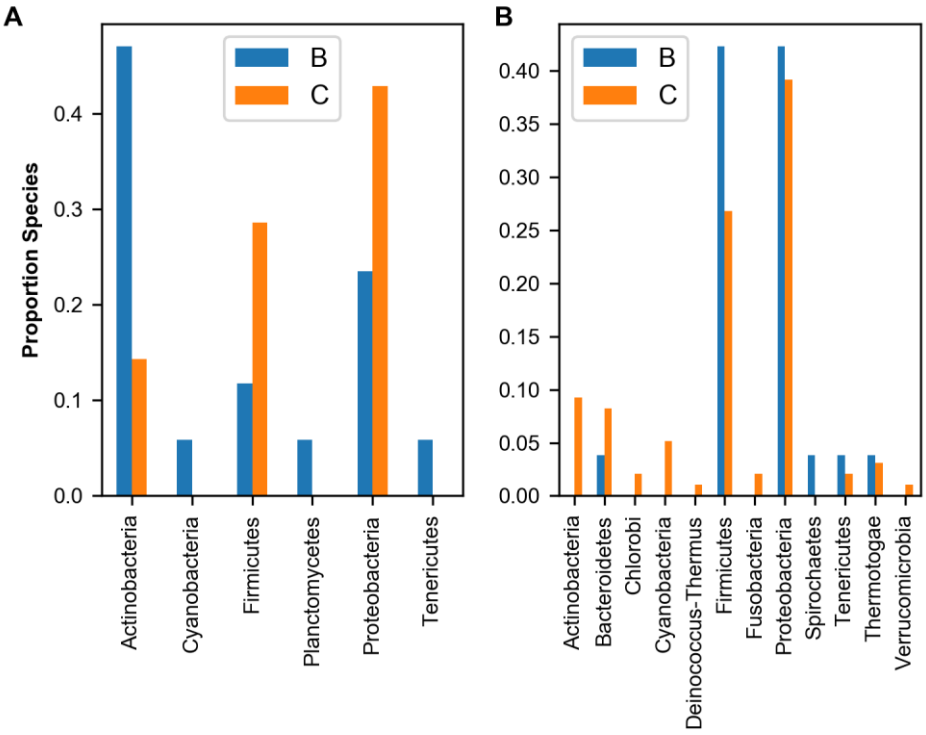
At 42 days, most of the species with increased abundances belonged to the phyla Actinobacteria, Firmicutes and Proteobacteria (Figure 19 A).

Figure 19. Phylum classification of the species with significantly (A) increased and (B) decreased abundance levels in the comparison between diets B and C with the control diet (T-test) at 42 days.



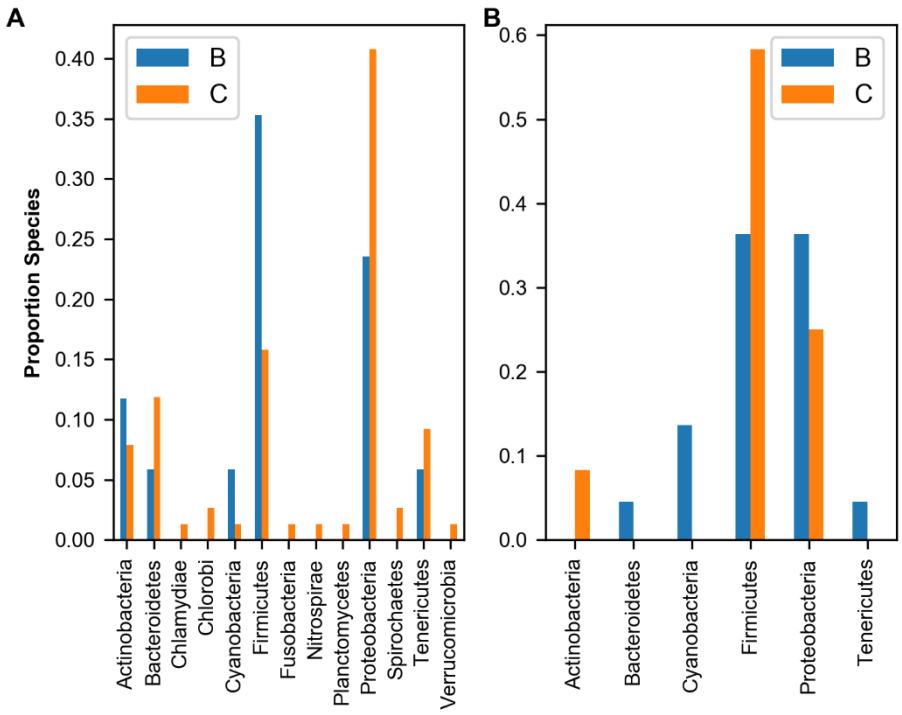
Overall, the number of species with significantly changed abundances in ceca of broilers fed with diet B in comparison to the control diet was similar at both sampling time (i.e., 43 and 49 species at 14 and 42 days, respectively). At 14 days, most of the species with increased abundances in comparison to the control diet belonged to the phyla Actinobacteria, Cyanobacteria and Planctomycetes (Figure 20 A), whereas most of the species with decreased abundances belonged to the phyla Bacteroidetes, Spirochetes and Thermotogae (Figure 20 B).

Figure 20. Proportion of species that differ between treatments and control in each phylum at 14 days. (A) Proportion of increased species; (B) Proportion of decreased species.



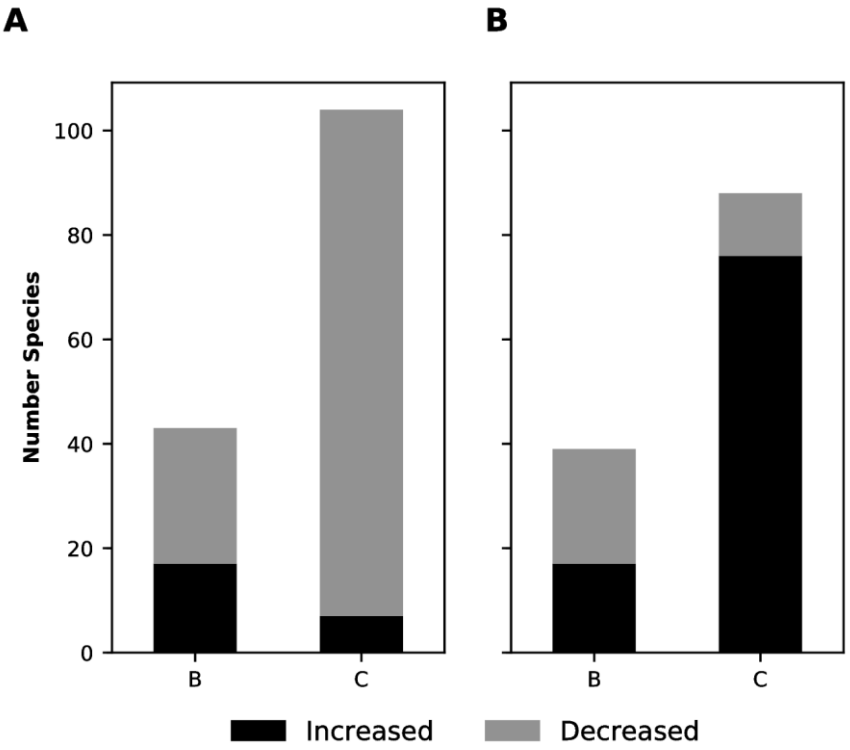
At 42 days, most of the species with increased abundances belonged to the phyla Actinobacteria, Firmicutes and Proteobacteria (Figure 21 A).

Figure 21. Proportion of species that differ between treatments and control in each phylum at 42 days. (A) Proportion of increased species; (B) Proportion of decreased species.



The number of species with significantly increased abundances in the ceca of broilers fed with the serine protease in comparison to the control was higher at 42 than 14 days (i.e., 76 vs 7, respectively), whereas the number of species with decreased abundances was higher a 14 than 42 days (i.e., 97 vs 12, respectively) (Figure 22).

Figure 22. Number of species significantly changed in the comparison between diets B and C with the control diet (T-test) at (A) 14 days and (B) 42 days. Black: number of species with increased abundance levels; grey: number of species with decreased abundance levels.



At 14 days, the species with significantly increased abundances belonged to the phyla Actinobacteria, Firmicutes, and Proteobacteria, whereas those with significantly decreased abundances belonged to the phyla Bacteroidetes, Chlorobi, Cyanobacetria, Deinococcus-Thermus, Fusobacteria, Tenericutes, and Thermotogae (Figure 20).

At 42 days, the species with increased abundances belonged to the phyla Bacteroidetes, Chlamydiae, Chlorobi, Cyanobacetria, Fusobacteria, Nitrospirae, Planctomycetes, Tenericutes, and Verrucomicrobia, whereas those with decreased abundances belonged to phyla Actinobacteria, Firmicutes, and Proteobacteria (Figure 21 B). The classification at the class level of the significantly increased and decreased groups are shown in Figure 23 and 24.

Figure 23. Class classification of the species with significantly different abundance levels in the comparison between diets B and C with the control diet at 14 days. (A) Increased species; (B) Decreased species.

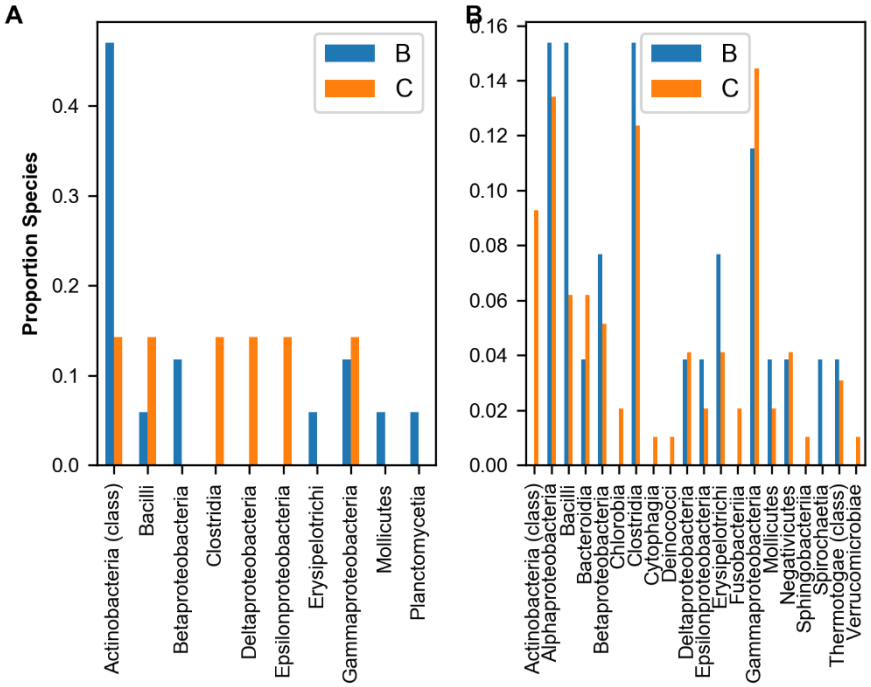
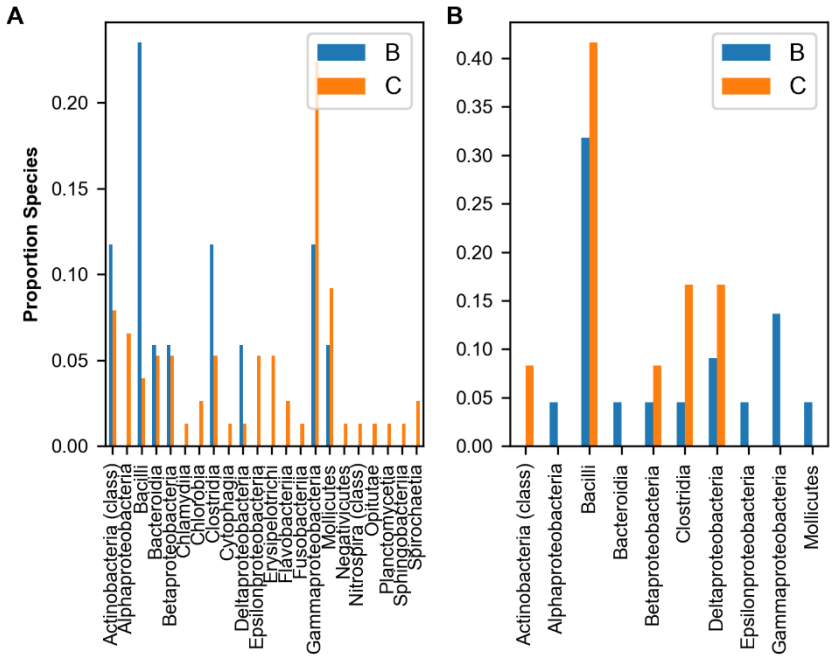


Figure 24. Class classification of the species with significantly different abundance levels in the comparison between diets B and C with the control diet at 42 days. (A) Increased species; (B) Decreased species.



3.2.3.4 Identification of signature species

At each sampling time, the species showing in the t test a p value < 0.05 in comparison to the control (i.e., diet A) and an average abundance ≥ 0.025% were considered signature species. At 14 days, five signature species were identified in the caeca of broilers fed with the diet containing -7% protein (i.e., diet B) (Table 9). All those species belonged to the phylum Firmicutes; *Eubacterium cylindroides* was significantly higher in comparison to the control, whereas the other signature species were significantly lower (Table 9). All signature species identified in the group fed with -7% protein and serine proteases, belonging to Bacteroidetes and Proteobacteria, were significantly lower in comparison to the control diet. At the end of the rearing period, the diet with -7% protein showed a significantly lower abundance of *Bacillus licheniformis, Lactococcus lactis and Lactobacillus ruminis* in comparison to the control diet. When serine protease was added in the same kind of diet (i.e., -7% protein) the abundance of *Solobacterium moorei, Lactobacillus lactis, Turicibacter* sp. HGF1 and *Acholeplasma laidlawii* were significantly higher, whereas those of *Subdoligranum variabile* and *Lactobacillus delbrueckii* decreased in comparison to the control diet (Table 9).

Table 9. Species assessed as signature, identified in the caeca of broilers fed with diets B and C with p values < 0.05 in comparison to group A and with an average abundance ≥ 0.025% within the three most abundant phyla colonising the birds at 14 and 42 days.

Phylum	Species	T-test p-value	SIMPER	Increase(+) /Decrease (-)
	Diet B 14 days			
Firmicutes	*Lactobacillus salivarius*	0.0368	0.5499	-
	Lactobacillus fermentum	0.0419	0.8324	-
	Eubacterium cylindroides	0.0451	0.8596	+
	Erysipelotrichaceae bacterium 3 1 53	0.0399	0.8998	-
	Lactobacillus brevis	0.0408	0.9033	-
	Diet C 14 days			
Bacteroidetes	*Prevotella ruminicola*	0.0208	0.9439	-
	Eubacterium dolichum	0.0465	0.8344	-
	Erysipelotrichaceae bacterium 3 1 53	0.0190	0.8497	-
	Acidaminococcus fermentans	0.0374	0.8687	-
	Eubacterium biforme	0.0355	0.8885	-
	Syntrophomonas wolfei	0.0482	0.8937	-
	Pelotomaculum thermopropionicum	0.0487	0.9048	-
	Carboxydothermus hydrogenoformans	0.0414	0.9099	-
	Phascolarctobacterium succinatutens	0.0079	0.9364	-
	Thermoanaerobacter brockii	0.0495	0.9377	-
Proteobacteria	*Bacillus pumilus*	0.0489	0.9422	-
	Candidatus Desulforudis audaxviator	0.0442	0.9515	-
	Desulfuromonas acetoxidans	0.0451	0.9552	-
	Ralstonia solanacearum	0.0488	0.9710	-
	Diet B 42 days			
Firmicutes	*Bacillus licheniformis*	0.0471	0.9192	-
	Lactococcus lactis	0.0471	0.9141	-
	Lactobacillus ruminis	0.0385	0.9023	-
	Diet C 42 days			
Firmicutes	*Subdoligranulum variabile*	0.0311	0.3739	-
	Lactobacillus delbrueckii	0.0335	0.8158	-
	Solobacterium moorei	0.0405	0.8743	+
	Lactococcus lactis	0.0475	0.8918	+
	Turicibacter sp. HGF1	0.0368	0.8955	+
Tenericutes	*Acholeplasma laidlawii*	0.0389	0.8646	+

SIMPER[§] - ordered cumulative contribution to the observed dissimilarity between the diet and the control

3.2.3.5 Caeca metabolic genes composition

The average abundances of level one metabolic and functional protein categories were similar among all tested groups, with the highest abundances of genes coding for carbohydrates metabolism, followed by genes for clustering-based subsystems (Figure 25).

Figure 25. Heatmap showing the average relative abundance of metagenomic reads annotated to SEED Subsystems (Level 1). The columns represent the diet types at each sampling time, the rows represent the SEED subsystems (Level 1), and the colorbar represent the range of average relative abundance of reads annotated to each category.

At 14 days, at level one functions, virulence, disease and defence genes, as well as cell wall and capsule genes, were higher in the ceca of broilers fed with serine protease in comparison to the ceca of broilers fed with -7% protein (Table 10). At the same sampling time, the main differences in abundances between level 2, level 3 and level function genes were identified in ceca of broilers belonging to groups B and C (Table 10). Moreover, the abundance of Protein secretion system Type VI (T6SS) was significantly higher in the ceca of broilers belonging to group B in comparison to the control.

