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APPLICATION OF TRADITIONAL AND INNOVATIVE TECHNIQUES TO INVESTIGATE PRODUCTIVE EFFICIENCY AND RELATED MOLECULAR TRAITS IN BROILER CHICKENS

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ABSTRACT

One of the most complex and tough challenges that the livestock industry will face in the next future is to fulfill the food requirement of a growing world population. In addition, the limited availability of arable land, continuous climate changes, the increasing cost of agricultural commodities used for bio-fuel production, as well as animal welfare and environmental impact concerns will inevitably strengthen the importance of replying to the increasing food demand in an efficient and sustainable way. As feeding represents the greatest cost in livestock, improving the exploitation of nutrients provided with the diet will positively impact on the overall productive efficiency. In livestock, feed efficiency (FE) could be roughly defined as the ability of an animal to convert feed into food outcomes and, in the specific case of broiler chickens, it represents the capacity of converting ingested feed into body mass. Due to the moderate heritability of FE-related parameters, important results have been obtained through the application of selective breeding programs. Alongside with genetic aspects, a better knowledge of the nutritional and environmental requirements of broiler chickens significantly contributed to the improvements in FE as well. However, the selection methods aimed at improving FE in modern fast-growing broiler lines were mostly performed without considering the potential alterations induced on the molecular mechanisms and on the physiological/metabolic features of the animals, resulting in undesirable side-effects such as an increased proneness to obesity and muscle disorders, as well as an hyperphagic feeding behavior. In addition, it has been reported that biological limits and animal welfare concerns will limit further genetic improvements of FE in broilers, suggesting that the biological potential for genetic improvement is minimal compared to the progresses already obtained. Therefore, a more detailed comprehension of nutritional and metabolic aspects involved in FE is necessary to continuously improving this trait while minimizing drawback effects.

From a homeostatic perspective, FE could be considered as the net result among energy intake, which is determined by the voluntary feed intake and its complex regulatory mechanisms, and energy expenditure, which is affected by the maintenance metabolism, the rate of anabolic

processes and the intermediary metabolisms in different tissues and organs. Several external factors, such as diet, diseases, and environmental conditions, are also involved in the phenotypic expression of FE. In particular, the diet can be considered one of the most important factors affecting the rate of the intermediary metabolisms in animal tissues (muscle, adipose tissue, liver, etc.) which can influence overall FE in broilers. In addition, also the possible interaction between dietary treatments and animal's genetic background should be considered when studying FE.

In this scenario, FE appears as a very complex and multifaceted trait regulated by intrinsic (e.g., physiological, metabolic and immunological aspects of the animal) and extrinsic factors (e.g., dietary treatments, environmental conditions, or diseases) that must be taken into account in a holistic approach. Indeed, any nutritional treatment will inevitably integrate with the metabolic aspects of the animal and hence a detailed comprehension of these complex interactions appears necessary for further improvements of FE. This is currently possible by the development of several analytical techniques, identified as *omics* technologies, able to assess the global variation of genes, proteins and metabolites expression levels in animal tissues in response to different stimuli.

Therefore, the present PhD program aimed at evaluating nutritional and physiological aspects involved in overall productivity of broiler chickens, with special regard to FE, combining both traditional and innovative approaches. Two main researches were included in this final dissertation. In the first trial it was investigated whether the currently adopted dietary arginine to lysine (Arg:Lys) ratios are sufficient to meet the modern broiler requirement in arginine, an essential amino acid involved in important functions in poultry. The first study aimed, therefore, to evaluate the effects of the dietary supplementation of L-arginine in a commercial broiler diet on productive performance, with special regard to FE, breast meat quality attributes, incidence and severity of breast muscle myopathies and foot pad dermatitis (FPD), and plasma and muscle metabolomics profile in fast-growing broilers. A total of 1,170 1-d-old Ross 308 male chicks was divided in two experimental groups of 9 replicates each fed either a commercial basal diet (CON, digestible Arg:Lys ratio of 1.05, 1.05, 1.06, 1.07 in each feeding phase, respectively) or the same