Table 10. Pairwise t-test results on metabolic and functional protein categories at 14 days

	Comparison	p-val	mean A	mean B	mean C
Level 1 - results with p-values <0.1					
Virulence, Disease and Defense	(B, C)	0.0210	0.02254017	0.02158902	0.02466282
Amino Acids and Derivatives	(B, C)	0.0856	0.08447138	0.08992435	0.08461438
Cell Wall and Capsule	(B, C)	0.0998	0.04244985	0.04057576	0.04486254
Level 2 - results with p-values <0.05 and abundance mean values ≥ 0.001					
Selenoproteins	(A, C)	0.0059	0.00165232	0.00195313	0.00205538
Sugar Phosphotransferase Systems, PTS	(B, C)	0.0077	0.00244844	0.00228124	0.00304398
D-tyrosyl-tRNA(Tyr) deacylase (EC 3.1.-.-) cluster	(B, C)	0.0176	0.00022085	0.00019047	0.00021945
Regulation of virulence	(B, C)	0.0180	0.00218882	0.00204319	0.00223771
Electron accepting reactions	(A, C)	0.0201	0.00461309	0.00465974	0.00578723
Sugar alcohols	(B, C)	0.0223	0.00729531	0.00692066	0.00842076
Electron accepting reactions	(B, C)	0.0251	0.00461309	0.00465974	0.00578723
Protein secretion system, Type VI	(A, B)	0.0286	0.00103303	0.00124519	0.00122071
Resistance to antibiotics and toxic compounds	(B, C)	0.0301	0.01564567	0.01445885	0.01687134
Pyridoxine	(B, C)	0.0418	0.00237646	0.00249819	0.00218212
Polysaccharides	(B, C)	0.0439	0.00490108	0.00524122	0.00426978
Level 3 – results with p-values <0.05 and abundance mean values ≥ 0.0005					
pVir Plasmid of Campylobacter	(B, C)	0.0018	0.00111436	0.00132148	0.00101498
At4g38090	(B, C)	0.0021	0.00059273	0.00046618	0.00068250
Sex pheromones in Enterococcus faecalis and other Firmicutes	(B, C)	0.0055	0.00093652	0.00068214	0.00120058
Beta-lactamase	(B, C)	0.0066	0.00063350	0.00051703	0.00083367
Galactose-inducible PTS	(B, C)	0.0088	0.00062466	0.00056931	0.00085701
rRNA modification Bacteria	(A, B)	0.0095	0.00361442	0.00334855	0.00337693
LMPTP YfkJ cluster	(B, C)	0.0148	0.00011742	$7.67\ 10^{-5}$	0.00013129
Pyruvate metabolism II: acetyl-CoA, acetogenesis from pyruvate	(B, C)	0.0160	0.00312521	0.00300561	0.00338952
Glycogen metabolism cluster	(B, C)	0.0172	0.00328702	0.00370479	0.00288228
Transport of Iron	(B, C)	0.0188	0.00173977	0.00191605	0.00153969
YgfZ	(B, C)	0.0195	0.01079670	0.01117850	0.01037185
Glycerol and Glycerol-3-phosphate Uptake and Utilization	(B, C)	0.0212	0.00201134	0.00199045	0.00240512
Fructose and Mannose Inducible PTS	(B, C)	0.0222	0.00059605	0.00058210	0.00083710
Histidine Biosynthesis	(B, C)	0.0225	0.00236479	0.00261008	0.00192856
Polyadenylation bacterial	(B, C)	0.0230	0.00120542	0.00128014	0.00103187
Arginine Biosynthesis extended	(B, C)	0.0239	0.00362080	0.00393469	0.00304773
rRNA modification Bacteria	(A, C)	0.0240	0.00361442	0.00334855	0.00337693
Glycogen metabolism	(B, C)	0.0248	0.00418025	0.00455074	0.00360966
CBSS-203122.12.peg.188	(B, C)	0.0250	0.00090132	0.00099590	0.00078267
Campylobacter Iron Metabolism	(B, C)	0.0252	0.00173004	0.00189640	0.00147344
CBSS-262719.3.peg.410	(A, B)	0.0271	0.00133850	0.00110650	0.00120509
Type VI secretion systems	(A, B)	0.0286	0.00103303	0.00124519	0.00122071
Terminal cytochrome oxidases	(A, C)	0.0291	0.00153680	0.00155148	0.00193620
Staphylococcus aureus hypothetical repetitive gene loci	(B, C)	0.0291	0.00136710	0.00135979	0.00158032
ATP-dependent RNA helicases, bacterial	(B, C)	0.0319	0.00062048	0.00052645	0.00070976
Protein degradation	(B, C)	0.0337	0.00086754	0.00084366	0.00108971
Leucine Biosynthesis	(B, C)	0.0374	0.00216641	0.00240628	0.00186562

Methylthiotransferases	(A, C)	0.0374	0.00098375	0.00089894	0.00072909
Succinate dehydrogenase	(B, C)	0.0381	0.00070802	0.00048619	0.00064609
Predicted secretion system W clustering with cell division proteins	(A, C)	0.0394	0.00063271	0.00057492	0.00047790
polyprenyl synthesis	(B, C)	0.0400	0.00100794	0.00113698	0.00085796
COG1836	(B, C)	0.0404	0.00074630	0.00077397	0.00065285
Protein chaperones	(B, C)	0.0407	0.00361011	0.00388526	0.00313795
Terminal cytochrome oxidases	(B, C)	0.0409	0.00153680	0.00155148	0.00193620
Housecleaning nucleoside triphosphate pyrophosphatases	(B, C)	0.0416	0.00060388	0.00051051	0.00059403
Pyridoxin (Vitamin B6) Biosynthesis	(B, C)	0.0418	0.00237646	0.00249819	0.00218212
Ammonia assimilation	(B, C)	0.0420	0.00374305	0.00430987	0.00360616
Fructose and Mannose Inducible PTS	(A, C)	0.0424	0.00059605	0.00058210	0.00083710
Transcription factors cyanobacterial RpoD-like sigma factors	(A, C)	0.0445	0.00080135	0.00073515	0.00056196
RNA modification cluster	(B, C)	0.0448	0.00070776	0.00063224	0.00074654
Mannose Metabolism	(A, C)	0.0451	0.00162792	0.00155888	0.00184369
Phage capsid proteins	(A, C)	0.0455	0.00060711	0.00070375	0.00092889
Branched-Chain Amino Acid Biosynthesis	(B, C)	0.0461	0.00511578	0.00564862	0.00457491
USS-DB-7	(A, B)	0.0477	0.00099220	0.00120424	0.00107820
Ethanolamine utilization	(A, C)	0.0481	0.00190448	0.00198283	0.00220315
CBSS-56780.10.peg.1536	(B, C)	0.0482	0.00059039	0.00061727	0.00054014
RNA methylation	(B, C)	0.0498	0.00341701	0.00327438	0.00353359
Level functions – results with p-values <0.05 and abundance mean values ≥ 0.0002					
PTS system, ,mannose-specific IIB component (EC 2.7.1.69)	(B, C)	0.0000	0.00020476	0.00014238	0.00036685
PTS system, mannose-specific IIA component (EC 2.7.1.69)	(B, C)	0.0003	0.00017017	0.00012239	0.00032328
Mannose-6-phosphate isomerase (EC 5.3.1.8)	(B, C)	0.0004	0.00042406	0.00027185	0.00043636
Cold-shock DEAD-box protein A	(B, C)	0.0007	0.00023546	0.00019995	0.00028155
Cell division protein FtsA	(B, C)	0.0017	0.00028104	0.00022496	0.00050602
5, 10-methylenetetrahydrofolate reductase (EC 1.5.1.20)	(A, C)	0.0018	0.00087568	0.00094374	0.00070525
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	(B, C)	0.0022	0.00115640	0.00094497	0.00163735
D-serine/D-alanine/glycine transporter	(B, C)	0.0029	0.00030117	0.00012210	0.00043357
Maltose phosphorylase (EC 2.4.1.8)	(B, C)	0.0030	0.00022293	0.00010109	0.00027986
Chorismate synthase (EC 4.2.3.5)	(B, C)	0.0030	0.00089952	0.00107742	0.00071337
Trehalose phosphorylase (EC 2.4.1.64)	(B, C)	0.0031	0.00044144	0.00019944	0.00054887
2-C-methyl-D-erythritol 2, 4-cyclodiphosphate synthase (EC 4.6.1.12)	(B, C)	0.0069	0.00053948	0.00066055	0.00044620
GTP-binding protein HflX	(B, C)	0.0073	0.00043417	0.00058593	0.00044268
2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (EC 2.7.7.60)	(A, C)	0.0084	0.00068442	0.00070114	0.00053022
DNA recombination protein RmuC	(B, C)	0.0089	0.00021964	0.00028578	0.00018107
6-phosphogluconate dehydrogenase, decarboxylating (EC 1.1.1.44)	(B, C)	0.0091	0.00047310	0.00028605	0.00066625
Rhodanese-related sulfurtransferase	(A, C)	0.0098	0.00018873	0.00023808	0.00028937
Gluconokinase (EC 2.7.1.12)	(B, C)	0.0102	0.00022773	0.00016595	0.00033474
Glycogen phosphorylase (EC 2.4.1.1)	(B, C)	0.0112	0.00215596	0.00235219	0.00171623
PTS system, mannose-specific IIB component (EC 2.7.1.69)	(A, C)	0.0114	0.00020476	0.00014238	0.00036685
2', 3'-cyclic-nucleotide 2'-phosphodiesterase (EC 3.1.4.16)	(B, C)	0.0115	0.00027059	0.00019471	0.00043095
PTS system, cellobiose-specific IIC component (EC 2.7.1.69)	(A, B)	0.0126	0.00032120	0.00017186	0.00025744
1-deoxy-D-xylulose 5-phosphate reductoisomerase (EC 1.1.1.267)	(B, C)	0.0128	0.00097032	0.00101755	0.00072552

Phosphoribosylformylglycinamidine synthase, glutamine amidotransferase subunit (EC 6.3.5.3)	(B, C)	0.0132	0.00126705	0.00150265	0.00106036
N-acetyl-gamma-glutamyl-phosphate reductase (EC 1.2.1.38)	(B, C)	0.0147	0.00026229	0.00032044	0.00022526
tRNA-i(6)A37 methylthiotransferase	(A, C)	0.0167	0.00337435	0.00318570	0.00237999
ClpB protein	(B, C)	0.0167	0.00368464	0.00431777	0.00340495
Phosphoribosylformylglycinamidine cyclo-ligase (EC 6.3.3.1)	(B, C)	0.0168	0.00062865	0.00069966	0.00054665
N-acetylglutamate synthase (EC 2.3.1.1)	(B, C)	0.0169	0.00030433	0.00032330	0.00024260
Methionine ABC transporter substrate-binding protein	(B, C)	0.0170	0.00061480	0.00049193	0.00072964
PTS system, mannose-specific IID component (EC 2.7.1.69)	(B, C)	0.0181	0.00027274	0.00026422	0.00043959
DNA topoisomerase I (EC 5.99.1.2)	(B, C)	0.0183	0.00261068	0.00287428	0.00206854
LSU ribosomal protein L11p (L12e)	(A, B)	0.0187	0.00055244	0.00044698	0.00042106
Phosphoribosylformylglycinamidine synthase, synthetase subunit (EC 6.3.5.3)	(B, C)	0.0190	0.00127648	0.00151254	0.00109266
Citrate synthase (si) (EC 2.3.3.1)	(B, C)	0.0198	0.00127890	0.00159784	0.00118206
Dipeptide transport system permease protein DppC (TC 3.A.1.5.2)	(A, B)	0.0199	0.00023559	0.00031684	0.00029518
Phosphoserine aminotransferase (EC 2.6.1.52)	(B, C)	0.0199	0.00105236	0.00114909	0.00084413
Putative Dihydrolipoamide dehydrogenase (EC 1.8.1.4)	(B, C)	0.0200	0.00012946	0.00011659	0.00025526
Rod shape-determining protein MreC	(B, C)	0.0201	0.00020421	0.00015158	0.00030797
GTP-binding protein HflX	(A, B)	0.0203	0.00043417	0.00058593	0.00044268
Hypothetical radical SAM family enzyme in heat shock gene cluster, similarity with CPO of BS HemN-type	(B, C)	0.0203	0.00029641	0.00038577	0.00022327
Glycerol-3-phosphate ABC transporter, periplasmic glycerol-3-phosphate-binding protein (TC 3.A.1.1.3)	(B, C)	0.0206	0.00038193	0.00022729	0.00054628
Beta-lactamase	(B, C)	0.0209	0.00032376	0.00027470	0.00040593
Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)	(B, C)	0.0209	0.00031347	0.00021530	0.00045976
Cytochrome d ubiquinol oxidase subunit II (EC 1.10.3.-)	(B, C)	0.0214	0.00038740	0.00020205	0.00060347
Acetylglutamate kinase (EC 2.7.2.8)	(B, C)	0.0222	0.00033701	0.00037355	0.00025759
N-acetylglutamate synthase (EC 2.3.1.1)	(A, C)	0.0222	0.00030433	0.00032330	0.00024260
23S rRNA (Uracil-5-) -methyltransferase RumA (EC 2.1.1.-)	(B, C)	0.0226	0.00072926	0.00053494	0.00073096
Phage major capsid protein	(A, C)	0.0236	0.00096949	0.00120269	0.00141684
Intramembrane protease RasP/YluC, implicated in cell division based on FtsL cleavage	(A, C)	0.0243	0.00034234	0.00034893	0.00041106
LSU ribosomal protein L22p (L17e)	(A, C)	0.0248	0.00017756	0.00015809	0.00013506
PTS system, mannose-specific IID component (EC 2.7.1.69)	(A, C)	0.0258	0.00027274	0.00026422	0.00043959
Ornithine decarboxylase (EC 4.1.1.17)	(B, C)	0.0264	0.00056055	0.00027304	0.00066510
Nicotinamidase (EC 3.5.1.19)	(B, C)	0.0279	0.00037653	0.00031431	0.00044666
Histidinol-phosphate aminotransferase (EC 2.6.1.9)	(B, C)	0.0284	0.00021720	0.00026506	0.00017673
Polyribonucleotide nucleotidyltransferase (EC 2.7.7.8)	(B, C)	0.0292	0.00364486	0.00378304	0.00274906
Xanthine/uracil/thiamine/ascorbate permease family protein	(B, C)	0.0292	0.00053745	0.00049061	0.00063072
Biotin-protein ligase (EC 6.3.4.15)	(A, C)	0.0304	0.00027108	0.00031636	0.00041320
3-isopropylmalate dehydratase small subunit (EC 4.2.1.33)	(B, C)	0.0306	0.00043568	0.00050108	0.00035614
UDP-N-acetylmuramoylalanyl-D-glutamyl-2, 6-diaminopimelate--D-alanyl-D-alanine ligase (EC 6.3.2.10)	(A, B)	0.0338	0.00105446	0.00083749	0.00093248
N-acetyl-L,L-diaminopimelate aminotransferase (EC 2.6.1.-)	(A, B)	0.0349	0.00020640	0.00013967	0.00020738
Ribosomal protein L11 methyltransferase (EC 2.1.1.-)	(B, C)	0.0349	0.00018907	0.00017648	0.00023043
Ribonuclease BN (EC 3.1.-.-)	(B, C)	0.0356	0.00018542	0.00012731	0.00035373
Thymidylate kinase (EC 2.7.4.9)	(A, C)	0.0357	0.00046558	0.00046620	0.00037458
Biotin operon repressor	(A, C)	0.0361	0.00016821	0.00019333	0.00026738
Acetylornithine aminotransferase (EC 2.6.1.11)	(B, C)	0.0362	0.00096226	0.00106153	0.00080046
Methylmalonyl-CoA decarboxylase, beta chain (EC 4.1.1.41)	(B, C)	0.0363	0.00044429	0.00051250	0.00029878

Pyrimidine-nucleoside phosphorylase (EC 2.4.2.2)	(A, C)	0.0370	0.00066343	0.00054688	0.00040286
Ubiquinone/menaquinone biosynthesis methyltransferase UbiE (EC 2.1.1.-)	(B, C)	0.0376	0.00025994	0.00016084	0.00034155
Phosphoribosyl-AMP cyclohydrolase (EC 3.5.4.19)	(A, C)	0.0377	0.00092342	0.00084429	0.00062097
Acetyl-coenzyme A carboxyl transferase alpha chain (EC 6.4.1.2)	(B, C)	0.0387	0.00035764	0.00039926	0.00029211
Glutamate 5-kinase (EC 2.7.2.11)	(B, C)	0.0387	0.00103425	0.00112361	0.00086500
High-affinity branched-chain amino acid transport system permease protein LivH (TC 3.A.1.4.1)	(B, C)	0.0388	0.00026786	0.00027168	0.00019846
Phospho-N-acetylmuramoyl-pentapeptide-transferase (EC 2.7.8.13)	(A, C)	0.0393	0.00027953	0.00027189	0.00022468
Cytochrome d ubiquinol oxidase subunit I (EC 1.10.3.-)	(B, C)	0.0395	0.00025666	0.00015817	0.00042143
3-isopropylmalate dehydrogenase (EC 1.1.1.85)	(B, C)	0.0395	0.00125259	0.00140642	0.00097793
3-ketoacyl-CoA thiolase (EC 2.3.1.16)	(A, C)	0.0411	0.00227912	0.00224503	0.00315085
Glutamate synthase [NADPH] small chain (EC 1.4.1.13)	(B, C)	0.0412	0.00146336	0.00176306	0.00121559
UTP--glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	(B, C)	0.0414	0.00047714	0.00038620	0.00047192
Hydroxymethylglutaryl-CoA synthase (EC 2.3.3.10)	(B, C)	0.0416	0.00020442	$6.73\ 10^{-5}$	0.00026246
Ribosomal RNA small subunit methyltransferase B (EC 2.1.1.-)	(B, C)	0.0418	0.00044192	0.00036550	0.00053045
3'-to-5' exoribonuclease RNase R	(B, C)	0.0434	0.00052974	0.00039537	0.00051405
Glycerate kinase (EC 2.7.1.31)	(A, C)	0.0443	0.00112512	0.00137300	0.00160909
Phosphoesterase, DHH family protein	(A, B)	0.0444	0.00036586	0.00026323	0.00032499
1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase (EC 1.17.7.1)	(B, C)	0.0448	0.00130742	0.00134226	0.00096096
2-isopropylmalate synthase (EC 2.3.3.13)	(B, C)	0.0449	0.00112915	0.00131132	0.00099531
Lacto-N-biose phosphorylase (EC 2.4.1.211)	(A, C)	0.0449	0.00057665	0.00067762	0.00031051
RNA polymerase sigma factor RpoD	(A, C)	0.0451	0.00320061	0.00291986	0.00224315
23S rRNA (Uracil-5-) -methyltransferase RumA (EC 2.1.1.-)	(A, B)	0.0453	0.00072926	0.00053494	0.00073096
Imidazole glycerol phosphate synthase amidotransferase subunit (EC 2.4.2.-)	(A, C)	0.0458	0.00016647	0.00022371	0.00012259
Butyrate kinase (EC 2.7.2.7)	(B, C)	0.0463	0.00039588	0.00048732	0.00025642
GTPase and tRNA-U34 5-formylation enzyme TrmE	(B, C)	0.0472	0.00294506	0.00228020	0.00310661
Arginine/ornithine antiporter ArcD	(A, C)	0.0482	0.00035904	0.00057199	0.00063394
Histidinol dehydrogenase (EC 1.1.1.23)	(B, C)	0.0488	0.00040510	0.00046680	0.00033088
D-tyrosyl-tRNA(Tyr) deacylase	(A, C)	0.0490	0.00034900	0.00031730	0.00026203
ATP-dependent Clp protease ATP-binding subunit ClpA	(B, C)	0.0491	0.00140518	0.00098455	0.00146038
Maltose O-acetyltransferase (EC 2.3.1.79)	(A, C)	0.0492	0.00027105	0.00025471	0.00021634
tRNA-i(6)A37 methylthiotransferase	(B, C)	0.0493	0.00337435	0.00318570	0.00237999
2, 3-bisphosphoglycerate-independent phosphoglycerate mutase (EC 5.4.2.1)	(A, C)	0.0497	0.00229392	0.00211853	0.00171048

At 42 days, statistically significant differences were observed between genes coding for motility and chemotaxis, potassium metabolism, cell division and cell cycle, stress response and iron acquisition and metabolism, between ceca of broilers fed with diets B and C (Table 11).

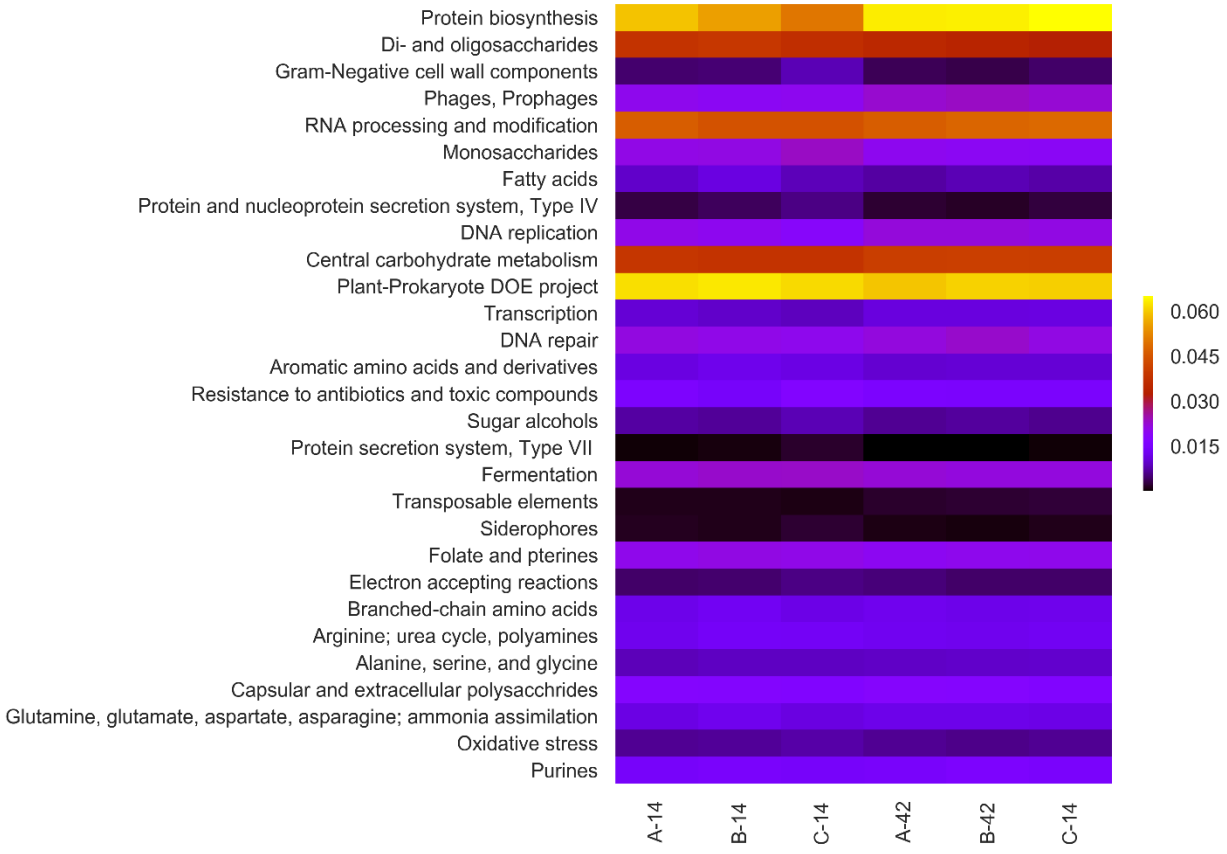
Table 11. Pairwise t-test results on metabolic and functional protein categories at 42 days

Function	Comparison	p-val	mean A	mean B	mean C
Level 1 – results with p-values <0.1					
Motility and Chemotaxis	(B, C)	0.0021	0.00300	0.00261	0.00362
Potassium metabolism	(B, C)	0.0303	0.00232	0.00225	0.00262
Cell Division and Cell Cycle	(B, C)	0.0354	0.01773	0.01810	0.01704
Stress Response	(B, C)	0.0683	0.01489	0.01410	0.01527
Iron acquisition and metabolism	(B, C)	0.0829	0.00972	0.00930	0.01070
Level 2 – results with p-values <0.05 and abundance mean values ≥ 0.001					
Protein secretion system, Type VI	(B, C)	0.0004	0.00122	0.00115	0.00149
Flagellar motility in Prokaryota	(B, C)	0.0036	0.00219	0.00185	0.00267
Protein secretion system, Type VI	(A, C)	0.0086	0.00122	0.00115	0.00149
DNA repair	(B, C)	0.0107	0.02190	0.02293	0.02139
Gram-Negative cell wall components	(B, C)	0.0129	0.00371	0.00329	0.00443
DNA uptake, competence	(B, C)	0.0131	0.00412	0.00410	0.00332
DNA uptake, competence	(A, C)	0.0194	0.00412	0.00410	0.00332
Sugar alcohols	(B, C)	0.0357	0.00665	0.00712	0.00625
Purines	(A, B)	0.0386	0.01472	0.01558	0.01486
Gram-Positive cell wall components	(B, C)	0.0458	0.00247	0.00303	0.00198
CRISPs	(A, B)	0.0460	0.00177	0.00145	0.00144
Level 3 – results with p-values <0.05 and abundance mean values ≥ 0.0005					
USS-DB-7	(B, C)	0.0003	0.00119	0.00112	0.00145
Type VI secretion systems	(B, C)	0.0004	0.00122	0.00115	0.00149
Recycling of Peptidoglycan Amino Acids	(B, C)	0.0015	0.00051	0.00045	0.00064

Function	Comparison	p-val	mean A	mean B	mean C
Level 1 – results with p-values <0.1					
One-carbon metabolism by tetrahydropterines	(A, B)	0.0022	0.00165	0.00185	0.00170
tRNA-methylthiotransferase containing cluster	(B, C)	0.0022	0.00079	0.00068	0.00083
CBSS-306254.1.peg.1508	(A, B)	0.0025	0.00049	0.00056	0.00050
Flagellum	(B, C)	0.0050	0.00139	0.00114	0.00162
Heme, hemin uptake and utilization systems in GramNegatives	(B, C)	0.0065	0.00048	0.00039	0.00056
Sporulation Cluster	(A, B)	0.0074	0.00148	0.00163	0.00151
At2g23840	(B, C)	0.0075	0.00205	0.00199	0.00218
Resistance to Vancomycin	(A, C)	0.0079	0.00073	0.00066	0.00057
CBSS-342610.3.peg.1794	(A, C)	0.0084	0.00064	0.00071	0.00073
Type VI secretion systems	(A, C)	0.0086	0.00122	0.00115	0.00149
USS-DB-7	(A, C)	0.0112	0.00119	0.00112	0.00145
Murein Hydrolases	(B, C)	0.0126	0.00093	0.00084	0.00101
Level functions – results with p-values <0.05 and abundance mean values ≥ 0.0002					
Stage V sporulation protein D	(B, C)	0.00004	0.00050	0.00043	0.00059
DNA-directed RNA polymerase alpha subunit (EC 2.7.7.6)	(B, C)	0.00008	0.00138	0.00122	0.00151
site-specific recombinase, phage integrase family	(A, C)	0.0001	0.00036	0.00027	0.00028
site-specific recombinase, phage integrase family	(A, B)	0.0017	0.00036	0.00027	0.00028

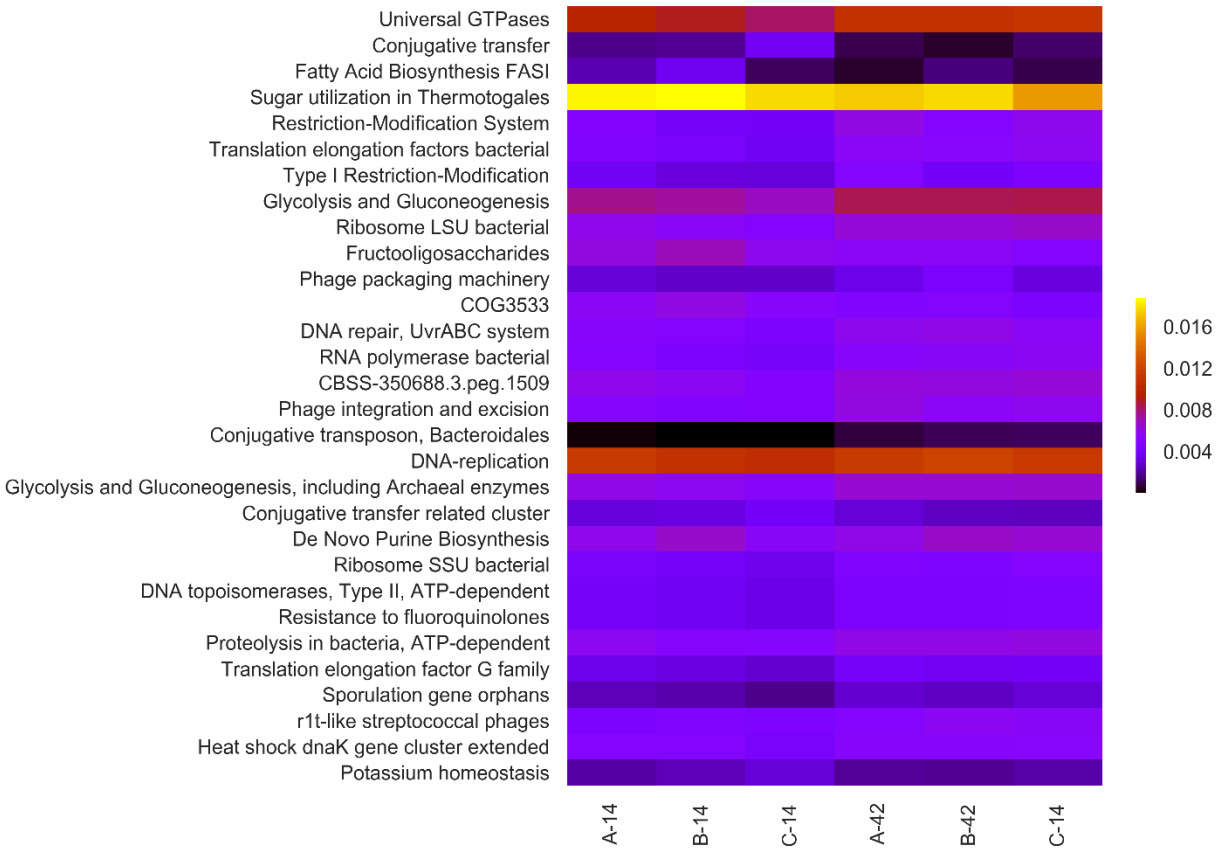
The average abundance of the 30 most variable level 2 functions was similar among all tested groups, with dominance of genes coding for protein biosynthesis, followed by genes included in a miscellaneous SEED category, comprising a diverse set of genes identified during investigation of plant-prokaryote interactions by a project at the Department of Energy (DOE), USA (Figure 26). Differences between level 2 functional categories were identified between groups B-C ad A-C in relation to T6SS genes. In fact, such genes were significantly lower in the -7% protein group in comparison to the control, but significantly higher in the ceca of birds treated with serine protease in comparison to both control group and -7% protein group. Additional differences concerned genes for DNA repair in groups B-C and genes for purines in groups A-B. Finally, genes for sugar alcohols were significantly higher in the ceca of chickens fed with serine protease in comparison to the ceca of chickens fed with -7% protein and no protease at 14 days, whereas at 42 days were significantly higher in ceca of broilers fed with diet B (Table 11).

Figure 26. Heatmap showing the average relative abundance of metagenomic reads annotated to SEED Subsystems (Level 2). The columns represent the diet types at each sampling time, the rows represent the SEED subsystems (Level 2), and the colour bar represent the range of average relative abundance of reads annotated to each category.



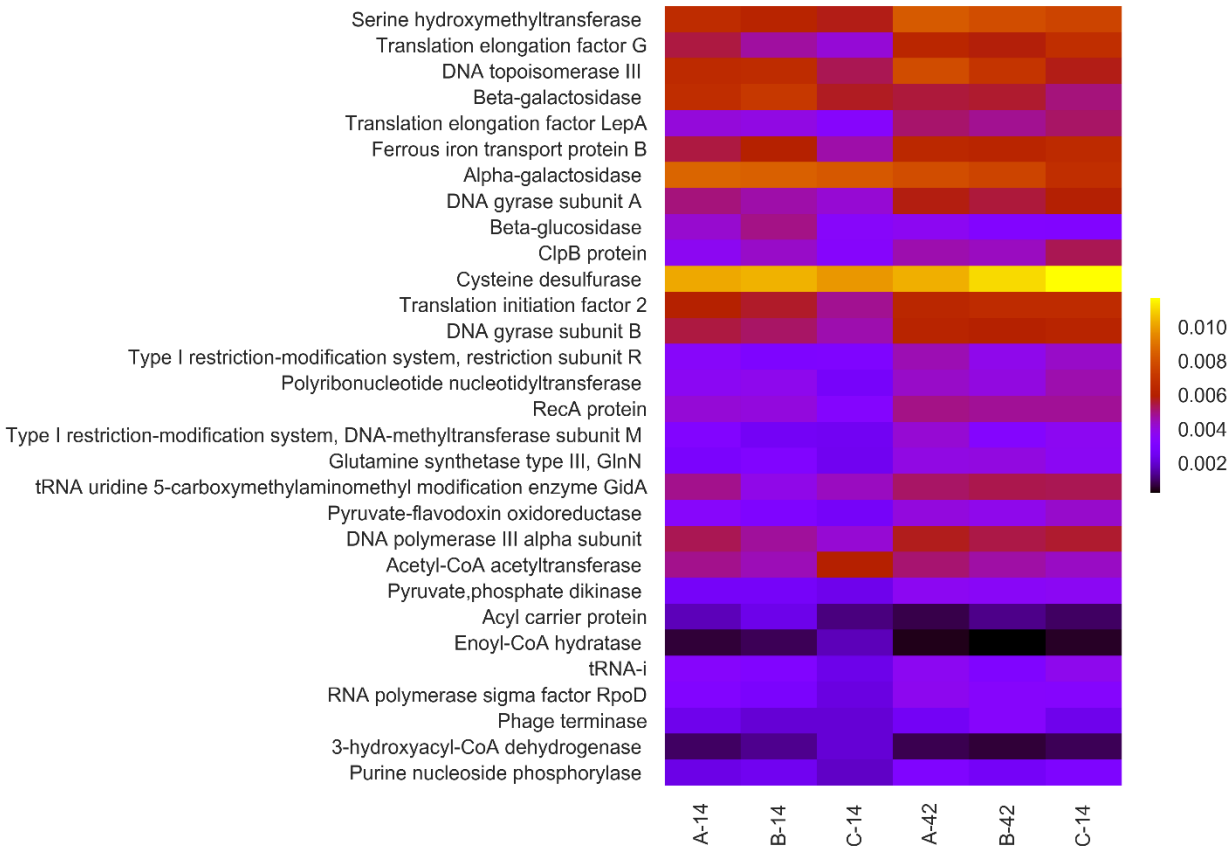
The most abundant genes coding for level 3 functions were those for sugar utilization in Thermotogales, followed by genes for DNA-replication and universal GTPases (Figure 27).

Figure 27. Heatmap showing the average relative abundance of metagenomic reads annotated to SEED Subsystems (Level 3). The columns represent the diet types at each sampling time, the rows represent the SEED subsystems (Level 3), and the colorbar represent the range of average relative abundance of reads annotated to each category.



The most abundant functional genes were cystine desulfurase, followed by alpha-galactosidase and serine hydroxymethyltransferase (Figure 28).

Figure 28. Heatmap showing the average relative abundance of metagenomic reads annotated to SEED Subsystems (Function). The columns represent the diet types at each sampling time, the rows represent the SEED subsystems (Function), and the colorbar represent the range of average relative abundance of reads annotated to each category.



The most abundance genes significantly different between groups B-C were stage V sporulation protein D and DNA-direct RNA polymerase alpha subunit. Moreover, the most abundant genes significantly different in groups A-C and A-B were site-specific recombinase, phage integrase family (Table 11).

3.3 Trial on metagenomic investigation of caeca of chickens fed with phytase and corresponding carcasses

3.3.1 Background

Feed enzymes for poultry have made the most progress and impact in the past decade and, in particular, phytase currently dominates this market, taking a 60 % share among all biotechnological additives (Adeola and Cowieson, 2011). Rapid growth in the last decade has been associated with the acceptance of phytase in replacing inorganic phosphates to mitigate spiralling feed costs and minimise nutrient excretion (Kiarie and Nyachoti, 2009). Phosphorous is the third most expensive nutrient in diets for non-ruminants; however, the majority (65 %) of the phosphorous in feedstuffs of plant origin is bound in mixed salts of phytic acids and is unavailable to the animal without phityse's dephosphorylation (Kiarie and Nyachoti, 2009). The phytic acid, myo-inositol hexaphosphate, is the major phosphate source in feedstuff of plant origin. About 75% of total phosphorus in cereals and legumes is present as phytate, salt of phytic acid, forms not readily available for monogastric (Wodzinsky and Ullah, 1996). Phytic acid consists of a sugar, myo-inositol (similar to glucose), to which are covalently bound phosphate groups (PO4), and it is present as a salt of mono and divalent cations (potassium, calcium and magnesium) that is rapidly accumulated in seeds during the ripening period. It is generally considered as the primary source of inositol and phosphorus reserve in plant seeds used in animal's nutrition (seed flour, cereal grains and legumes) (Maga, 1982). Furthermore, phosphorus, under the phytic acid form, has an anti-nutritional effect on ilea digestibility, reducing minerals and dietary protein availability and increasing endogenous secretions as mucin (Cowieson et al., 2004). In fact, phytic acid appears to have a strong anti-nutritional effect due to its unusual molecular structure in which, in the complete dissociation, the six phosphate groups lead to a total of twelve negative charges. That is why phytic acid can bind various mono, di and tri cations forming insoluble complexes containing especially potassium and magnesium and in small quantities calcium, zinc, iron or copper. The solubility and stability of such complexes decreases when the number of residues decrease. Therefore, the removal of phosphate residues on the myo-inositol ring lead to a greater availability of essential minerals (Sandberg et al., 1999; Han et al., 1994). The phytate binds also proteins and amino acids decreasing their digestibility. In fact, phytic acid, in acidic pH conditions, can firmly bind the vegetal protein until their isoelectric point is generally around pH 4.0-5.0 decreasing their solubility, digestibility and nutritional value. In addition, other than creating complexes with minerals and protein, phytic acid interacts with some enzymes involved in the digestion process such as trypsin, pepsin, α-amylase and β-galactosidase, decreasing their activity (Kerovuo, 2000).

An approach to provide adequate phosphorous to poultry, since the avian intestinal and pancreatic secretions of phytase are insufficient, could be to include feedstuffs with high phosphorous availability, such as inorganic supplements, as dicalcium phosphate, in the diet (Selle and Ravindran, 2007). However, this addition, even if it could provide the adequate phosphorus requirement, raises the cost of the diet and can lead to an excessive excretion of phosphorous in the manure. It also has a considerable impact on non-renewable global reserves for rock phosphate (Kiarie and Nyachoti, 2009). Another approach could be to improve the use of phytic acid in the basal vegetable feedstuffs using exogenous phytase. This approach is widely adopted especially in poultry feeds (Adeola and Cowieson, 2011; Slominski, 2011; Woyengo & Nyachoti, 2011). Other than making the phosphorus available for the host, the supplementation of phytase could avoid the anti-nutritional effect of phytic acid reducing endogenous losses and increasing protein digestibility and, consequently, limiting protein supply in the diet and modulating microbiota.