basal diet supplemented on-top with crystalline L-arginine (ARG, digestible Arg:Lys ratio of 1.15, 1.15, 1.16, 1.17, respectively). Productive parameters, such as body weight (BW), daily feed intake (DFI) and feed conversion ratio (FCR), were determined on a pen basis at the end of each feeding phase (12, 22, 33, 43 d). At slaughter (43 d), the incidence and severity of FPD and breast myopathies were assessed, while plasma and breast muscle samples were collected and analyzed by proton nuclear magnetic resonance-spectroscopy. The dietary supplementation of arginine significantly reduced cumulative FCR compared to the control diet at 12 d (1.352 vs. 1.401, $P<0.05$), 22 d (1.398 vs. 1.420; $P<0.01$) and 33 d (1.494 vs. 1.524; $P<0.05$), and also tended to improve it in the overall period of trial (1.646 vs. 1.675; $P=0.09$). BW was significantly increased in ARG compared to CON group at 33 d (1,884 vs. 1829 g; $P<0.05$). No significant effect was observed on meat quality attributes, breast myopathies and FPD occurrence. ARG birds showed significantly higher plasma concentration of arginine and leucine, and lower of acetoacetate, glutamate, adenosine and proline. Arginine and acetate concentrations were higher, whereas acetone and inosine levels were lower in the breast of ARG birds ($P<0.05$). Based on our experimental conditions, the Arg:Lys ratios currently adopted at least in Countries where the animal protein sources are not allowed in feed formulation (i.e. European Union) appear to be inadequate to exploit the maximum productive potential of modern fast-growing broilers. The Arg:Lys ratios tested in this trial had positive effects on FE without showing any negative implication on meat quality attributes, foot pad condition and incidence of breast meat abnormalities. Furthermore, plasma and muscle metabolome showed significant alterations in response to the arginine supplementation. According to this analysis, the improvements in FE observed in the supplemented group might be likely ascribed to a potential modulatory effect of arginine on energy and protein metabolism and hence on the overall energy homeostasis in broiler chickens.

The second study was undertaken to characterize productive traits and intestinal transcriptomic profile of two fast-growing chicken hybrids in order to better understand the metabolic dynamics occurring in a key organ such as the small intestine, which is involved in

important immunological, endocrine, and regulatory functions able to influence FE, feeding behavior, and overall energy homeostasis in broilers. A total of 1,170 one-day-old female chicks (n = 585 per genotype) were weighed and randomly divided into 18 pens (9 replications/group). Both the groups received the same commercial diet (starter, 0-9 d; grower I, 10-21 d; grower II, 22-34 d; and finisher, 35-43 d). BW, DFI and FCR were determined on a pen basis at the end of each feeding phase. At processing (43 d), the incidence and severity of FPD were evaluated on all the processed birds and ileum mucosa was collected from 1 bird/replication. Total mRNA was extracted to perform microarray analysis (Chicken Gene 1.1^{ST} Array Strip) and an exploratory pathway analysis was then conducted (Gene Set Enrichment Analysis software). The two genotypes showed different growth patterns throughout the study. HA birds exhibited higher BW and better FCR than HB after 9 d (228 vs. 217 g and 1.352 vs. 1.419, respectively, $P<0.05$). At 21, 34 and 43 d, HB birds reported higher BW (807 vs. 772 g; 1,930 vs. 1,857 g and 2,734 vs. 2,607 g, respectively; $P<0.01$), DFI (74.9 vs. 70.6 g/bird/d, $P<0.01$; 144.4 vs. 139.6 g/bird/d, $P=0.06$; and 196.5 vs 182.4 g/bird/d, $P<0.01$) and similar FCR compared to HA ones. HB group showed a higher percentage of birds with no FPD (75 vs. 48%; $P<0.001$). Regarding gene expression profile, a total of 114 and 179 gene sets resulted significantly enriched in the ileum mucosa of HA and HB broilers, respectively. In the HA group, a high percentage of biological gene sets involved in cellular energy metabolism and mitochondria structure and functionality was observed (43 and 23% of the total, respectively). Other gene sets that were significantly enriched in the ileum mucosa of HA birds were related to ribosome structure and protein synthesis (11%), cell structure and integrity (8%), as well as antioxidant and detox mechanisms (6%). On the other hand, a significant enrichment in gene sets related to the immune system activation (28% of the total) was observed in the ileum mucosa of the HB birds. Moreover, an increased expression of gene sets involved in signal transduction and cell signaling (20%), DNA remodeling and replication – chromatin/histone modification (15%), cell activation, migration and adhesion (12%), inflammation (7%) and bone remodeling (4%) was detected in the HB group. Taken together, the transcriptomic analysis revealed that HA broilers

might be characterized by a healthier condition of the intestinal mucosa likely supported by an increased mitochondria functionality and antioxidant capacity. On the other hand, HB chickens reported a potential inflammatory condition in the gut coupled with a marked activation of the immune system. The factors involved in the onset of this condition, as well as the determinants of the differences observed in feeding behavior in the two genotypes, are not clear and other scientific insights are necessary to better understand these aspects.

Overall, the results obtained combining both traditional and innovative techniques (transcriptomics and metabolomics) in a holistic approach can shed some light on important nutritional and molecular aspects involved in productive efficiency of broiler chickens, confirming the usefulness of these analytical platforms in investigating the molecular mechanisms in response to dietary treatments or associated with different genetic backgrounds in poultry. A better and detailed knowledge regarding nutritional and metabolic features of modern fast-growing broiler chicken hybrids may allow an optimization of productive strategies to efficiently sustain the increasing demand of poultry meat while improving animal welfare, product quality, and environmental sustainability.