Ptak et al. (2015) investigated the phytase effect on the microbial ecology of the gastrointestinal tract of chickens. The chickens were fed with 4 different diets, with the factors being adequate, or insufficient calcium and digestible phosphor and with or without 5000 phytase units (FTU)/kg of *Escherichia coli* 6-phytase. The reduction in calcium and digestible phosphorus resulted in a significant reduction of ilea total bacteria count, while phytase supplementation increased ileal total bacterial counts. Additionally, the deficient diet reduced butyrate- but increased lactate-producing bacteria. The addition of phytase increased *Lactobacillus* sp./*Enterococcus* sp. whereas in case of *Clostridium leptum* subgroup, *Clostridium coccoides - Eubacterium rectale* cluster, *Bifidobacterium* sp. and *Streptococcus/Lactococcus* counts a significant insufficient calcium and digestible phoshor level x phytase interaction was found. Furthermore, the reduction of calcium and digestible phosphorus levels lowered *Clostridium perfringens* and *Enterobacteriaceae* counts. The analysis of fermentation products showed that reducing the calcium and digestible phosphorus content in the diet reduced the total amount of short chain fatty acids, DL-lactate, and acetic acid in the ileum, suggests that phosphorus is a factor which limits fermentation in the ileum, while phytase increased concentrations of these acids in the digestible phosphorus deficient diet group.

However, Smulikowska et al. (2010) reported an increase of caecal acetate in broiler chickens fed with phytase. Furthermore, it was shown by Lumpkins et al. (2009) that phytase reduces intestinal 5AC mucin mRNA abundance in broiler chickens and the reduction in mucin levels could correspond to a reduction in *Clostridium perfringens* and the occurrence of necrotic enteritis in chickens, since this bacterium prospers on mucin (Cooper and Songer, 2009). It might be concluded that the addition of phytase can play a role in modulating the gut microbiota of chicken.

For this reason, the present project aims to characterize the gut microbiota modulation of chicken fed with phytase, alone and combined with inositol, with special emphasis on the single population changes.

3.3.2 Methodology

3.3.2.1 Animals and diet groups

In the trials with phyase, a total of 1755 one-day-old male chicks (Ross 308), obtained in September 2012 from the same breeder flock and hatching session, were used. The chicks were housed in a poultry house containing 27 pens of 6 m^2 each. Before housing, birds were individually weighed and divided according to their live weight in 3 classes: 42-44 g, 45-47 g, 48-50 g. The groups were distributed in 27 pens at the stocking density of about 10 chicks/m2 (i.e., 65 birds/pen), while maintaining the same class distribution of live-weight of the population placing in each pen an equal number of chicks belonging to the three classes. The 27 pens were divided into five diet groups of 9 replicates each. Pens labelled as *group A* hosted birds fed with the basal diet summarised in Table 12 (i.e., diet A); those of *group B*, birds fed with a basal diet supplemented with phytase at 500 FTU/kg feed (i.e., diet B); those of *group C*, birds fed with a basal diet supplemented with phytase at 1500 FTU/kg (i.e., diet C); those of *group D*, birds fed with a basal diet supplemented with phytase at 500 FTU/kg feed and 3g/kg inositol (i.e., diet D); those of *group E*, birds fed with a basal diet supplemented with phytase at 1500 FTU/kg feed and 3g/kg inositol (i.e., diet E) (Table 12). Feeds, formulated according to the five different diets, were supplied *ad libitum* in mash form throughout the experiment. The feeding program included four phases: starter (0-10 day), grower first period (11-21 day), grower second period (22-35 day) and finisher (36-42 day). The experiment lasted 35 days, when birds reached the slaughter weight of about 2.8 kg of live weight. Photoperiod and temperature programs were set up according to the European welfare regulation 43/2007 (European Union, 2007).

Table 12 Basal diets composition (%) of the feed administered to the chicks belonging to the trial with serine protease

	0-10 days					11-21 days					22-35 days					36-42 days				
Diet	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Corn	29.02	29.02	29.02	29.02	29.025	36.98	36.98	36.98	36.98	36.98	18.64	18.64	18.64	18.64	18.64	15.01	15.01	15.01	15.01	15.01
Soybean meal	25.17	25.17	25.17	25.17	25.173	22.01	22.01	22.01	22.01	22.01	17.31	17.31	17.31	17.31	17.31	14.32	14.32	14.32	14.32	14.32
Wheat	20	20	20	20	20	15	15	15	15	15	15	15	15	15	15	21.19	21.19	21.19	21.19	21.19
Sunflower	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Sorghum	20	20	20	20	20	15	15	15	15	15	15	15	15	15	15	21.19	21.19	21.19	21.19	21.19
Expanded soybean	10	10	10	10	10	10	10	10	10	10	15	15	15	15	15	15	15	15	15	15
Gluten Corn	3	3	3	3	3	2	2	2	2	2	.	.	.	.	.	.	.	.	.	.
Vegetable oils	3.65	3.65	3.65	3.65	3.65	3.49	3.49	3.49	3.49	3.49	4.12	4.12	4.12	4.12	4.12	4.86	4.86	4.86	4.86	4.86
Dicalcium phosphate	1.10	1.10	1.10	1.10	1.10	0.68	0.68	0.68	0.68	0.68	0.33	0.33	0.33	0.33	0.33	0.21	0.21	0.21	0.21	0.21
Calcium Carbonate	0.94	0.94	0.94	0.94	0.94	0.84	0.84	0.84	0.84	0.84	0.78	0.78	0.78	0.78	0.78	0.81	0.81	0.81	0.81	0.81
White corn	.	.	.	.	.	.	.	.	.	.	15	15	15	15	15	15	15	15	15	15
Dl Methionin	0.29	0.29	0.29	0.29	0.29	0.26	0.26	0.26	0.26	0.26	0.12	0.12	0.12	0.12	0.12	0.29	0.29	0.29	0.29	0.29
Salt	0.22	0.22	0.22	0.22	0.22	0.20	0.20	0.20	0.20	0.20	0.16	0.16	0.16	0.16	0.16	0.23	0.23	0.23	0.23	0.23
Protease	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Vitamin-mineral premix	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.42	0.42	0.42	0.42	0.42	0.27	0.27	0.27	0.27	0.27
Threonin	0.13	0.13	0.13	0.13	0.13	0.12	0.12	0.12	0.12	0.12	0.10	0.10	0.10	0.10	0.10	0.09	0.09	0.09	0.09	0.09
Sodium bicarbonate	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.1	0.1	0.1	0.1	0.1	.	.	.	.	.
Phytase	0.0	500	1500	500	1500	0.0	500	1500	500	1500	0.0	500	1500	500	1500	0.0	500	1500	500	1500
Colin	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Lysine sulphate	0.60	0.60	0.60	0.60	0.60	0.56	0.56	0.56	0.56	0.56	0.43	0.43	0.43	0.43	0.43	0.40	0.40	0.40	0.40	0.40
Dry matter	88.53	88.53	88.53	88.50	88.26	88.39	88.39	88.39	88.12	88.13	88.23	88.23	88.23	87.97	87.97	88.17	88.17	88.17	87.90	87.91
Protein	22.88	22.88	22.87	22.87	22.80	20.99	20.99	20.98	20.92	20.92	19.40	19.40	19.39	19.34	19.34	18.28	18.28	18.27	18.22	18.22
Lipid	7.21	7.21	7.21	7.20	7.18	7.26	7.26	7.26	7.24	7.24	8.74	8.74	8.73	8.71	8.71	9.40	9.40	9.39	9.37	9.36
Fiber	3	3	3	3	3	3	3	3	3	3	3	3	3.06	3.05	3.05	2.98	2.98	2.98	2.97	2.97
ash	5	5	5	5	5	5	5	5	5	5	4	4	4.36	4.33	4.35	4.05	4.06	4.07	4.05	4.06
Ca tot	0.87	0.87	0.87	0.87	0.86	0.71	0.71	0.71	0.70	0.70	0.58	0.58	0.58	0.58	0.58	0.53	0.53	0.53	0.53	0.53
P tot	0.57	0.57	0.57	0.57	0.57	0.48	0.48	0.48	0.48	0.48	0.41	0.41	0.41	0.41	0.41	0.38	0.38	0.38	0.38	0.38
ZN tot	116	116	115.98	115.97	115.64	114.59	114.60	114.57	114.25	114.23	100.89	100.88	100.86	100.58	100.56	74.46	74.45	74.44	74.23	74.21
CU tot	21.82	21.82	21.82	21.81	21.75	21.26	21.27	21.26	21.20	21.20	18.89	18.89	18.88	18.83	18.83	14.09	14.09	14.09	14.05	14.04

3.3.2.2 Sample collection

In the trial with phytase nine chickens were randomly selected and humanely euthanized at day 35 days from each diet group (i.e., A, B, C, D, E). The entire gastrointestinal tract of each individual selected bird was dissected out and a small sample (i.e., 0.5-2 g) of caecum content was collected from both caeca into 2 ml sterile plastic tubes. Furthermore, to characterize the skin microbiota of the chicken after slaughter, plucking, an evisceration, 15 samples of 15 g each of skin and 300 ml of carcass washing water were collected from corresponding carcasses at the slaughterhouse.

The samples were collected from 15 fresh poultry carcasses belonging to three different groups of 5 animals each, within groups A, C and E (5+5 samples for each group). Each carcass was processed in a sterile environment as follows: a 10-g sample of skin was removed by tissue excision from the neck and the breast of each carcass using a sterile scalpel and then placed in a sterile bag with 40 mL of sterile saline solution. Tissue samples were homogenized for 1 minute using the Pulsifier® (Microgen Bioproducts Ltd, Cambrige, UK) and the whole rinse fluid was placed in a 50-ml falcon tube and then centrifuged at 4500 Xg for 8 min to pellet the bacteria. After tissue removal, each chicken carcass was placed in sterile heavy plastic bags with 300 ml sterile physiologic solution to sample the carcass washing water. The bags were sealed and vigorously shaken by hand for 3 min. After shaking, each carcass was removed and the rinse fluid was first poured into a new sterile bag and then divided into 6 falcon tubes (50 ml).

All the tubes (6 for each sample) were centrifuged at 4500 Xg for 8 minutes to pellet the bacteria. The supernatant fluids were removed by suction and all the 6 pellets were transferred together in a new falcon tube with the few remaining supernatant (total volume of each carcass washing water sample was 20 ml). All caecum, skin and carcass washing water samples collected (e.g. 45, 15 and 15 respectively) were stored at -80°C until further testing.

3.3.2.3 DNA extraction from the chicken caecum contents

The DNA was extracted from each sample of caecum content using a bead-beating procedure (Danzeisen et al., 2011). Briefly, 0.25 g of cecal content were suspended in a 1 ml lysis buffer (500 mM NaCl, 50 mM Tris-Cl, pH 8.0, 50 mM EDTA, 4 % SDS) with MagNA Lyser Green Beads (Roche, Milan, Italy) and homogenized on the MagNA Lyser (Roche) for 25 secs at 6500 rpm. The samples were then heated at 70°C for 15 min., followed by centrifugation to separate the DNA from the bacterial cellular debris. This process was repeated with a second 300 µl aliquot of lysis buffer. The samples were then subjected to 10 M v/v ammonium acetate (Sigma, Milan, Italy) precipitation, followed by isopropanol (Sigma) precipitation and a 70% ethanol (Carlo Erba, Milan, Italy) wash and re-suspended in 100 ul 1X Tris-EDTA (Sigma). The samples were treated with DNase-free RNase

(Roche) and incubated overnight at 4°C, before being processed through the QIAmp® DNA Stool Mini Kit (Qiagen, Milan, Italy) according to manufacturer's directions with some modifications. Samples were measured on a BioSpectrometer® (Eppendorf, Milan, Italy) to assess DNA quantity and quality.

3.3.2.4 DNA extraction from chicken carcass skin and washing water

The DNA was extracted from each sample using a two-step bead beating protocol developed in our laboratory. 500 µL aliquots of each sample were placed in 2 mL microtubes with a 600 µL lysis buffer (500 mM NaCl, 50 mM Tris-Cl, pH 8.0, 50mM EDTA, 4 % SDS) and 20 µl of lysozime (20mg/ml) and heated at 37°C for 30 min. The samples were homogenized on MagNA Lyser (Roche) for 20 secs at 900 xg, after the addition of 2 MagNA Lyser Green Beads (Roche, Milan, Italy). After the beads' removal, the samples were incubated with 180 µl Buffer AL and 20 µl proteinase K (Kit dnEASY Blood and Tissue) at 56°C overnight. Each sample was then processed using the DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy) according to manufacturer's directions with some modifications. DNA was measured on a BioSpectrometer® (Eppendorf, Milan, Italy) to assess its quantity and quality.

3.3.2.5 Library preparation and metagenomic sequencing

The DNA extracted from each sample were quantified on a BioSpectrometer® (Eppendorf, Milan, Italy) to assess DNA yield, in terms of quantity and quality. Moreover, DNA purity was assessed in terms of absence of contaminants according to the value of the A260 / A280 nm ratio.

The DNA extracted and assessed for quality and quantity was submitted to the library preparation procedure with the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA). Nextera technology provides an input DNA fragmentation and transposase mediated ligation of oligo-adapters, essentials to anchor the amplified DNA fragment (around 500 bp) to the sequencer flow cell and to amplify the insert DNA by PCR. Illumina's preparation procedure was chosen because it improves traditional protocols by combining DNA fragmentation, end-repair, and adaptor-ligation into a single step using an engineered enzyme (Head et al., 2014). The PCR reaction also adds index (barcode) sequences. However, the use of an engineered enzyme makes this protocol very sensitive to the amount of DNA input compared with other fragmentation methods (Head et al., 2014). Since the ratio of transposase complexes to sample DNA is critical and the subsequent fragment size is also dependent on the reaction efficiency, DNA concentration was evaluated other than by BioSpectrometer (Eppendorf) even using fluorimetric analysis by Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen).

In particular, according to the first quantification obtained through BioSpectrometer (Eppendorf), the DNA was brought to the concentration required (0.2 ng/µl) through other intermediate dilutions (i.e., 25, 10 and 2 ng/µl) all quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) on the Infinite 200 PRO (Tecan) instrument. All libraries were validated according to Illumina's protocol. To determin the nanomolarity of each library, the concentration and average length of the DNA fragments were checked through fluorimetric analysis by Quant-iT™ PicoGreen® dsDNA Assay Kit and through Chip DNA Hi Sensitivity analysis on Bioanalyzer 2100 (Agilent Technologies). Each library pool of 24 samples was adjusted to a micro molarity between 1.3 to 2 (depending on the pool's library with the lower molarity) to be sequenced. A total of 5 µl of each library (1.3-2 nM) were pooled together. Each pool of 24 libraries was loaded into a flow cell of a glass slide. Each fragment of DNA library was anchored on complementary oligo-adapters placed on the flow cell and clonally amplified through a solid-phase amplification called bridge amplification and then sequenced by synthesis. Whole genome sequencing was performed using the HiScanSQ sequencer (Illumina) at 100 bp in paired-end mode. Metagenomic sequencing yielded an average of 6.841 million mapped reads/sample, with a Phread quality score always higher than 30.

3.3.2.6 Sequences analysis

The metagenomic sequences belonging to the phytase trial were analysed using a pipeline developed at the Technical University of Denmark (DTU). The quality trimming process was performed using the wrapper tool Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Trim Galore! was set to trim low-quality base calls off from the 3' end of the reads with a Phred score threshold of 20, to filter all the reads shorter than 50 bp, to discharge the trimmed paired-end reads (R1 and R2) if at least one of them became shorter than the set threshold (50 bp), to retain the unpairs in a new file and to automatically generate a new FastQC report of the trimmed sequences. The trimming process, with the previously mentioned options, allowed the removal of the biases and the discharge of short reads optimizing the quality of the data and reducing the potential crashes of programs which require sequences with a certain minimum length as the one used in this pipeline, MGmapper (70bp).

After the new generated FastQC report check, the reads were mapped to reference sequence databases using MGmapper version 2.2. The pre-processing step was skipped because all the reads were already checked and trimmed. The features for the mapping process were set as follows: the threshold for fraction of matches+mismatches (FMM) in relation to the full length of a read was set to 0.8 and the minimal alignment score (MAS) was set to 30 (default values). The sequences were mapped against the Nt database in (1) full mode (i.e., accepting as true hit all the primary hit to the following

databases: Bacteria, MetaHitAssembly, HumanMicrobiome, Bacteria_draft, Plant, Common_animals) and (2) best mode (i.e., accepting as true hit when the alignment score of a sequence to a database was higher than all the other alignment scores of the same sequence to other databases).

3.3.2.7 Statistical analysis

The results regarding the relative abundances of bacterial taxa and functional groups were compared through White's non-parametric t-test, using Statistical Analysis of Metagenomic profile Software v 2.0.9 (STAMP) (Parks et al., 2014). After removing non-bacterial species, taxa abundances obtained from MG-RAST were normalized so that each sample total abundance resulted 1.

3.3.3 Results

3.3.3.1 Sequences obtained

All samples with a A260 / A280 nm ratio value out of the range between 1.7 and 1.9 were excluded from the analysis and the DNA was extracted again from the original sample. The quantity and quality parameters of the DNA samples sequenced in this project along with corresponding library parameters abd reads achieved for each individual sample are described in Table 13.

To characterize the chicken skin microbiota DNA was extracted from the homogenized skin samples.

Table 13. Parameters of the samples and libraries sequenced in the project.