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^{1}H-NMR proton nuclear magnetic resonance; **3HADH** 3-hydroxyacyl-CoA dehydrogenase; **AMPK** adenosine monophosphate AMP-activated protein kinase; **Arg:Lys** arginine to lysine; **avANT** avian adenine nucleotide translocator; **avTOR** avian target of rapamycin; **avUPC** avian uncoupling protein; **BW** body weight; **CART** cocaine- and amphetamine-regulated transcript; **COX** III cytochrome oxidase III; **CP** crude protein; **CPT**1 carnitine palmitoyl transferase1; **DFI** daily feed intake; **DWG** daily weight gain; **DWG** daily weight gain; **ETC** electron transport chain; **EU** European Union; **FAS** fatty acid synthase; **FCR** feed conversion ratio; **FE** feed efficiency; **FI** feed intake; **FPD** Foot pad dermatitis; **GH** growth hormone; **GLUT2** glucose transporter 2; **GLUT5** glucose transporter 5; **GO** Gene Ontology; **IGF** insulin-like growth factor; **IGFs/PI3K/Akt** insulin-like growth factor-I/phosphatidylinositol 3-kinase/protein kinase B; **iNOS** inducible nitric oxide synthase; **Jnk** c-Jun NH(2)-terminal protein kinase; **MAP** mitogen-activated protein; **MAP4K4** mitogen-activated protein kinase 4; **MOS** mannan-oligosaccharides; **MS** mass spectrometry; **mTOR** mechanistic target of rapamycin; **NES** normalized enrichment score; **NMR** nuclear magnetic resonance; **NO** nitric oxide; **NPY** neuropeptide Y; **NRC** National Research Council; **PGC-1α PPAR-γ** coactivator-1α; **PI3Ks** phosphatidylinositol-3-kinases; **PKA** protein kinase-A; **POMC** proopiomelanocortin neurons; **PPAR-γ** peroxisome proliferator-activated receptor-γ; **PRKAγ2** protein kinase AMP-activated non-catalytic subunit gamma 2; **QTL** Quantitative Trait Loci; **RAR-RXR** retinoic acid and retinoid X receptor; **RFI** residual feed intake; **RICTOR** rapamycin independent companion of target of rapamycin; **RNA-seq** RNA-sequencing; **ROS** reactive oxygen species; **rPCA** Robust principal component analysis; **RT-PCR** real-time quantitative reverse transcription polymerase chain reaction; **SGLT1** Na+-dependent glucose transporter 1; **SGLT5** Na+-dependent glucose transporter 5; **SM** spaghetti meat; **WB** woody breast; **WS** white striping.

1. INTRODUCTION

1.1. Productive efficiency in broiler chickens: overview of classical aspects and innovative approaches

According to the FAO estimates (FAO, 2009), world's population is going to reach 9.1 billion in 2050, 34% higher than today. In order to fulfill the food requirement of this growing population, global food production (net of food used for biofuels) must rise by about 70%, which means that annual cereal and meat production need to reach approximately 3 billion and 470 million tons, respectively (FAO, 2009). In addition, the limited availability of arable land, as well as climate changes and the increased utilization of agricultural commodities for bio-fuel production, will increase the pressure on the agriculture production system posing serious risks regarding food security and availability in the next future. Therefore, an appropriate and efficient use of the agricultural commodities is necessary to improve food production and sustainability, to reduce environmental impact and pollution, as well as to guarantee food supply, both from a qualitative and quantitative point of view, to an increasing world population (FAO, 2009).

Among livestock, broiler chickens showed the highest efficiency in converting feed into body weight (Willems et al., 2013) which is a fundamental aspect for maintaining a sustainable agriculture (Dridi et al., 2015). In addition, poultry meat is highly appreciated from different classes of consumers as indicated by the continuous increase in its consumption from 1970 to today (FAO, 2013), which is likely ascribable to its healthy profile (high protein, low fat, balanced n-6 to n-3 PUFA ratio, low levels of sodium and cholesterol), its relatively low price, and the absence of religious limitations related to its consumption (Petracci et al., 2015).

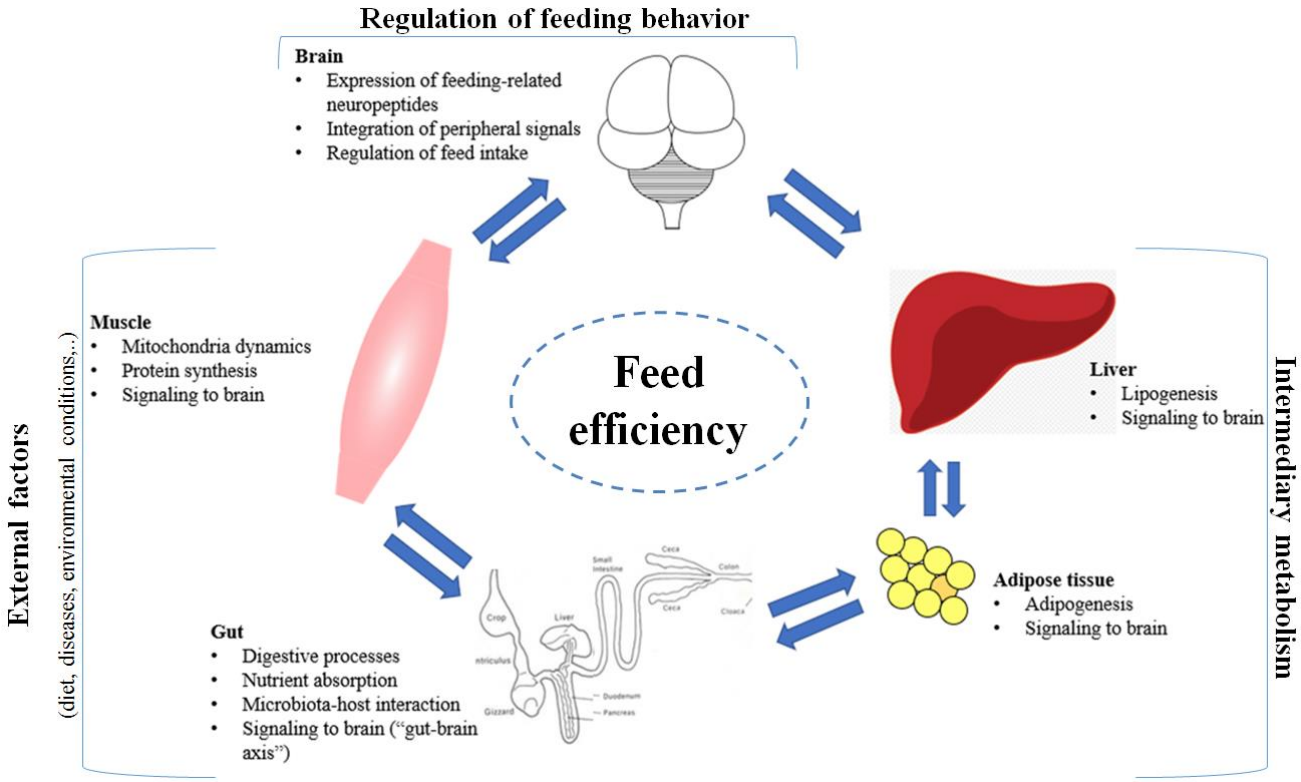
Feed efficiency (FE) could be roughly defined as the ability of an animal to convert feed into food outcomes (e.g., meat, eggs, milk, etc.) and therefore it represents the productive parameter which correlates inputs to outputs. Among the substantial and different challenges that the poultry industry is currently facing, improving FE probably represents the most important one since up to 70% of total production costs is given by feed. In broilers, FE represents the capacity of the animal

to convert feed into body mass. Animals considered to have a better FE typically show lower proportion of feed intake to body weight gain (Willems et al., 2013). FE could be assessed using different parameters such as feed conversion rate (FCR, also referred as gross efficiency), which represents the ratio between feed intake and body weight gain for a specific period of growth, or residual feed intake (RFI) (Koch et al., 1963), which is the variation between actual and expected feed intake of an animal based on the estimated requirement for its maintenance and growth/production (Aggrey et al., 2010). Therefore, the lowest FCR and RFI values, the more efficient the animal. Recently, other alternative measures of FE such as residual maintenance energy (Romero et al., 2009), residual gain, and residual intake and gain (Romero et al., 2009; Berry and Crowley, 2012) have been proposed. However, FCR and RFI are still the most used parameters to express FE in poultry (Dridi et al., 2015). It should be noted that FCR and RFI represent different strategies for the genetic improvement of FE, each one with its advantages and drawbacks. Indeed, FCR could be considered as a composite trait, including both feed intake and daily weight gain, while RFI is based only on the measurement of feed consumption. Considering practical aspects, both FCR and RFI require individual feed intake measurement, which is not easily assessable in field conditions unless using individual cages or automated electronic feeding systems (Willems et al., 2013). Estimated FCR and RFI heritability coefficients range from 0.47 to 0.51 and 0.45 to 0.46 during 28-35 d of bird age, and from 0.41 to 0.43 and 0.42 to 0.43 during 35-42 d, respectively (Aggrey et al., 2010), indicating a moderate heritability for FE parameters. Therefore, important results have been obtained through the application of selection programs aimed at improving FE in broilers (Siegel, 2014), resulting in a 50% reduction of FCR over the last 50 years (Zuidhof et al., 2014). Similarly, comparing breeding lines from 1957 and 1991, significantly improvements in FCR (from 3.00 to 2.04) were observed at both constant age and weight (Havenstein et al., 1994a; Emmerson, 1997). Nevertheless, a better knowledge of the nutritional and environmental requirements of broiler chickens significantly contributed to the improvements in FE as well. Overall, it was estimated that from 85 to 90% of the improvements in FE in broilers could