Sample			Library			
ID	Conc (ng/ml)	Ratio 260/280 nm	Conc (ng/µl)	Fragment lenght	mM	Reads (n)
Phytase-caceca						
XT_307	1210.10	1.83	3.42	662	7.94	5325585
XT_308	177.70	1.88	4.86	900	8.30	9765424
XT_309	1232.20	1.83	4.74	941	7.75	6226416
XT_310	762.60	1.84	4.19	774	8.33	4036908
XT_311	487.10	1.84	4.38	1263	5.33	8738856
XT_312	1068.10	1.86	4.46	927	7.40	6292292
XT_313	1111	1.84	2.23	772	4.44	5607068
XT_314	820.80	1.85	2.96	717	6.35	6157612
XT_315	1177.90	1.86	6.39	912	10.78	7419414
XT_316	276.30	1.83	4.63	1256	5.67	6949576
XT_317	1090.30	1.82	3.54	1250	4.36	6470484
XT_318	1293.60	1.84	3.53	642	8.47	4346994
XT_319	1024.80	1.84	3.28	986	5.12	5608516
XT_320	898.40	1.84	4.23	1053	6.19	6806712
XT_321	400.00	1.83	3.51	1113	4.85	6274678
XT_322	543.00	1.81	4.87	1267	5.91	7830358
XT_323	1439.60	1.84	4.57	1120	6.28	6903988
XT_324	1239.90	1.84	3.01	1110	4.17	8899214
XT_325	1187.30	1.82	3.66	1072	5.26	7857888
XT_326	1033.20	1.84	3.85	1064	5.57	7963304
XT_327	1110.80	1.85	2.14	979	3.37	8322336
XT_328	1218.50	1.84	3.76	1391	4.16	8396650
XT_329	501.80	1.83	3.27	758	6.63	8057404
XT_330	752.20	1.84	4.89	1166	6.31	8493560
XT_331	1119.90	1.84	3.85	950	6.23	6486162
XT_332	420.30	1.82	2.78	539	7.93	6661378
XT_333	1360.00	1.82	3.64	1177	4.76	8473134
XT_334	1311.10	1.85	5.16	993	8.00	7308470
XT_335	1408.80	1.84	2.61	1122	3.58	7636498
XT_336	504.80	1.82	4.23	1046	6.22	5310828
XT_337	1386.20	1.84	3.19	1128	4.36	7693028
XT_338	813.30	1.85	2.44	1000	3.76	4308024
XT_339	1248.50	1.84	4.50	1079	6.42	7708538
XT_340	1344.30	1.84	3.51	974	5.55	7503362
XT_341	1232.10	1.84	4.87	1128	6.64	8347030
XT_342	891.80	1.82	4.03	991	6.25	5921694

XT_343	719.80	1.84	5.77	1162	7.63	9915124
XT_344	508.70	1.83	4.77	1018	7.21	5146582
XT_345	756.20	1.84	4.47	1130	6.08	5989196
XT_346	908.90	1.84	4.71	1075	6.75	7393520
XT_347	954.10	1.84	5.03	1219	6.35	7234728
XT_348	1426.20	1.84	3.77	938	6.19	8242848
XT_349	793.50	1.85	4.90	1032	7.30	7754078
XT_350	818.50	1.84	5.69	1430	6.12	10894772
XT_351	1335.40	1.84	5.27	939	8.64	7310282
Phytase-carcasses						
XT_357	55.80	1.89	7.06	1150	9.45	9445326
XT_358	70.40	1.97	3.66	1256	4.49	7636834
XT_359	20.40	1.75	4.73	1235	5.89	7728898
XT_360	35.90	1.95	4.23	1066	6.10	6850052
XT_361	19.90	1.99	2.20	1238	2.73	6664934
XT_367	11	1.73	3.95	1465	4.15	5226314
XT_368	80.00	1.99	5.60	1206	7.14	6363476
XT_369	37.20	1.95	4.88	1313	5.72	6544524
XT_370	32.80	1.83	3.43	1285	4.10	5160700
XT_371	14.60	1.91	7.39	1315	8.65	5485326
XT_377	36.90	1.89	3.23	1551	3.20	7974236
XT_378	20.10	1.97	2.29	1501	2.35	5492496
XT_379	17.80	1.90	1.97	1322	2.30	7335068
XT_380	24.00	1.90	2.83	1444	3.02	7171638
XT_381	25.30	1.90	2.53	1549	2.51	7168624

3.3.3.2 Caeca microbiota composition

In the trial with phytase the microbiota composition of the chicks fed with the basal diet (group A) was compared with those of chicks fed with a basal diet and commercial phtase without (group B) and with inositol (group D) and triple concentration of commercial phythase without (group C) and with (group E) inositol. The microbiota composition in the caeca collected from nine birds for each group is summarized in Table 14 and shows that Firmicutes and Bacteroidetes represented more than 90% of microbiota in all tested groups. The most represented phylum in Group A, B, C and D was Firmicutes showing a percentage of abundance of 56.68, 50.14, 51.41 and 54.40, respectively, followed by Bacteroidetes with abundances of 37.15, 41.72, 39.74 and 43.20, respectively. In Group E the most represented phylum was Bacteroidetes (i.e., 47.95% of abundance) followed by Firmicutes (i.e., 44.77% of abundance). The relative frequency of abundance of Firmicutes in Group A was significantly higher than that observed in Group E (P=0.024), whereas that of Bacteroidetes was significantly lower (P=0.031).

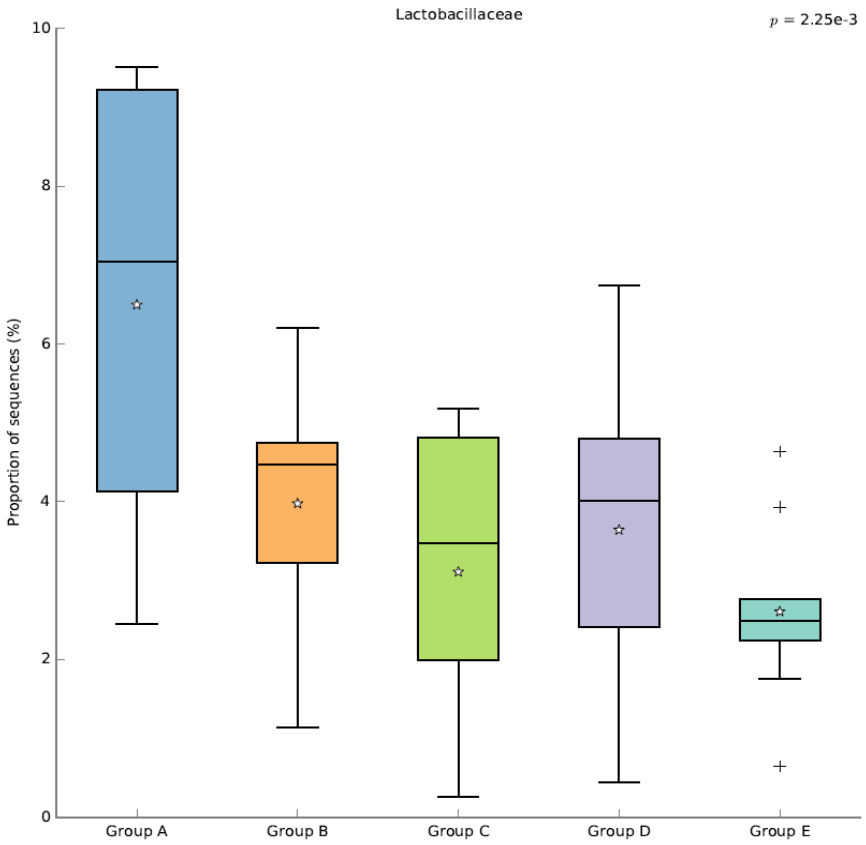
Within the phylum of Firmicutes, Clostridia was the most abundant class in all groups (41.98, 38.07, 39.80, 43.02, 36.05%), followed by Bacilli (8.16, 5.23, 5.20, 4.67, 3.64%), Erysipelotrichi (2.08, 2.06, 1.80, 2.36, 1.69% of abundance) and Negativicutes (0.80, 0.40, 0.47, 0.55, 0.21%). The most represented families in the Clostridia class were Lachnospiraceae (11.22, 10.21, 10.19, 11.83, 9.29%), Ruminococcaceae (9.89, 8.48, 9.18, 9.46, 8.44%) and Clostridiaceae (9.14, 7.83, 8.69, 9.13, 7.07%) followed by Eubacteriaceae (2.44, 2.38, 2.27, 2.77, 2.26%), Oscillospiraceae (1.92, 1.82, 2.28, 2.01, 1.84%), Peptostreptococcaceae (0.60, 0.61, 0.58, 0.68, 0.53%) and Acidaminococcaceae (0.73, 0.34, 0.41, 0.47, 0.15%). The distribution of the families belonging to the Clostridia class was similar among all five Groups, except for Acidaminococcaceae that showed a lower main relative frequency of abundance in Group E (0.036) compared to Group A. The mean relative abundance of Bacilli was significantly higher in Group A (P<0.05) in comparison to all the treated groups. Within the Bacilli class, the most represented family was Lactobacillaceae (6.50, 3.98, 3.64, 3.11, 2.61%), followed by Enterococcaceae (0.59 0.44, 0.45, 0.47, 0.40%), Bacillaceae (0.56, 0.27, 0.65, 0.45, 0.18%), Streptococcaceae (0.37, 0.42, 0.36, 0.51, 0.32%) and Staphylococcaceae (0.08, 0.09, 0.06, 0.09, 0.08%) (Table 14). The main relative frequency of abundance of the families, belonging to the Bacilli class, was similar among all groups, except for Lactobacillaceae that showed a relative frequency of abundance significantly higher (P=0.022) in group A compared to the treated groups (Figure 29).

Table 14. Mean relative frequency of abundance (%) of Phyla, Classes and Families of caecum bacteria in Groups A, B, C, D and E.

Phylum	Class	Family	Group A	Gruop B	Group C	Group D	Group E
Firmicutes			56.68	50.14	51.41	54.40	44.77
	Clostridia		41.98	38.07	39.80	43.02	36.05
		Lachnospiraceae	11.22	10.21	10.19	11.83	9.29
		Ruminococcaceae	9.89	8.48	9.18	9.46	8.44
		Clostridiaceae	9.14	7.83	8.69	9.13	7.07
		Eubacteriaceae	2.44	2.38	2.27	2.77	2.26
		Oscillospiraceae	1.92	1.82	2.28	2.01	1.84
		Peptostreptococcaceae	0.60	0.61	0.58	0.68	0.53
		Acidaminococcaceae	0.73	0.34	0.41	0.47	0.15
	Bacilli		8.16	5.23	5.20	4.67	3,64
		Lactobacillaceae	6.50	3.98	3.64	3.11	2.61
		Enterococcaceae	0.59	0.44	0.45	0.47	0.40
		Bacillaceae	0.56	0.27	0.65	0.45	0.18
		Staphylococcaceae	0.08	0.09	0.06	0.09	0.08
		Streptococcaceae	0.37	0.42	0.36	0.51	0.32
	Erysipelotrichia		2.08	2.06	1.80	2.36	1.69
		Erysipelotrichaceae	2.08	2.06	1.80	2.36	1.69
	Negativicutes		0.80	0.40	0.47	0.55	0.21
Bacteroidetes			37.15	41.72	43.20	39.74	47.95
	Bacteroidia		37.08	41.63	43.13	39.68	47.89
		Rikenellaceae	19.09	13.68	14.11	15.80	16.38
		Bacteroidaceae	11.54	21.60	17.35	22.53	24.22
		Porphyromonadaceae	6.34	6.21	6.33	6.38	7.11
Proteobacteria			4.75	6.34	4.31	4.60	5.40
	Gammaproteobacteria		1,54	3.53	1.51	0.66	0.76
		Enterobacteriaceae	1.46	3.48	1.45	0.60	0.70
	Epsilonproteobacteria		1,22	1.33	0.85	0.39	2.16
	Deltaproteobacteria		0,83	0.64	0.82	2.61	0.88

		Desulfovibrionaceae	0.56	0.39	0.41	0.40	0.54
	Betaproteobacteria		0,22	0.23	0.18	0.32	0.24
	Alphaproteobacteria		0,13	0.06	0.10	0.08	0.27
		Bradyrhizobiaceae	0.03	0.02	0.02	0.03	0.03
Actinobacteria			0.78	0.67	0.63	0.79	0.56
	Coriobacteriia		0,48	0.43	0.39	0.41	0.33
	Actinobacteria		0,31	0.23	0.24	0.38	0.23
		Bifidobacteriaceae	0.13	0.09	0.09	0.19	0.08
Fusobacteria			0.19	0.07	0.12	0.08	0.13
	Fusobacteriia		0.19	0.07	0.12	0.08	0.13
		Fusobacteriaceae	0.19	0.07	0.12	0.08	0.13
Spirochaetes			0.16	0.12	0.12	0.13	0.13
	Spirochaetia		0.16	0.12	0.12	0.13	0.13
Tenericutes			0.16	0.13	0.10	0.13	0.13
	Mollicutes		0.16	0.13	0.10	0.13	0.13

Figure 29. Box Plot showing the abundance (%) of Lactobacillaceae among tested Groups



Within the Phylum Firmicutes, Erysipelotrichia class shows a comparable abundance in the control group (i.e., 2.08 %) and in the groups treated with normal concentration of phytase with and without inositol (i.e., 2.06 and 2.36% in groups B and D, respectively) but it tends to decrease in the groups treated with triple concentration of phytase with and without inositol (i.e., 1.80 and 1.69% in groups C and E, respectively). In particular, the relative frequency of abundance of Erysipelotrichi in Group D was significantly higher (P=0.0275) compared to Group E. The same trend was observed for Erysipelotrichaceae, the only represented family of this class. Finally, the relative abundance of Negativicutes was significantly higher in Group A compared to Group E (P=0.0352) (Table 14).

In all the five Groups, Bacteroidia was the most representative class of the Bacteroidetes phylum and it was significantly lower in Group A in comparison to Group E (P=0.031). Moreover, in Group A Rikenellaceae was the most represented family (19.09%) in comparison to the other groups, where the relative abundances of the same family were 13.68, 14.11, 15.80, 16.38% respectively (Table 14). On the contrary, in groups B, C, D and E Bacteroidaceae was the most represented family (21.60, 17.35, 22.53, 24.22% respectively), belonging to the Bacteroidia class, compared to Group A where

the relative abundance of this family was (11.54%). Gammaproteobacteria was the most represented class belonging to the Proteobacteria phylum in group A, B, C, E (1.54, 3.53, 1.51, 0.76%), while in Group D within the Proteobacteria class, Deltaproteobacteria showed a higher relative frequency of abundance than Gammaproteobateria (2.61 and 0.66% respectively) compared to the other Groups (0.83, 0.64, 0.82, 0.88%). The other most represented class in the Proteobacteria phylum was Epsilonproteobacteria(1.22, 1.33, 0.85, 0.39, 2.16%) followed by Betaproteobacteria (0.22, 0.23, 0.18, 0.32, 0.24%) and Alphaproteobacteria (0.13, 0.057, 0.10, 0.082, 0.27%).

It is remarkable that, even if not statistically significant, the abundances of Gammaproteobacteria and, in particular, of the Enterobacteriaceae family, tend to decrease in groups D and E treated with normal and triple concentration of phytase supplemented with inositol, in comparison with the control group. On the contrary, the abundance of Alfaproteobacteria was statistically lower ($P < 0.005$) in groups B and C (i.e., 0.057, 0.082%) in comparison to group A (0.13%). The same trend was observed in the Fusobacteria phylum, Fusobacteriia class and Fusobacteriaceae family that showed a significant higher abundance in the control group in comparison to all other groups ($P < 0.005$).

Actinobacteria abundance was comparable between groups A, B and C while in groups D and E it was significantly lower ($P=0.035$) compared to the control. Within the Actinobateria phylum, the most represented classes were Coriobacteriia followed by Actinobacteria and within the Actinobacteria class, the Bifidobacteriaceae was the most represented family.

Tables 15 summarises the top 30 bacterial species identified in the control group in comparison to groups treated with commercial phytase at the standard and triple concentration. Moreover, Table 16 summarises the top 30 bacterial species identified in the control group in comparison to groups treated with phytase and inositol. In all tested groups the top five species are represented by genus *Alistipes*, *Faecalibacterium* and *Bacteroides.*

Table 15. Mean relative frequency of abundance (%) of the 30 most representative species (MRS) of caecum bacteria in Groups A, B and C.

MRS	Group A species	Mean	Group B species	Mean	Group C species	Mean
1	*Alistipes sp. AG:268*	5.31	*Bacteroides dorei*	7.4	*Bacteroides dorei*	5.9
2	*Alistipes shahii*	4.34	*Bacteroides fragilis*	7.27	*Bacteroides sp. CAG:20*	4.6
3	*Bacteroides sp. CAG:20*	4.01	*Alistipes sp. CAG:268*	3.79	*Alistipes sp. CAG:268*	4.42
4	*Bacteroides fragilis*	3.92	*Bacteroides sp. CAG:20*	3.6	*Bacteroides fragilis*	3.83
5	*Faecalibacterium prausnitzii*	3.77	*Faecalibacterium prausnitzii*	3.53	*Faecalibacterium prausnitzii*	3.7
6	*Alistipes senegalensis*	3.52	*Escherichia coli*	3.29	*Alistipes shahii*	3.57
7	*Pseudoflavonifractor capillosus*	3.32	*Pseudoflavonifractor capillosus*	3.14	*Pseudoflavonifractor capillosus*	3.35
8	*Lactobacillus crispatus*	2.9	*Alistipes shahii*	3.08	*Alistipes senegalensis*	2.91
9	*[Clostridium] saccharolyticum*	2.84	*[Clostridium] saccharolyticum*	2.61	*[Clostridium] saccharolyticum*	2.91
10	*Barnesiella intestinihominis*	2.23	*Alistipes senegalensis*	2.49	*Barnesiella intestinihominis*	2.6
11	*Subdoligranulum variabile*	1.92	*Barnesiella intestinihominis*	2.07	*Eubacterium sp. ER2*	1.86
12	*Eubacterium sp. ER2*	1.71	*Lactobacillus crispatus*	1.85	*Butyricicoccus pullicaecorum*	1.79
13	*Alistipes finegoldii*	1.58	*Firmicutes bacterium CAG:94*	1.67	*Lactobacillus crispatus*	1.44
14	*Tannerella sp. 6_1_58FAA_CT1*	1.48	*Eubacterium sp. ER2*	1.62	*Subdoligranulum variabile*	1.42
15	*Escherichia coli*	1.39	*Subdoligranulum variabile*	1.33	*Lachnospiraceae bacterium 7_1_58FAA*	1.35
16	*Firmicutes bacterium CAG:94*	1.37	*Odoribacter splanchnicus*	1.3	*Firmicutes bacterium CAG:94*	1.34
17	*Butyricicoccus pullicaecorum*	1.33	*Helicobacter pullorum*	1.3	*Tannerella sp. 6_1_58FAA_CT1*	1.3
18	*Lachnospiraceae bacterium 7_1_58FAA*	1.22	*Butyricicoccus pullicaecorum*	1.28	*Alistipes finegoldii*	1.3
19	*Bacteroides dorei*	1.2	*Tannerella sp. 6_1_58FAA_CT1*	1.18	*Clostridium sp. ATCC 29733*	1.24
20	*Helicobacter pullorum*	1.19	*Lachn.bacterium 7_1_58FAA*	1.11	*Oscillibacter sp. KLE 1745*	1.21
21	*Clostridium sp. CAG:678*	1.19	*Oscillibacter sp. KLE 1745*	1.11	*Odoribacter splanchnicus*	1.03
22	*Odoribacter splanchnicus*	1.17	*Clostridium sp. ATCC 29733*	1.1	*[Clostridium] methylpentosum*	0.99
23	*Alistipes sp. CAG:29*	1.16	*Alistipes finegoldii*	1.1	*Alistipes sp. CAG:29*	0.95
24	*Clostridium sp. ATCC 29733*	1.16	*Odorib. splanchnicus CAG:14*	0.94	*Flavonifractor plautii*	0.89
25	*Oscillibacter sp. KLE 1745*	1.12	*[Clostridium] methylpentosum*	0.86	*Alistipes obesi*	0.81
26	*Alistipes obesi*	0.97	*Alistipes sp. CAG:29*	0.8	*Odoribacter splanchnicus CAG:14*	0.73
27	*[Clostridium] methylpentosum*	0.95	*Flavonifractor plautii*	0.73	*Lactobacillus salivarius*	0.65
28	*Lactobacillus salivarius*	0.9	*Alistipes obesi*	0.69	*Escherichia coli*	0.56
29	*Odoribacter splanchnicus CAG:14*	0.84	*Lactobacillus salivarius*	0.62	*Clostridium sp. CAG:678*	0.55
30	*Flavonifractor plautii*	0.81	*Clostridium sp. CAG:678*	0.26	*Helicobacter pullorum*	0.38

Table 16. Mean relative frequency of abundance (%) of the 30 most representative species (MRS) of caecum bacteria in Group A, Group D and E.