be related to genetics, while the remaining part to a better comprehension of their environmental and nutritional needs (Sherwood, 1977; Havenstein et al., 1994a,b, 2003a,b). However, Tallentire et al. (2018) recently reported that the artificial selection for efficiency in modern broiler chickens will inevitably face biological limits and animal welfare concerns. Based on their estimates, they predicted that the biological potential for genetic improvements is minimal compared to the progresses already obtained (Tallentire et al., 2018), suggesting that a more detailed comprehension of environmental factors, such as nutritional aspects, may play a much more important role for further improvements of FE in broiler chickens. In addition, the selection methods finalized at improving FE were mostly performed without considering the potential changes induced on the molecular mechanisms and on the physiological/metabolic features of broilers (Dridi et al., 2015). Consequently, alongside with the improvements in feed efficiency, undesirable changes have been observed in modern fast-growing broiler lines, such as an increased proneness to obesity and muscle disorders as well as a hyperphagic feeding behavior (Zampiga et al., 2018).

From a homeostatic perspective, FE could be considered as the net result among energy intake, which is determined by the voluntary feed intake and its complex regulatory mechanisms (Richards, 2003; Richards and Proszkowiec-Weglarz, 2007), and energy expenditure, which is affected by the maintenance metabolism, the rate of anabolic processes and the intermediary metabolisms in different tissues and organs (Zampiga et al., 2018). Several external factors, such as diet, diseases, and environmental conditions, are also involved in the phenotypic expression of FE (Zampiga et al., 2018). An overview of the factors affecting FE in broiler chickens is shown in Figure 1.1.

Figure 1.1. Overview of the factors affecting feed efficiency in broiler chickens.



In particular, diet can be considered one of the most important factors affecting the rate of intermediary metabolisms in animal tissues (muscle, adipose tissue, liver, etc.) which can influence overall FE in broilers. In addition, also the possible interaction between dietary treatments and animal's genetic background should be considered.

According to the scenario described by Tallentire et al. (2018), a much more detailed comprehension of the environmental and physiological factors involved in FE, both applying traditional and innovative approaches, appears necessary to continuously improving this trait as well as to promote the sustainability of the whole production system. This is currently possible by the development of several analytical techniques, identified as omics technologies, able to assess the global variation of genes, proteins and metabolites expression levels. These analytical platforms take advantage from the extraordinary results obtained by the animal genome sequencing in the past

decade (Zampiga et al., 2018). Indeed, whereas genomic information remains constant during the lifespan of an animal, gene products such as proteins and metabolites change their expression levels in a rapid and dynamic manner being regulated by a plethora of different environmental and physiological factors. Transcriptomics, proteomics and metabolomics are the main omics technologies currently used to investigate the expression profile of genes, proteins, and metabolites, respectively. Briefly, transcriptomic aims to identify the expression levels of genes in mRNA transcripts in response to different environmental stimuli or during specific pathophysiological conditions, as well as to identify genes underlying specific traits. Northern blotting, real-time quantitative reverse transcription PCR (RT-PCR), microarray and RNA-sequencing (RNA-seq) are the main analytical platforms currently applied in transcriptomics studies. However, it's known that the mRNA levels in a cell do not necessarily reflect those of the corresponding protein. Therefore, it might be useful to study the proteome, which is defined as the global set of proteins and all their post-translation modifications expressed in a cell/tissue/organ at a given time during specific conditions (Liebler, 2001; Twyman, 2013). Due to the wide differences in chemical and physical properties of proteins, and because no amplification method is provided for them, proteomic studies mainly rely on several chromatographic and electrophoretic methods to separate proteins (Picard et al., 2010), which can be subsequently identified using mass spectrometry (MS) combining soft-ionization techniques with different mass analyzers (Soares et al., 2012). Other analytical techniques available for proteins are nuclear magnetic resonance (NMR) approaches or immunological methods such as Western blot. Finally, metabolomics represents the quali-quantitative study of a wide range of small biological metabolites (Adamski and Suhre, 2013), either deriving from the genome expression (endogenous metabolites) or not (e.g., xenobiotic metabolites, such as environmental pollutants or drugs) (Junot et al., 2014). Usually, different biological samples can be analyzed through NMR or MS approaches in order to identify metabolites showing differential expression in relation to different conditions or stimuli (e.g., diseases or dietary treatments) or to discover biomarkers useful for discriminating animals or animal products with

different characteristics (Fontanesi, 2016). Although each of the above-mentioned analytical platforms provides very useful outputs, they are only able to describe "a part of the entire biological picture" if considered singularly. Liebler (2001) reported that "each protein, regardless its role and form, expresses a function that assumes significance only in the context of all the other functions and activities also being expressed in the same cell". Therefore, the next step is to integrate all the information obtained by the different omics platforms using appropriate bioinformatics and statistical tools. This relatively new approach, called system biology, provides a holistic and methodological overview of the entire biological system rather than its singular components alone (D'Alessandro and Zolla, 2013).