MRS	Group A species	Mean	Group D species	Mean	Group E species	Mean
1	*Alistipes sp. CAG:268*	5.31	*Bacteroides dorei*	9.41	*Bacteroides dorei*	12.55
2	*Alistipes shahii*	4.34	*Bacteroides fragilis*	4.82	*Alistipes sp. CAG:268*	4.6
3	*Bacteroides sp. CAG:20*	4.01	*Bacteroides sp. CAG:20*	4.7	*Bacteroides sp. CAG:20*	3.84
4	*Bacteroides fragilis*	3.92	*Alistipes sp. CAG:268*	3.96	*Alistipes shahii*	3.79
5	*Faecalibacterium prausnitzii*	3.77	*Faecalibacterium prausnitzii*	3.84	*Bacteroides fragilis*	3.62
6	*Alistipes senegalensis*	3.52	*Alistipes shahii*	3.25	*Pseudoflavonifractor capillosus*	3.2
7	*Pseudoflavonifractor capillosus*	3.32	*Pseudoflavonifractor capillosus*	3.17	*Alistipes senegalensis*	3.07
8	*Lactobacillus crispatus*	2.9	*Barnesiella intestinihominis*	2.69	*Faecalibacterium prausnitzii*	2.88
9	*[Clostridium] saccharolyticum*	2.84	*Alistipes senegalensis*	2.62	*[Clostridium] saccharolyticum*	2.26
10	*Barnesiella intestinihominis*	2.23	*[Clostridium] saccharolyticum*	2.6	*Barnesiella intestinihominis*	2.18
11	*Subdoligranulum variabile*	1.92	*Butyricicoccus pullicaecorum*	2	*Helicobacter pullorum*	2.11
12	*Eubacterium sp. ER2*	1.71	*Lactobacillus crispatus*	1.89	*Tannerella sp. 6_1_58FAA_CT1*	1.48
13	*Alistipes finegoldii*	1.58	*Firmicutes bacterium CAG:94*	1.56	*Butyricicoccus pullicaecorum*	1.46
14	*Tannerella sp. 6_1_58FAA_CT1*	1.48	*Eubacterium sp. ER2*	1.54	*Odoribacter splanchnicus*	1.43
15	*Escherichia coli*	1.39	*Oscillibacter sp. KLE 1745*	1.48	*Lactobacillus crispatus*	1.4
16	*Firmicutes bacterium CAG:94*	1.37	*Escherichia coli*	1.39	*Subdoligranulum variabile*	1.37
17	*Butyricicoccus pullicaecorum*	1.33	*Subdoligranulum variabile*	1.35	*Eubacterium sp. ER2*	1.36
18	*Lachnospiraceae bacterium 7_1_58FAA*	1.22	*Tannerella sp. 6_1_58FAA_CT1*	1.25	*Alistipes finegoldii*	1.36
19	*Bacteroides dorei*	1.2	*Lachn.bacterium 7_1_58FAA*	1.25	*Lachn.bacterium 7_1_58FAA*	1.15
20	*Helicobacter pullorum*	1.19	*Alistipes finegoldii*	1.16	*Firmicutes bacterium CAG:94*	1.11
21	*Clostridium sp. CAG:678*	1.19	*Clostridium sp. ATCC 29733*	1.12	*Oscillibacter sp. KLE 1745*	1.09
22	*Odoribacter splanchnicus*	1.17	*Odoribacter splanchnicus*	0.93	*[Clostridium] methylpentosum*	1.04
23	*Alistipes sp. CAG:29*	1.16	*[Clostridium] methylpentosum*	0.92	*Odoribacter splanchnicus CAG:14*	1.01
24	*Clostridium sp. ATCC 29733*	1.16	*Alistipes sp. CAG:29*	0.84	*Alistipes sp. CAG:29*	1
25	*Oscillibacter sp. KLE 1745*	1.12	*Helicobacter pullorum*	0.83	*Clostridium sp. ATCC 29733*	0.91
26	*Alistipes obesi*	0.97	*Flavonifractor plautii*	0.82	*Alistipes obesi*	0.85
27	*[Clostridium] methylpentosum*	0.95	*Alistipes obesi*	0.73	*Flavonifractor plautii*	0.78
28	*Lactobacillus salivarius*	0.9	*Odori.splanchnicus CAG:14*	0.65	*Escherichia coli*	0.65
29	*Odoribacter splanchnicus CAG:14*	0.84	*Clostridium sp. CAG:678*	0.56	*Lactobacillus salivarius*	0.43
30	*Flavonifractor plautii*	0.81	*Lactobacillus salivarius*	0.53	*Clostridium sp. CAG:678*	0.23

In relation to the bacterial species with abundances higher than 0.020 in at least one group and significantly different among groups, the relative frequencies of abundance of *Lactobacillus johnsonii* and *Lactobacillus amylovorus* were significantly higher in group A in comparison to groups B and C (Table 17) as well as D and E (Table 18). *Clostridium* species abundances were significantly higher in group A compared to groups B and C (Table 17) as well as group E (Table 18).

Table 17. Statistically significant differences between means of relative frequency of abundance (%) of caecum bacterial species in Group A, Group B and Group C.

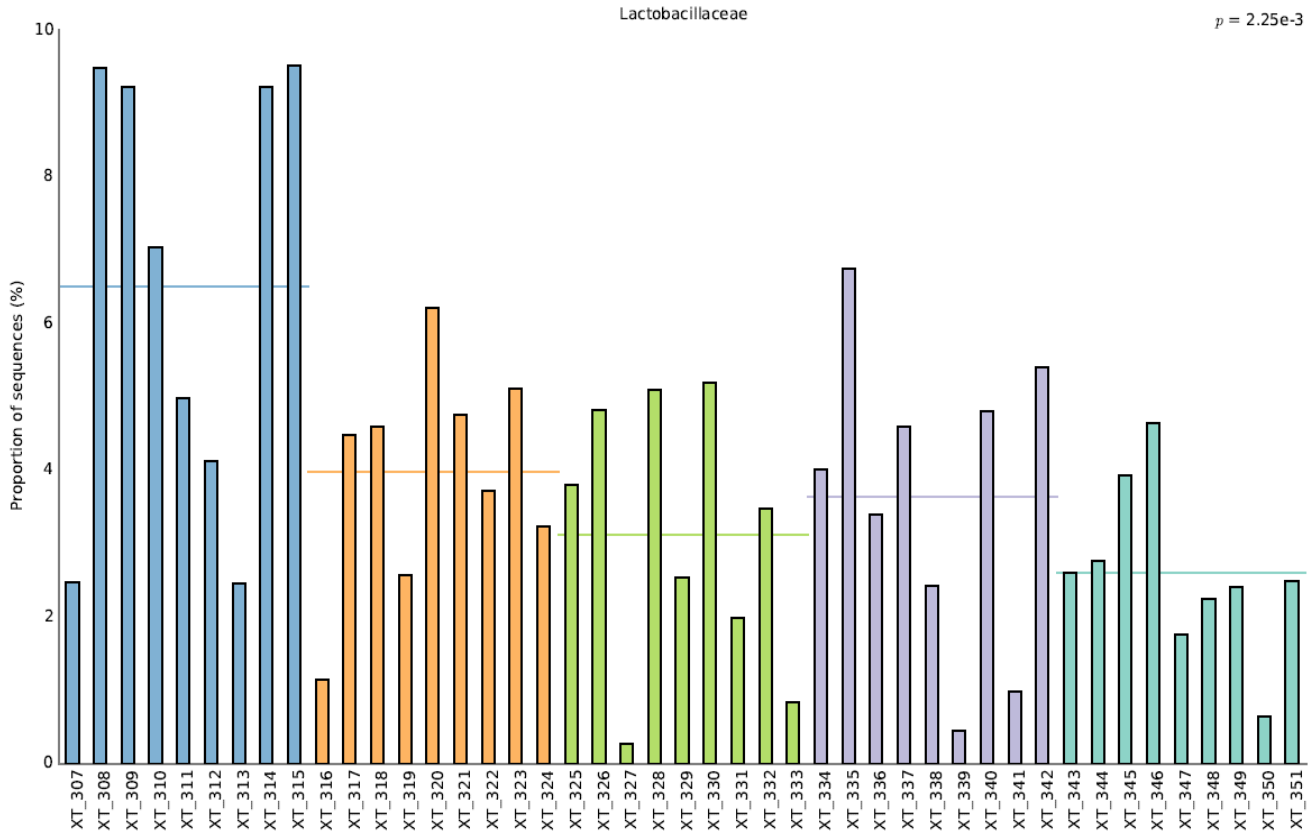
	Group A	Group B	Group C	
Species		Mean		P-values
Lactobacillus johnsonii	0.58	0.21	0.19	0.02385
Clostridium sp. CAG:354	0.46	0.15	0.18	0.04859
Lactobacillus amylovorus	0.29	0.16	0.12	0.04451
Fusobacterium sp. CAG:439	0.17	0.06	0.06	0.00107
Azospirillum sp. CAG:260	0.05	0.01	0.01	0.00024
Brachyspira sp. CAG:484	0.05	0.02	0.02	0.00404
Bacteroides sp. 2_1_56FAA	0.04	0.08	0.04	0.00899
Lactobacillus gigeriorum	0.03	0.02	0.01	0.02854
Clostridium sp. CAG:768	0.03	0.01	0.02	0.03194
Clostridium sp. KNHs214	0.03	0.01	0.01	0.04000
Streptococcus mitis	0.03	0.02	0.02	0.04177
Lactobacillus acidophilus	0.02	0.01	0.01	0.04244

Table 18. Statistically significant differences between means of relative frequency of abundance (%) of caecum bacterial species in Group A, Group D and Group E.

	Group A	Group D	Group E	
Species		Mean		P-values
Faecalitalea cylindroides	0.68	0.53	0.49	0.04053
butyrate-producing bacterium SM4/1	0.59	0.53	0.43	0.03415
Lactobacillus johnsonii	0.58	0.18	0.22	0.02009
Clostridium sp. M62/1	0.46	0.54	0.36	0.02645
Lactobacillus amylovorus	0.29	0.13	0.10	0.00837
Eubacterium desmolans	0.28	0.30	0.51	0.00923
Blautia obeum	0.28	0.25	0.20	0.02819
Firmicutes bacterium CAG:114	0.20	0.17	0.14	0.04204
Bacteroides cellulosilyticus	0.13	0.18	0.21	0.01780
Firmicutes bacterium CAG:110	0.11	0.37	0.13	0.02141
Clostridium sp. KLE 1755	0.10	0.08	0.06	0.01052
Clostridium sp. CAG:58	0.09	0.07	0.05	0.00043
Ruminococcus sp. SR1/5	0.06	0.05	0.04	0.00989
Clostridium clostridioforme CAG:132	0.05	0.05	0.03	0.03618

Overall, it should be noticed that, in this as in previous trials, a consistent variation in the relative abundance of predominant phyla, classes and families was observed between the same replicates. One example is reported for the Lactobacillaceae family (Figure 30). This result underlines the importance to test a representative number of birds within each treated group and nine chickens should be a suitable number.

Figure 30. Abundance variation of Lactobacillaceae family between cickens of the same treatment.



3.3.3.3 Caeca metabolic genes composition

The comparison between abundance of level function genes in the tested groups showed a number of significantly different functions, mainly belonging to carbohydrate metabolism, amino acid metabolism and membrane transport. All significantly different functions were lower in the treated groups in comparison to the control and were mainly associated to glutathione metabolism, pyruvate metabolism and phosphotransferase system (Table 19). The only exception was represented by pepN; aminopeptidase N [EC:3.4.11.2] significantly higher in the group treated with commercial phytase at triple concentration. The PTS components, significantly lower in the treated groups, were found to regulate numerous cellular functions not or only indirectly related to carbon metabolism and transport. In fact, PTS components control nitrogen and phosphate metabolism as well as potassium transport, antibiotic resistance, biofilm formation, and endotoxin production and also regulate the virulence of several pathogens. Given the high physiological impact of its regulatory functions, the PTS can no longer be considered merely a sugar transport and phosphorylation system but plays some regulatory roles (Deutscher et al., 2014).

Table 19. Metabolic functions significantly different between control and treated groups.

Metabolism	Patways	Function	P value	Control	Group B	Group C	Group D	Group E
Metabolism of other amino acids	Glutathione metabolism	pepN; aminopeptidase N [EC:3.4.11.2]	0,031	1,857	1,314	2,045	1,163	1,308
Carbohydrate metabolism	Starch and sucrose metabolism	E2.4.1.8, mapA; maltose phosphorylase [EC:2.4.1.8]	0,012	0,742	0,396	0,473	0,499	0,445
Carbohydrate metabolism	Pyruvate metabolism	ppc; phosphoenolpyruvate carboxylase [EC:4.1.1.31]	0,016	0,652	0,323	0,350	0,379	0,275
Carbohydrate metabolism	Pyruvate metabolism	E1.2.3.3, poxL; pyruvate oxidase [EC:1.2.3.3]	0,000	0,493	0,236	0,233	0,286	0,232
Carbohydrate metabolism	Pentose phosphate pathway	E1.1.1.44, PGD, gnd; 6-phosphogluconate dehydrogenase [EC:1.1.1.44]	0,014	0,457	0,189	0,213	0,211	0,174
Carbohydrate metabolism	Starch and sucrose metabolism	E3.2.1.122, glvA; maltose-6'-phosphate glucosidase [EC:3.2.1.122]	0,044	0,453	0,230	0,169	0,282	0,228
Cancers	Pathways in cancer	E4.2.1.2B, fumC; fumarate hydratase, class II [EC:4.2.1.2]	0,016	0,416	0,211	0,349	0,235	0,362
Amino acid metabolism	Arginine and proline metabolism	E4.1.1.17, ODC1, speC, speF; ornithine decarboxylase [EC:4.1.1.17]	0,003	0,375	0,167	0,192	0,116	0,272
Amino acid metabolism	Lysine biosynthesis	patA; aminotransferase [EC:2.6.1.-]	0,044	0,374	0,179	0,147	0,216	0,127
Membrane transport	Phosphotransferase system (PTS)	PTS-Man-EIIB, manX; PTS system, mannose-specific IIB component [EC:2.7.1.69]	0,008	0,358	0,150	0,182	0,217	0,162
Membrane transport	Phosphotransferase system (PTS)	PTS-Man-EIIA, manX; PTS system, mannose-specific IIA component [EC:2.7.1.69]	0,003	0,338	0,106	0,137	0,180	0,097
Membrane transport	Phosphotransferase system (PTS)	PTS-Arb-EIIB, glvB; PTS system, arbutin-like IIB component [EC:2.7.1.69]	0,010	0,331	0,139	0,164	0,178	0,120
Membrane transport	Phosphotransferase system (PTS)	PTS-Arb-EIIC, glvC; PTS system, arbutin-like IIC component	0,010	0,325	0,135	0,121	0,155	0,086
Amino acid metabolism	Arginine and proline metabolism	pip; proline iminopeptidase [EC:3.4.11.5]	0,001	0,325	0,135	0,121	0,155	0,086

3.3.3.4 Chicken carcass microbiota composition

In the trial with phytase, other than the caecal microbiota, skin microbiota composition of chicken carcasses collected at the slaughterhouse at the end of the cooling tunnel was investigated. The microbiota composition of the skins belonging to carcasses collected from group A (i.e., control diet) was compared with those of carcasses fed with a triple concentration of commercial phytase without (group C) and with (group E) inositol. The microbiota of the skins belonging to each group is summarised in Table 20. For all groups Proteobacteria followed by Firmicutes represented more than 90% of bacterial populations. Both these Phyla were largely represented in all groups. However, the relative frequency of abundance of Proteobacteria was higher in Group A compared to the other groups. On the contrary, the abundance of Firmicutes was higher in the treated groups (Group C and E) in comparison to group A. Gammaproteobacteria was the most abundant class within Proteobacteria, followed by Betaproteobacteria and Alphaproteobacteria (Table 20). Enterococcaceae and Moraxellaceae were the most rapresented families in the Proteobacteria class, followed by Aeromonadaceae, Shewanellaceae, Pseudomonadaceae, Pasteurellaceae, Idiomarinaceae and Vibrionaceae (Table 20). Enterococcaceae showed a significantly lower ($P=0.029$) relative abundance in group A (i.e., 22.84%) in comparison to groups C and E (i.e., 42.00 and 37.56%, respectively). The same trend was observed in the relative frequency of abundance of Vibrionaceae that was significantly lower in the control group compared to the others ($P=0.010$). On the contrary, the abundances of Moraxellaceae, Shewallanaceaea and Idiomondaceae were significantly higher in group A (i.e., $P<0.05$) compared to the treated groups. The Phylum Firmicutes was mostly represented by Bacilli and Clostridia. In particular, both Clostridia ($P=0.011$) and Clostridiaceae ($P<0.05$) were significantly more abundant in group B compared to Group A. Within the Bacilli class, the most represented families were Planococcaceae, Bacillaceae, Paenibacillaceae, Staphylococcaceae, Enterococcaceae, Lactobacillaceae and Streptococcaceae (Table 20). In the distribution of Bacillaceae, Lactobacillaceae, Streptococcaceae, Staphylococcaceae, between all groups, significant differences were not observed, while the other families showed statistically significant differences. In particular, the abundance of Eterococcaceae was significantly higher ($P=0.04$) in group E compared to groups A and C, while the Paenibacillaceae abundance was significantly higher in group A compared to the others groups (Table 20). In the Bacilli class, group E showed a significantly higher relative frequency of abundance of the Enterococcaceae family ($P=0.029$) compared to the other groups and a significantly lower relative frequency of abundance of Planococcaceae compared to group C ($P<0.05$).

Table 20. Mean relative frequency of abundance (%) of Phyla, Classes and Families of carcass skin microbiota in chickens belonging to groups A, C, and E.