In this scenario, FE appears as a very complex and multifaceted trait regulated by intrinsic (e.g., physiological, metabolic, and immunological aspects of the animal) and extrinsic factors (e.g., dietary treatments, environmental conditions, or diseases) that must be taken into account in a holistic approach. Indeed, any nutritional treatment will inevitably integrate with the metabolic and physiological aspects of the animal and hence a detailed comprehension of the complex interactions between these aspects appears necessary. This approach, combining both traditional and innovative techniques, may allow a better understanding of the different aspects associated with productive efficiency in broiler chickens, which might be useful to improve the sustainability of the poultry industry as well as animal welfare, health and well-being, antibiotic consumption and environmental impact and pollution. In addition, the application of innovative analytical techniques will add important information about the molecular mechanisms in different tissues of broilers which may exert a major effect on FE.

1.2. Anatomic and physiological features of the chicken's gastrointestinal tract

In order to understand important aspects related to FE in broilers, it is fundamental to evaluate the anatomic and physiological features of the gastrointestinal tract of the chickens. Indeed, a wide range of enzymatic and biochemical reactions necessary for nutrient digestion and

absorption takes place in this organ. In addition, the gastrointestinal tract shows important immunological and regulatory mechanisms (which will be discussed in detail in the relative chapter). Therefore, the gastrointestinal tract could be considered one of the most important organs able to influence feed efficiency and growth performance in broilers and thereby a detailed evaluation of the physiological aspects occurring in this organ is necessary.

Digestive processes include all the chemical and physical mechanisms necessary to reduce the dietary macronutrients into simple compounds which can be easily absorbed by the intestinal mucosa. A brief overview of the chicken's enzymes involved in the digestion of dietary compounds is reported in the Table 1.2.

Table 1.2. Enzymes involved in the digestive processes in chickens (adapted from Denbow, 2015).

Secretion	Organ	Enzyme	Substrate	Product
Saliva	Mouth and crop	Alfa amylase	Starch	Dextrin
Gastric secretion	Proventriculus	Pepsinogen – Pepsin	Protein	Polypeptides
Pancreatic secretion	Pancreas	Procarboxypeptidase (A and B)	Di- and tripeptides	Amino acids
		Amylase	Starch	Oligosaccharides
		Chymotrypsinogen (A, B, and C)	Polypeptides	Oligopeptides
		Trypsinogen - Trypsin	Polypeptides	Oligopeptides
		Endopeptidases	Polypeptides	Oligopeptides
		Lipase	Triglycerides	Monoglycerides and fatty acids
Intestinal secretion	Small intestine	Maltase	Maltose	Glucose
		Isomaltase	Dextrins	Glucose
		Sucrase	Sucrose	Glucose
		Trypsin	Polypeptides	Oligopeptides
		Peptidases	Di- and tripeptides	Amino acids
		Amylase	Starch	Oligosaccharides
		Lipase	Triglycerides	Monoglycerides and fatty acids
	Gross intestine	Bacterial cellulases	Cellulose	Glucose

From an anatomical perspective, chickens present some peculiarities which differentiates them from mammals. As birds need to fly, their gastrointestinal tract is shorter than that of mammals (Denbow, 2015). However, the reduced length is compensated by the reverse peristalsis (also defined as gut reflux), a unique feature of birds which represents a backward movement of the ingesta in 3 different sections of the gastrointestinal tract (i.e., gastric flux, small intestine flux, and cloaca-cecal flux (Duke, 1986; 1994)) finalized at increasing the retention time and then to allow a proper digestion of nutrients (Ravindran et al., 2016). In addition, birds lack teeth and heavy jaw muscles while showing a bill or a beak which presents huge anatomical differences according to the feeding behavior of each specie (Denbow, 2015).

Although the upper part of the gastrointestinal tract of the chicken plays a modest role in nutrient digestion, it could be stated that the digestive processes start in the mouth. The main role of the mouth is to facilitate the movement of food to the esophagus, which is supported by the release of a watery secretion containing mucus from the salivary glands (Scanes and Pierzchala-Koziec, 2014). It has also been demonstrated that the salivary glands release alpha amylase, a starch digesting enzyme (Jerrett and Goodge, 1973; Rodeheaver and Wyatt, 1986) which may enhance starch digestion during transit and storage time of the ingesta in the crop and proventriculus (Scanes and Pierzchala-Koziec, 2014). However, Denbow (2015) reported that amylase is not present in the salivary secretion of *Gallus* and *Meleagris spp*.

The esophagus is thin-walled, distensible tube which main function is to carry the ingested food from the pharynx to the stomach by means of peristaltic contractions of its circular smooth muscles and mucus secretion (Scanes and Pierzchala- Koziec, 2014; Denbow, 2015). However, the role of the esophagus in nutrient digestion and absorption is very limited (Denbow, 2015).

The ingested food can be stored in the crop, a ventral diverticulum of the esophagus of chickens and other birds. Its primary role is not nutrient absorption but mainly food storage and fermentation. In addition, some immunological functions of the crop have been delineated (Scanes and Pierzchala-Koziec, 2014). Here, endogenous (mainly alpha amylase) and eventual exogenous

enzymes (Oluski et al., 2007), may start digestive processes of specific dietary compounds. Then, esophagus continues as thoracic esophagus and connects with the proventriculus (Denbow, 2015).