Phylum	Class	Family	Group A	Group C	Group E
Proteobacteria			94.99	92.68	92.81
	Gammaproteobacteria		94.61	92.15	92.51
		Enterobacteriaceae	22.84	42.00	37.56
		Moraxellaceae	51.36	23.71	22.05
		Aeromonadaceae	19.48	25.18	30.95
		Shewanellaceae	0.47	0.82	1.48
		Pseudomonadaceae	0.31	0.30	0.33
		Idiomarinaceae	0.03	0.02	0.01
		Pasteurellaceae	0.07	0.05	0.03
		Vibrionaceae	0.02	0.02	0.03
	Betaproteobacteria		0.35	0.51	0.26
		Comamonadaceae	0.29	0.45	0.19
		Neisseriaceae	0.04	0.02	0.03
	Alphaproteobacteria		0.02	0.02	0.03
Firmicutes			4.01	6.46	6.10
	Bacilli		3.14	3.21	3.42
		Planococcaceae	0.57	0.68	0.36
		Bacillaceae	0.24	0.11	0.08
		Paenibacillaceae	0.11	0.06	0.06
		Staphylococcaceae	0.19	0.56	0.18
		Enterococcaceae	0.33	0.45	1.17
		Lactobacillaceae	0.02	0.38	0.46
		Streptococcaceae	1.66	0.96	1.09
	Clostridia		0.85	3.24	2.67
		Clostridiaceae	0.77	3.18	2.61
		Peptostreptococcaceae	0.05	0.03	0.02
Bacteroidetes			0.96	0.79	0.97
	Bacteroidia		0,05	0.23	0.12
		Bacteroidaceae	0.04	0.21	0.09
	Flavobacteriia		0,90	0.55	0.83
		Flavobacteriaceae	0.90	0.55	0.83
Actinobacteria			0.04	0.06	0.10
	Actinobacteria		0,04	0.06	0.10
		Micrococcaceae	0.01	0.03	0.02
		Bifidobacteriaceae	0.003	0.004	0.03

Whithin the phylum Bacteroidetes, the Flavobacteriia class was the most represented in all groups followed by Bacteroidia (Table 20). The abundance of the Flavobacteria class and Flavobacteriaceae was significantly lower ($P<0.05$) in group C in comparison to the control group. On the contrary, the Bacteroidia class was significantly higher in group C compared to the control group ($P<0.05$).

Bacteroidaceae was the most represented family in the Bacteroidia class and showed a significantly higher abundance in group E compared to the control group ($P<0.05$).

Finally, phylum Actinobacteria showed a frequency of abundance significantly higher ($P=0.045$) in the control group in comparison to groups A and E. The same trend was observed for the class Actinobacteria. In Group E, Bifidobacteriaceae was the most represented family and it showed a significant higher abundance ($P<0.01$) in Group E in comparison to groups A and C, where the relative abundances of the same family were as low as 0.003 and 0.004% respectively (Table 20), The Micrococcaceae abundance was significantly higher ($P=0.029$) in group C in comparison to the control group.

Table 21 summarises the top 30 bacterial species identified in the control group in comparison to the treated groups (Group C and E). Among these species, both Group C and E showed a higher abundance of some pathogenic bacteria compared to Group A. Group C and E presented a higher abundance of *Escherichia coli* and *Clostidium perfringens* compared to Goup A, where the relative abundances of the same species were 16.27 and 0.73% respectively. The same trend was observed for *Salmonella enterica* that was present as one of the top 30 species only in groups C and E, while it was absent in Group A. Comparing the top 30 bacteria species reported in Table 21 it can be noticed that the composition of group A showed a prevalence of Acinetobacter and Aeromonas genera, while the other two groups showed a more diversified composition (Table 21). Among these genera, potential pathogenic species present in Group A, such as *Acinetobacter baumannii* and *Acinetobacter haemolyticus* showed a higher abundance compared to the other groups. Moreover, other than potential pathogens belonging to Acinetobacter genus, even *Klebsiella oxytoca* and *Morganella morganii* had a higher relative frequency of abundances in group A compared to group C and E, while on the contrary, *Aeromonas hydrophila* and *Aeromonas veronii* had a higher relative frequency of abundances in Group C and E compared to Group A (Table 21).

Table 21. Mean relative frequency of abundance (%) of the 30 top representative species (MRS) of skin microbiota in Groups A, C and E.

MRS	Group A species	Mean	Group C species	Mean	Group E species	Mean
1	*Acinetobacter johnsonii*	32.86	*Escherichia coli*	36.80	*Escherichia coli*	30.47
2	*Escherichia coli*	16.27	*Aeromonas veronii*	18.65	*Aeromonas veronii*	22.28
3	*Aeromonas veronii*	13.83	*Acinetobacter johnsonii*	14.25	*Acinetobacter johnsonii*	14.21
4	*Acinetobacter lwoffii*	9.84	*Aeromonas hydrophila*	4.46	*Aeromonas hydrophila*	5.77
5	*Aeromonas hydrophila*	3.78	*Acinetobacter lwoffii*	4.35	*Acinetobacter lwoffii*	4.23
6	*Citrobacter freundii*	1.32	*Clostridium perfringens*	3.15	*Clostridium perfringens*	2.57
7	*Aeromonas media*	1.07	*Aeromonas media*	1.28	*Citrobacter freundii*	2.53
8	*Morganella morganii*	1.05	*Citrobacter freundii*	0.97	*Aeromonas media*	1.67
9	*Acinetobacter junii*	0.99	*Morganella morganii*	0.74	*Enterococcus cecorum*	0.90
10	*Acinetobacter towneri*	0.85	*Acinetobacter junii*	0.67	*Shewanella baltica*	0.85
11	*Acinetobacter baumannii*	0.80	*Kurthia sp. 11kri321*	0.66	*Aeromonas salmonicida*	0.81
12	*Klebsiella oxytoca*	0.74	*Psychrobacter sp. P11F6*	0.52	*Morganella morganii*	0.54
13	*Clostridium perfringens*	0.73	*Aeromonas salmonicida*	0.46	*Klebsiella oxytoca*	0.45
14	*Providencia rustigianii*	0.70	*Shewanella baltica*	0.44	*Acinetobacter junii*	0.39
15	*Streptococcus iniae*	0.67	*Acinetobacter towneri*	0.43	*Streptococcus iniae*	0.38
16	*Kurthia sp. 11kri321*	0.56	*Providencia rustigianii*	0.42	*Empedobacter brevis*	0.37
17	*Aeromonas salmonicida*	0.53	*Acinetobacter baumannii*	0.40	*Lactobacillus salivarius*	0.35
18	*Acinetobacter bereziniae*	0.53	*Staphylococcus aureus*	0.37	*Providencia rustigianii*	0.35
19	*Empedobacter brevis*	0.50	*Streptococcus iniae*	0.36	*Kurthia sp. 11kri321*	0.35
20	*Moraxella bovoculi*	0.49	*Acinetobacter bouvetii*	0.36	*Acinetobacter baumannii*	0.34
21	*Acinetobacter bouvetii*	0.48	*Klebsiella oxytoca*	0.35	*Acinetobacter bouvetii*	0.29
22	*Streptococcus dysgalactiae*	0.43	*Lactobacillus salivarius*	0.35	*Streptococcus parauberis*	0.29
23	*Acinetobacter gerneri*	0.41	*Acinetobacter bereziniae*	0.30	*Citrobacter sp. FDAARGOS_156*	0.25
24	*Acinetobacter haemolyticus*	0.39	*Comamonas aquatica*	0.29	*Hafnia alvei*	0.24
25	*Acinetobacter sp. TTH0-4*	0.39	*Empedobacter brevis*	0.27	*Acinetobacter bereziniae*	0.24
26	*Acinetobacter venetianus*	0.38	*Klebsiella pneumoniae*	0.26	*Aeromonas molluscorum*	0.24
27	*Acinetobacter radioresistens*	0.37	*Salmonella enterica*	0.25	*Salmonella enterica*	0.23
28	*Acinetobacter tandoii*	0.37	*Enterococcus cecorum*	0.24	*Acinetobacter towneri*	0.22
29	*Acinetobacter parvus*	0.32	*Acinetobacter gerneri*	0.21	*Proteus mirabilis*	0.21
30	*Enterobacter cloacae*	0.27	*Proteus mirabilis*	0.21	*Klebsiella pneumoniae*	0.21

The bacterial species with abundances higher than 0.025% in at least one group and significantly different between groups A and E as well as A and C are summarized in Tables 22 and Table 23. Group A showed significant higher abundances of the species belonging to Acinetobacter, Moraxella and Enterobacter genera compared to both the treated groups, i.e. group C and group E (Table 22 and 23). The same trend was observed for Pseudomonas genus. In particular, the *Pseudomonas pseudoalcaligenes* species was significantly higher in group A compared to groups C and E, while *Pseudomonas aeruginosa* was significantly higher compared only to group C. On the contrary, the species belonging to the Shigella and Shawanella genera were significantly lower in the control group in comparison to the treated groups. *Clostridium perfringens* abundance was significantly higher in Group C compared to Group A, while *Salmonella enterica* showed a significant difference only in the comparison between group E and A. Group E showed even a significant higher abundance of *Aeromonas schubertii* in comparison to Group A. Finally, group A showed higher abundances of *Citrobacter youngae* and *Citrobacter amalonaticus* compared to group C. On the contrary, the species *Klebsiella pneumoniae* and *Klebsiella variicola* were significantly higher in Group E compared to Group A.

Table 22. Statistically significant differences between means of relative frequency of abundance (%) of skin bacterial species in groups A and E.

	Group A	Group E	
Species	Mean		P-values
Acinetobacter johnsonii	32.86	14.21	0.0008
Acinetobacter lwoffii	9.84	4.23	0.0005
Acinetobacter junii	0.99	0.39	0.0035
Acinetobacter towneri	0.85	0.22	0.0024
Acinetobacter baumannii	0.80	0.34	0.0003
Klebsiella oxytoca	0.74	0.45	0.0394
Acinetobacter bereziniae	0.53	0.24	0.0001
Acinetobacter gerneri	0.41	0.16	0.0019
Acinetobacter haemolyticus	0.39	0.18	0.0006
Acinetobacter sp. TTH0-4	0.39	0.17	0.0005
Acinetobacter venetianus	0.38	0.17	0.0010
Acinetobacter radioresistens	0.37	0.14	0.0003
Acinetobacter tandoii	0.37	0.16	0.0021
Acinetobacter parvus	0.32	0.18	0.0033
Enterobacter cloacae	0.27	0.18	0.0014
Acinetobacter schindleri	0.26	0.07	0.0228
Enterobacter asburiae	0.26	0.13	0.0007
Shewanella baltica	0.23	0.85	0.0162
Acinetobacter ursingii	0.23	0.09	0.0003
Acinetobacter sp. Ver3	0.22	0.08	0.0017
Acinetobacter pittii	0.21	0.09	0.0013
Leclercia adecarboxylata	0.20	0.10	0.0027
Comamonas aquatica	0.19	0.10	0.0434
Moraxella osloensis	0.15	0.08	0.0147
Salmonella enterica	0.14	0.23	0.0292
Klebsiella pneumoniae	0.14	0.21	0.0015
Serratia liquefaciens	0.12	0.06	0.0066
Acinetobacter gyllenbergii	0.12	0.05	0.0007
Acinetobacter bohemicus	0.11	0.05	0.0015
Paenibacillus sophorae	0.10	0.06	0.0403
Acinetobacter sp. ATCC 27244	0.09	0.04	0.0012
Enterococcus cecorum	0.08	0.90	0.0222
Bacillus mycoides	0.08	0.03	0.0009
Aeromonas schubertii	0.07	0.12	0.0471
Enterobacter sp. E20	0.07	0.03	0.0002
Shewanella oneidensis	0.07	0.16	0.0002
Acinetobacter harbinensis	0.06	0.02	0.0020
Acinetobacter equi	0.05	0.02	0.0009
Shewanella putrefaciens	0.05	0.17	0.0027

Pseudomonas pseudoalcaligenes	0.05	0.03	0.0029
Acinetobacter sp. NIPH 298	0.05	0.02	0.0014
Shewanella sp. ANA-3	0.04	0.09	0.0004
Acinetobacter nosocomialis	0.04	0.02	0.0001
Macrococcus caseolyticus	0.04	0.07	0.0255
Enterobacter cancerogenus	0.04	0.02	0.0022
Plesiomonas shigelloides	0.04	0.01	0.0360
Comamonas kerstersii	0.04	0.02	0.0284
Bacteroides fragilis	0.03	0.05	0.0222
Shewanella sp. MR-7	0.03	0.06	0.0005
Shewanella sp. MR-4	0.03	0.06	0.0003
Edwardsiella tarda	0.03	0.04	0.0428
Idiomarina loihiensis	0.02	0.01	0.0197
Shewanella sp. W3-18-1	0.02	0.08	0.0042
Shigella sonnei	0.02	0.03	0.0446
Klebsiella variicola	0.01	0.04	0.0345
Bifidobacterium gallinarum	0.003	0.03	0.0281

Table 23. Statistically significant differences between means of relative frequency of abundance (%) of skin bacterial species in group A and Group C.

	Group A	Group C	
Species	Mean		P-values
Acinetobacter johnsonii	32.86	14.25	0.0024
Acinetobacter lwoffii	9.84	4.35	0.0021
Acinetobacter towneri	0.85	0.43	0.0435
Acinetobacter baumannii	0.80	0.40	0.0059
Klebsiella oxytoca	0.74	0.35	0.0098
Clostridium perfringens	0.73	3.15	0.0088
Acinetobacter bereziniae	0.53	0.30	0.0121
Empedobacter brevis	0.50	0.27	0.0134
Acinetobacter gerneri	0.41	0.21	0.0340
Acinetobacter haemolyticus	0.39	0.20	0.0090
Acinetobacter sp. TTH0-4	0.39	0.18	0.0023
Acinetobacter venetianus	0.38	0.18	0.0069
Acinetobacter radioresistens	0.37	0.20	0.0148
Acinetobacter tandoii	0.37	0.17	0.0084
Enterobacter cloacae	0.27	0.17	0.0072
Enterobacter asburiae	0.26	0.14	0.0024
Acinetobacter ursingii	0.23	0.13	0.0195
Acinetobacter sp. Ver3	0.22	0.10	0.0155
Acinetobacter pittii	0.21	0.10	0.0178
Leclercia adecarboxylata	0.20	0.09	0.0022
Moraxella osloensis	0.15	0.09	0.0231
Serratia liquefaciens	0.12	0.06	0.0025
Acinetobacter bohemicus	0.11	0.05	0.0061
Pseudomonas aeruginosa	0.11	0.08	0.0457
Paenibacillus sophorae	0.10	0.06	0.0322
Citrobacter youngae	0.10	0.07	0.0453
Enterobacter sp. 638	0.095	0.056	0.0122
Acinetobacter sp. ATCC 27244	0.090	0.047	0.0236
Bacillus mycoides	0.079	0.035	0.0041
Acinetobacter harbinensis	0.057	0.030	0.0337
Acinetobacter equi	0.054	0.023	0.0034
Pseudomonas pseudoalcaligenes	0.048	0.025	0.0025
Chryseobacterium gleum	0.047	0.030	0.0457
Acinetobacter sp. NIPH 298	0.047	0.026	0.0428
Acinetobacter nosocomialis	0.040	0.020	0.0037
Enterobacter cancerogenus	0.040	0.021	0.0016
Citrobacter amalonaticus	0.025	0.018	0.0367
Shigella flexneri	0.017	0.043	0.0320
Shigella sonnei	0.016	0.043	0.0379
Shigella dysenteriae	0.014	0.033	0.0238

4. DISCUSSION AND CONCLUSIONS

The animal gut microbiome describes the collective genomic content of the microbial community ihabiting the gut and the total genetic capacity of that community (Tremaroli and Bäckhed, 2012). Since the gut microbiome is involved in the regulation of multiple host metabolic pathways, a deep understanding of the relationships between microbiome and host provides new strategies to fight diseases, improve animal health and as a consequence safety of foods of animal origin. Using shotgun metagenomic sequencing the relationship between chickens and their microbiome has been investigated in the context of application of different feeding strategies specifically represented by the supplementation in a control diet of (1) *Lactobacillus acidophilus D2/CSL,* (2) serine protease, associated or not to a low level of proteins, (3) phytase at standard and triple concentration associated or not with inositol.

Overall, in the three feeding trials the gut populations were investigated in their natural habitats and in the context of their interrelationships with the host using shotgun metagenomic sequencing. Knowledge of the microorganisms having gene coding for enzymes associated to specific metabolic pathways allows improving (or decreasing) those pathways driving specific microbial populations by fit for purpose nutrition strategies and investigating how the metabolic pathways are correlated to one another in the different microorganisms. The variability between individuals treated with the same additive has been previously reported in other studies that characterized chicken caeca (Stanley et al., 2013b; Sergeant et al., 2014), these studies hypothesized that this great variation in the relative abundance of the bacterial community between individuals could depend on the initially colonizing microbiota and immune system of the host (Donaldson et al., 2015). To take into account such variability the number of individual chickens tested within each group, including controls, changed during the project and shoud be fixed at nine birds collected at least at twice along the rearing cycle.

4.1 Impact of feed supplemented with *Lactobacillus acidophilus D2/CSL* on chicken gastrointestinal tract

The administration of probiotic *Lactobacilli* has been demonstrated to stimulate immune responses (Haghighi et al., 2005; Brisbin et al., 2011), improve digestive health (Kim et al., 2012), as well as growth performance (Loh et al., 2010; Shim et al., 2012; Askelson et al., 2014) in poultry. *Lactobacillus* administration has also been shown to reduce colonization by *Campylobacter* (Ghareeb et al., 2012; Neal-McKinney et al., 2012), *Clostridium* (La Ragione et al., 2004), and *Salmonella* (Chen et al., 2012; Ghareeb et al., 2012), improving the microbial food safety of poultry meat.

The ability of *Lactobacillus* species to adhere to epithelial tissues and colonize poultry has been reported (Jin et al., 1996; Edelman et al., 2002; Bouzaine et al., 2005). However, microbial factors

important to gastro intestinal persistence of *Lactobacillus* in poultry are not well characterized. The lack of a species-specific cell culture model has been a problem to investigations of *Lactobacillus* adhesion and its contribution to gastro intestinal colonization in poultry. According to previous studies (Oakley et al., 2013) Firmicutes and Proteobacteria were the most common phyla identified in caeca tested in our research. At finer scales of taxonomic resolutions, the majority of sequences belonged to various members of Clostridia class. In the day-old chicks, the most represented bacteria genera were *Lactobacillus, Clostridium, Blautia, Escherichia, Enterococcus, Eubacterium* and *Ruminococcus*. This trend was partially observed also in the chickens at 41 days. However, at the end of the rearing period the most representative genera were *Faecalibacterium, Subdonigranulum, Roseburia* and *Eubacterium*. The presence of *Clostridium*-related species in the chicken caeca was observed by other authors (Bjerrum et al., 2006; Gong et al., 2007; Lund et al., 2010). *Clostridium* clusters IV (including *Faecalibacterium prausnitzzi, Subdoligranulum variabile* and *Anaerotruncus colihominis*) and XIVa (including *Roseburia intestinalis* and *Ruminococcus torques)* produce primarily butyrate (Van den Abbeele et al., 2010). In particular, *F. prausnitzzi* has a requirement for acetate, and produces butyrate, formate and lactate (Scupham, 2007). Butyric acid has been shown to have an important function in protection against pathogens in poultry (Fernandez-Rubio et al., 2009). Furthermore, it is involved in several intestinal functions, being an energy source stimulating the epithelial cells proliferation and differentiation, other than exerting an antimicrobial effect by promoting the production of peptides and stimulating the production of tight junction protein (Dalmasso et al., 2008). Overall, the microbiological profiles identified in day-old chicks, in CON and LA partially confirm those reported by Gong et al., (2008) showing that the first days after hatching the broiler caecum is colonized by facultative aerobes bacteria. Oxygen consumption by these bacteria alters the lower gut environment to more reducing conditions, which facilitates subsequent growth and colonization of extremely oxygen-sensitive obligates anaerobes (Barnes et al., 1972; Gong et al., 2002; Wise and Siragusa, 2007).