In birds, the stomach is essentially divided into 2 chambers: the proventriculus and the muscular gizzard. The proventriculus represents the avian counterpart of the glandular component of the mammalian stomach, which is constituted by deep gastric glands with lobules and multiple secretory tubules (Scanes and Pierzchala-Koziec, 2014). The main role of the proventriculus is to secrete the gastric acid which contains hydrochloric acid and pepsinogen. The latter, in presence of hydrochloric acid, is activated into pepsin which, in turn, initiates protein digestion (Scanes and Pierzchala-Koziec, 2014). Gastric acid secretion is stimulated by several neural transmitters and neuropeptides as elegantly reviewed by Scanes and Pierzchala-Koziec (2014). The gizzard is defined as the "muscular stomach" in which ingested food is subjected to mechanical processes able to physically reduce food particle size and dimension. These physical operations are determined by the contraction of the circular and concentric musculature (smooth muscle) of the gizzard (Scanes and Pierzchala-Koziec, 2014).

The gastrointestinal tract continues with the small intestine, which can be grossly divided into duodenum, jejunum, and ileum. In these parts, important processes regarding nutrient digestion and absorption take place. Several digestive enzymes, such as sucrase, isomaltase, amino-peptidase (Uni et al., 2003), lipase, amylase, maltase (Jamroz, 2005) and trypsin (Noy and Sklan, 1995), are produced in the small intestine of birds. At the duodenal level, the pancreatic secretion, mainly constituted by digestive enzymes and zymogens such as amylase, chymotrypsinogen, procarboxypeptidase (Marchaim and Kulka, 1967), lipase, and members of the cationic and anionic trypsin sub-families (Wang et al., 1995), is added to the digesta. Moreover, duodenum receives the hepato-enteric and the cystic-enteric duct, both carrying bile to the small intestine from the left lobe of the liver and the gall bladder, respectively (Scanes and Pierzchala-Koziec, 2014). Bile contains bile acids (mainly dihydroxycholanic and trihydroxycholanic acid) which are essential for lipid

digestion acting as emulsifier, as well as bile pigments, amylase, and proteins as immunoglobulins A (Scanes and Pierzchala- Koziec, 2014).

The distal part of the intestine, defined as gross intestine, could be roughly divided in ceca, colon, and cloaca, with the latter representing a common pathway for the excretory, reproductive and digestive systems (Denbow, 2015). Ceca and colon present a relevant number of flat villi and relatively few goblet cells (Denbow, 2015). The contribution of colon and cloaca to the digestive processes is very limited, although the former seems to be involved in water and electrolytes reabsorption (Scanes and Pierzchala- Koziec, 2014). Ceca are two finger-like blind-ending sacs originating from the ileum-colon junction which play a much more important role in chicken metabolism (Scanes and Pierzchala- Koziec, 2014). Indeed, specific peristaltic movements either from the ileum or by retrograde transport from the colon push digesta in the ceca where undigested nutrients can be fermented, and water absorbed. These fermentative processes are carried out by the bacterial communities colonizing the ceca, which shown the highest microbial diversity and abundance in comparison to the other sections of the gastrointestinal tract (Gong et al., 2007). Ceca microbes can ferment cellulose and complex carbohydrates which have not been enzymatically digested in the previous parts of the gastrointestinal tract producing volatile fatty acids (Gong et al., 2007).

The small intestinal is also the main site for nutrient absorption (Leeson and Summers, 2001). Indeed, the mucosa layer of the small intestine is characterized by the presence of villi and glandular crypts which can extend the absorptive surface. Essentially, all the glucose obtained from starch digestion can be absorbed there using both active and passive mechanisms (Denbow, 2015). Glucose is transported into the enterocytes using the Na+-dependent glucose transporters, SGLT1 and SGLT5. Similarly, fructose absorption is mediated by the apical GLUT5-type cells (Garriga et al., 2004). Within the epithelial cells, glucose and fructose are transported into the interstitial space by the basolateral GLUT-2 transporter (Denbow, 2015). In addition, an important role in glucose absorption is played by the ceca (Savory and Mitchell, 1991).

Dietary proteins are enzymatically broken down into amino acids, dipeptides and tripeptides, and then absorbed through the brush border membrane of the intestinal epithelial cells. Amino acids can be absorbed passively as free amino acids or through a wide number of amino acid transporters with different substrate specificity. According to Denbow (2015), amino acids transporters can be classified in 4 groups: for neutral amino acids, for basic amino acids, for acidic amino acids, and for proline, β-alanine, and related amino acids. On the other hand, amino acids can be absorbed in the form of di- and tri-peptides either by the peptide transporter PepT1, via the paracellular route in case of increased tight-junctions permeability, or by cell-penetrating peptides which are able of carrying cargo across the plasma membrane (Gilbert et al., 2008). Hurwitz et al. (1972) reported that jejunum could be considered as the primary site for the absorption of di- and tri-peptides, even though more recent studies have shown discordant results as stated by Denbow (2015). In addition, amino acids absorption can be relevant in the ceca (Denbow, 2015).