R. torques, which is known to degrade GI mucin (Wilson et al., 1997) was significantly higher in CON group in comparison to LA at 41 days. Degradation of mucin is regarded as a pathogenicity factor, since loss of the protective mucus layer may expose GI tract cells to pathogens (Ruseler-van Embden et al., 1995). However, mucin also constitutes a carbon and energy source for intestinal microbiome. It has been estimated that 1% of colonic microbiome is able to degrade host mucin using enzymes (e.g. glycosidases and sulfatases) that can degrade the oligosaccharide chains (Hoskins and Boulding, 1981). Despite the apparent low level of mucin degrading bacteria, these species provide nutrients for other resident bacteria, which can use the monosaccharides or amino acids released from

mucin degradation (Derrien et al., 2004). This result might be possibly related to the higher incidence of pasty vent observed in CON group, but this hypothesis needs to be confirmed.

In relation to the metabolic functions, LA group showed a significantly higher level of β-glucosidase. These enzymes contribute to the hydrolysis of glucose monomers from non-starch polysaccharides (e.g., cellulose, β-glucans), playing an important role in the fermentation of undigested carbohydrates and, ultimately, in animal performance and health. In particular, β-glucosidase (β-glucoside glucohydrolase; EC3.2.1.21) hydrolyzes alkyl- and aryl-β-glucosides, as well as diglucosides and oligosaccharides, to release glucose and an aglycone (Reese, 1977). It also hydrolyzes isoflavonal glycoside conjugates into isoflavone aglycones, such as genistein, daidzein, and glycitein. An increase of the concentrations of genistein and daidzein in soy milk has been reported by using strains of *Streptococcus thermophilus, L. acidophilus, L. delbrueckii ssp.bulgaricus, L. casei, L. plantarum, L. fermentum* and several *Bifidobacterium* species (Donkor and Shah, 2008; Rekha and Vijayalakshmi, 2011). These aglycones hydrolyzed by β-glucosidases from intestinal microorganisms are readily absorbed across the villi of the intestine (Ismail et al., 2005), possess greater bioavailability than the corresponding glycoside conjugates (Izumi et al., 2000) and a wide range of biological properties, such as antioxidant and anti-tumor activities (Fritz et al., 1998; Brouns et al., 2002). Qian and Sun (2009) confirmed that broilers fed with 0.2% β-glucosidase show a significantly increased average daily weight gain ($P<0.05$) and significant higher feed conversion ratios ($P<0.05$) than control.

In conclusion, the relative abundance of *Lactobacillus acidophylus* in the caeca of LA chickens was comparable with that of CON group. This result might be explained taking into account the colonization preference of the administered strain for the crop and the small intestine, even if this specific aspect was not investigated. Beside the lack of colonization of LA in broiler caeca, the results of this study seem to suggest that the metabolic activity of supplemented *Lactobacillus acidophilus,* and in particular the lactic acid production, positively affect the microbial species producing butyric acid by a cross feeding mechanism. Finally, a positive effect was observed in relation to the metabolic functions of the treated group, with particular reference to the higher abundance of the β-glucosidase, improving animal performance and health.

4.2 Impact of feed supplemented with serine protease on chicken gastrointestinal tract

Nutritionists are evaluating proteases for their ability to improve protein and amino acid digestibility in the chicken diets (Olukosi et al., 2015). Nonetheless, the understanding of the mode of action of proteases in the gastrointestinal tract of chickens is still limited (Olukosi et al., 2015). Diet associated differences in the composition of chicken ileum and cecum have been previously reported only in

relation to the association between abundance of Lactobacilli and Clostridium perfringens and supplementation of xylanase in a barley-basal diet (Totok et al., 2008). In this project, taxonomic and functional changes occurring at 14 and 42 days in ceca of chickens fed with a control diet in comparison to diets with a decreased content of protein (i.e., -7%) supplemented, or not supplemented, with an enzyme preparation of serine protease were investigated. Despite the strong relationship between age of the birds and microbiota composition, the microbiota compositional diversity did group the samples at family level according to their diet, at both 14 and 42 days. At phylum level, abundances of Actinobacteria and Proteobacteria were mainly affected by the diet, as well as interaction between diet and time. Abundances of Firmicutes and Cyanobacteria were mainly affected by age of the birds.

The lower level of protein in the diet decreased the abundance of Lactobacillaceae at 14 days but then they increased over time. Lactobacillaceae have been considered key players in host metabolic balance because may reduce the antigen load from gut bacteria to the host, and may alleviate certain inflammation responses (Zhu et al., 2015). The increase of Lactobacillaceae over time might explain the corresponding rise of Faecalibacterium prausnitzii in the ceca of broilers fed with -7% protein between 14 and 42 days. In fact, according to Miquel et al., 2014, the increase of Bifidobactirium longum and Lactobacilus spp might fed Faecalibaterium prausnitzii through the production of acetate. In a study supporting this hypothesis, Bifidobacterium longum BB536 intake (13 weeks treatment) enhanced Faecalibacterium prausnitzii 16S rRNA gene copy numbers in Japanese individuals with cedar pollinosis (Odamaki et al., 2007). Furthermore, an in vitro experiment conducted to quantify butyric-producing bacteria in a simulated broiler cecum, the supplementation of different Lactobacillus species, including Lactobacillus salivarius, after 24 h of incubation, significantly increased the number of Lactobacilli, Bifidobacteria and Faecalibacterium prausnitzii (Meimandipour et al., 2010). Faecalibacterium praustnizii, as well as Sudoligranum variabile representing one of the signature species decreasing in diet C in comparison to the control at 42 days, are producers of short-chain fatty acids (SCFA), such as butyric acid and formic acid (Bjerrum et al., 2006), having an important function in both growth performance (Garcia et al., 2007) and protection against pathogens (Fernandez-Rubio et al., 2009). All diet C signature species at 14 days belonged to Bacteroidetes and were significantly lower in the chickens fed with serine protease in comparison to the control. This result demonstrated the lower health status of ceca collected from animals fed with serine protease. In fact, Bacteroidetes are the main bacteria involved in producing SCFA and play an important role in breaking down complex molecules to simpler compounds, which are essential to the growth of the host and gut microbiota (Lan et al., 2006). The animals fed with serine protease showed also a higher abundance of Gammaproteobacteria at 14 days in comparison to control group and group -7% protein.

Gammaproteobacteria contain many pathogenic species, including *Escherichia coli*, which rinsed over time in the ceca of broilers fed with diet C and was significantly higher in animals fed with serine protease in comparison to the other groups at the end of the rearing period (i.e., 42 days). Some *E. coli* strains are capable of causing opportunistic secondary infections in birds, following other respiratory tract pathogens, such as infectious bronchitis virus or *Mycoplasma gallisepticum* (Smith et al., 1985; Gross, 1990) in response to high ammonia levels in poultry houses, or physiological changes in the avian host. Besides, its potential as zoonotic pathogen, there is evidence that intestinal microbiome, including *E. coli*, may serve as reservoirs for antibiotic resistance and spread of resistance to zoonotic pathogens, such as *Salmonella* (Nandi et al., 2004; Fricke et al., 2009). Therefore, the increasing trend of this species linked to the supplementation of proteases should be carefully taken into consideration.

The most abundant functional genes in all tested groups were cystine desulfurase, followed by alpha-galactosidase and serine hydroxymethyltransferase. Cysteine desulfurase is a pyridoxal 5′-phosphate (PLP)-dependent homodimeric enzyme that catalyzes the conversion of L-cysteine to L-alanine and sulfane sulfur via the formation of a protein-bound cysteine persulfide intermediate on a conserved cysteine residue. Increased evidence for the functions of cysteine desulfurases has revealed their important roles in the biosyntheses of Fe-S clusters, thiamine, thionucleosides in tRNA, biotin, lipoic acid, molybdopterin, and NAD. The enzymes are also proposed to be involved in cellular iron homeostasis and in the biosynthesis of selenoproteins (Mihara and Esaki, 2002). At 14 days, genes coding protein secretion system Type VI (T6SS) were significantly higher in the ceca of birds fed with -7% protein in comparison to the control group. However, at 42 days T6SS were significantly higher in the ceca of birds treated with serine protease in comparison to both control group and -7% protein group. Type VI secretion systems (T6SSs) are the most recently described specialized secretion systems. T6SSs are widely distributed in Gram-negative bacteria, especially in Proteobacteria, where type VI secretion gene clusters may be found in several copies on the chromosome (Cascales and Cambillau, 2012). First thought of as secretion systems dedicated to virulence towards eukaryotic host cells, recent data have shown unambiguously that these systems are regulating bacterial interactions and competition (Cascales and Cambillau, 2012). T6SSs are required to kill neighbouring, non-immune bacterial cells by secreting anti-bacterial proteins directly into the periplasm of the target cells upon cell-to-cell contact. This intense bacterial warfare indirectly contributes to pathogenesis in animals as T6SS facilitates the colonization of specific niches where pathogens then develop anti-host defences and toxins (Cascales and Cambillau, 2012).

In conclusion, the decrease level of protein in the diet negatively affected the abundance of Lactobacillaceae at 14 days. The supplementation of serine protease in the same feed (i.e., -7% protein) increased the level of Gammaproteobacteria at 14 days and the abundance of *E. coli* over time. Moreover, the supplementation of protease increased the abundances of T6SS genes contributing to pathogenesis in animals. Therefore, the supplementation of serine protease to improve protein and amino acid digestibility in chicken diets negatively affect the animal gut health.

4.3 Impact of feed supplemented with phytase on chicken gastrointestinal tract

Availability of the phosphorus fraction from phytic acid [myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); InsP6] present in plant seeds and feed, depends on the breakdown of $InsP_6$. Phytases catalyse the hydrolysis of $InsP_6$ to less-phosphorylated inositol phosphates, *myo*-inositol, and orthophosphates. Inositol phosphate isomers (InsP**s**) formed through the degradation action of different phytases have been assumed to possess physiological relevance in the digestive tract of broilers (Zyła et al., 2004). The supplementation of phytase in the diets have been showed to significantly affected growth performance of broiler chickens increasing final body weight feed consumption and body weight gain. Furthermore, an enhanced phosphorus availability can affect the structure of the microbial community in the digestive tract of broiler chickens (Zeller et al., 2015; Borda-Molina et al., 2016). However, the majority of the studies available focus on the effect of phytase on growth performance and body weight of the chickens, while few studies focused on the effect of this enzyme on the chicken microbiota.

This project aimed to distinguish the effects of the administration of a standard and triple concentration of a commercial phytase, alone and combined with inositol, on the composition of microbial communities present in the cecum content of broiler chickens. As commonly described in previous studies (Stanley et al., 2013a; Deusch et al., 2015), the majority of the microorganisms colonizing chicken caecum belong to the phylum Firmicutes. However, in the present study Firmicutes was the most abundant phylum in all groups except group E in which the birds were fed with commercial phytase at triple concentration and inositol. In particular, caeca of birds fed with diet E showed a higher abundance of Bacteroidetes in comparison to the control group and lower abundance of Clostridi, Lactobacillaceae, Negativicutes, Bacteroidia Fusobacteria and Actinobacteria.

The abundance of Erysipelotrichaceae, a family associated to butyryl-CoA production enzymes (Eeckhaut et al., 2011; De Maesschalck et al., 2014) was affected from the addition of phytase in triple concentration to the diet. On the contrary, Enterobacteriaceae abundance was reduced by the addition of inositol in comparison to the control group. The Lactobacillaceae family and

Lactobacillus sp. were negative affected from all the treatments in comparison to the control group. This result is in contrast with other studies where the addition of phytase leaded to a higher abundance of Lactobacillaceae (Borda-Molina et al., 2016). However, a study conducted by Witzig et al., (2015) reported that diets supplemented with phytase seem to support the abundance of Enterobacteriaceae while decreasing Lactobacillaceae in the ileum of chickens. Furthermore, it should be taken into account that the decrease of phytase efficacy in supporting positive populations like Lactobacillaceae might be due to high level of mineral phosphorus (Zeller et al., 2015).

4.4 Impact of feed supplemented with phytase on chicken carcass microbiota

The skin is the most exposed organ, responsible for providing a barrier to the external environment, however, despite a potent cutaneous immune system, many different microbial communities multiply on the skin surface. However, there are not studies, we are aware of, that analyse the skin microbiota of the chicken using metagenomic sequencing. In fact, authors have mainly focused on the research of photogenic bacteria on carcasses or on poultry meat products in order to avoid food born diseases. However, a better understanding of the dynamics between bacteria populations colonizing the chicken skin and of their modulation could, aside from improving the animal welfare, prevent the onset of foodborne pathogens and higher the food quality level of pouty meat products (Cogen et al., 2008; Grice and Segre, 2011; Sanford et al., 2013).

The result showed that the skin microbiota of chickens in both groups were mainly composed of Proteobacteria and Firmicutes. Among these phyla, some of the more represented classes in the control group in comparison to the treated groups were Moraxellaceae, Flavobacteriaceae while at a species level there was a prevalence of Acinetobacter and Pseudomonas species. Freeman et al. (1976) identified *Pseudomonas putrefaciens* and *Pseudomonas* species that were members of groups I and II of Shewan's classification, as well as Flavobacterium and oxidative Moraxella, as bacteria producing a number of the compounds found in the aroma of spoiled chickens as hydrogen cyanide, methyl isopropyl sulfide, 2-propane thiol, methyl propionate, ethyl benzene, and an unidentified compound. Even Acinetobacter, that is usually found on the feathers of the bird and may originate from the deep litter, have been identified as spoilage bacteria along with Flavobacterium.

On the contrary, *Shawanella* species, such as *Shawanella putrefaciens,* identified by Russell et al. (1995) as the bacterial species responsible for spoilage of poultry from various locations around the U.S, showed lower abundance in the control group in comparison to the treated groups.

Morover, results showed that Enterococcaceae, Clostridiaceae and Micrococcaceae and some enteric pathogenic species, such as *Salmonella enterica, Escherichia coli* and *Clostridium perfringens,* showed a statistically higher abundance in one or both the treated groups in comparison to the control

group. However, the higher abundance of these families and these pathogenic species in the treated groups in comparison to the control, was observed only in the skin samples but not in the fecal samples of the group fed with the same diet. Therefore, it could be hypothesized that this phenomenon could depend on a contamination at a slaughterhouse.

However, this principle can be applied even to others pathogenic species, such as Salmonella that was showed to increase during some de-feathering processes (Clouser et al., 1995). Smith et al., (2007) reported the effect of external or internal faecal contamination on the numbers and incidence of coliforms, *Escherichia coli* and *Campylobacter* after evisceration and passage through an inside-outside bird washer. The authors showed that carcasses with external contamination had the highest numbers of bacteria after washing compared to carcasses with internal contamination or carcasses without applied contamination.

Shigella species were also found to be significantly more abundant in the treated groups in comparison to the control group. Some species of Shigella genus are pathogenic bacteria causing Shigellosis and Shigella-contaminated food is often the source of infection. This study results show that, aside from the presence of some pathogenic bacteria contaminations caused from processing, the effect of phytase on chicken skin lead to mostly the reduction of spoilage genera and species in the treated groups compared to the control. The reduction of these genera and species, responsible of the spoilage of meat, could allow to heighten the quality of poultry meat product.

4.5 Future prospectives

In recent years the ability to obtain a through picture of gut microbial communities has been consistently improved by the introduction of molecular, culture-independent techniques based on ribosomal 16S rRNA gene sequencing, including fluorescent in situ hybridization (FISH), fragment restriction length polymorphism mapping, competitive and quantitative PCR, denaturating (or temperature) gradient gel electrophoresis (DGGE/TGGE), shotgun sequencing DNA, and whole metagenomic analyses (Deng et al., 2008). The greatest challenges in this research field, apart from pure metagenomic complexitity, relate to understanding the temporal dynamics of metabolic communication between the host and its gut microbiota on an evolutionary time scale, in relation to global changes in nutritional strategies as well as environmental stressors. Alterations to the gut microbiota affect chicken biological fitness at multiple levels that will need to be better understood if we want to elucidate the role of the gut microbiota and the best ways to manipulate the microbiota to gain animal health benefit and improve food safety.

Host–microbe interaction is currently a very active area of research and may help in identifying clusters of GIT bacteria that are consistently associated with better growth performance and health in

animals raised in varied environments (Torok et al 2011; Stanley et al., 2012). Highly productive animals have been developed by selection for elite genetic traits; it is possible that in the future, gains in productivity and health outcomes could be influenced by selection of elite GIT microbiota. Therefore, the role of the GIT microbiota in both productivity and health is subject to intensive study. Even if our knowledge of the gut microbiota composition, metabolic functions, and influence on animal health, welfare, and performance is far from complete, metagenomic sequencing results collected in this project should help to fill this gap. In this project, the comparison of multiple metagenomes from the chicken guts using MG-RAST reveled the relationships between dit and host at different time points based on taxonomic and functional profiling. Even if the project time frame did not allow to go further in the identification of the best diet to fight pathogens in the gut to improve poultry meat safety, the results achieved contribute to reach this aim.

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6. LIST OF PUBBLICATIONS AND ATTENDANCE TO CONFERENCES

Publications

- De Cesare A., do Valle I. F., Sala C., Moniaci P., Astolfi A., Castellani G., Manfreda G. Effect of protease administration in a low protein feed on chicken caeca microbiome. Accepted for publication in PlosOne.
- De Cesare A., Sirri F., Manfreda G., Moniaci P., Giardini A., Meluzzi A. Effect of Lactobacillus acidophilus D2/CSL (CECT 4529) supplementation on gut microbiome and broiler chicken performance. Submitted to Applied Enviromental Microbiology.

In preparation

- *Impact of supplementation of phytase at different concentration and in association with inositol on chicken caeca and carcasses microbiomes.*
- *Comparison between bioinformatics pipelines for metagenomic analysis.*

Conferences

- COMPARE, Annual General Meeting, 8-10 March 2016, Cophagen, Denmark.
- XXI Workshop on Developments in the Italian Ph.D. Research On Food Science, Technology and Biotechnology, University of Naples – Federico II, Portici, 13-16 September, 2016.