Fats digestion and absorption are relatively complex processes. Lipids are water-insoluble compounds and their digestion is due to the synergic action of bile salts and pancreatic lipase. Since lipids digestion takes place in the aqueous intestinal environment, bile salts ensure the emulsification of dietary fats allowing the pancreatic lipase to hydrolyse the triglycerides present on the water–oil interface, with the production of 2-monoglycerides and free fatty acids (Leeson and Summers 2001). Although it has no enzymatic function, co-lipase is necessary to initiates the activity of pancreatic lipase (Borgstrom and Erlanson, 1971). Once hydrolyzed, short-chain fatty acids and monoglycerides are passively absorbed by the intestinal mucosa (Pond et al., 2005). However, medium- and long-chain saturated fatty acids, diglycerides, fat soluble vitamins and cholesteryl esters need a further solubilisation in the hydrophobic core of mixed micelles prior to be absorbed (Davenport, 1980). In these micelles, hydrophobic molecules are kept in the inner part, while the hydrophilic ones face the aqueous intestinal environment (Ravindran et al., 2016). Bile salts, which are amphiphilic molecules, also play a key role in the formation of these mixed micelles (Krogdahl, 1985). Tancharoenrat et al. (2014) showed that fatty acids digestion and

absorption occur at different rate throughout the small intestine. These processes mainly take place in the jejunum (Hurwitz et al., 1973; Tancharoenrat et al., 2014) and, to a lesser extent, in the ileum (Tancharoenrat et al., 2014) while the contribution of the hindgut is negligible (Renner, 1965). Within the enterocytes, monoglycerides and long-chain fatty acids are re-esterified and together with cholesterol, lipoproteins, and phospholipids form chylomicrons which are directly released in the portal circulation as the poultry lymphatic system is poorly developed (Hermier, 1997). All these aspects related to the digestive physiology of the chicken should be considered during poultry feed formulation and diet manufacturing, whose basic concepts will be discussed in the next chapter.

1.3. Basic concepts of feed formulation

Nutrition has the primary aim of providing nutrients to meet the requirements for a proper growth as well as to maintain physiological and immunological functions. At the same time, nutrition plays a central role in preserving gut health and functionality, which are strictly associated with animal health, welfare and productivity, as well as to guarantee food safety and meat quality. As previously stated, nutrition can be likely considered one of the most important environmental factors affecting energy homeostasis and hence FE. Therefore, understanding important aspects related to poultry nutrition is fundamental to improve production efficiency in broilers.

Modern fast-growing broiler chickens can express their extraordinary growth potential as long as their nutritional and environmental requirements are guarantee. Formulation is a mathematical exercise of setting up a blend of ingredients to meet the nutritional requirements of the birds (Leeson and Summers, 2001). Therefore, according to Lesson and Summers (2001), the necessary steps for feed formulation include to:

- Establish which nutrients are essential for the bird
- Set requirement values for these nutrients using quantifiable units
- Identify which ingredients are available in the market

- Quantify inclusion levels and costs of each ingredient
- Find the mathematical blend of ingredients able to meet the nutritional requirements previously established.

According to these criteria, nutritionists should define a pool of raw materials able to fulfill the nutritional requirements of the birds at the minimum cost of formulation. Inadequately formulated diets can have a tremendous impact on broiler growth and FE, but also on animal health and welfare, carcass and meat quality, as well on feeding costs and environmental pollution.

The knowledge regarding nutritional requirements of broilers has been subjected to extraordinary improvements over the past 50 years. In current commercial practice, broilers usually receive from 3 to 5 types of feeds which shown different physical form (mash vs. crumble vs. pellet) and chemical composition according to the age of the birds and to the established slaughter weight (National Research Council, 1994; Aviagen, 2014; Cobb-Vantress, 2018). This practice is known as "phase-feeding" and it has been developed to fit the diet according to the requirements of these animals which change dramatically during their lifespan. In general, 3 different feeding phases, defined as "starter", "grower" and "finisher", are always provided. However, when male broilers are raised till 55-60 d of age, another finisher feed could be included (Schiavone et al., 2008). Regarding the physical form, starter diets are usually administered in a mash or crumble form while grower and finisher ones are pelleted (d = 2-3 mm) (Aviagen, 2014; Cobb-Vantress, 2018). Breeding companies provide nutritional specifications which can be used, alongside with the recommendations defined by the National Research Council (NRC, 1994), as a guide for a proper formulation. Considering the main aspects of the diet composition, the apparent metabolizable energy (AME) content tends to increase from starter to finisher phase (approximately from 3,000 to 3,200 Kcal/kg) while crude protein content follows an opposite trend (from 22-23% in starter to 17-18% in finisher). Calcium and available phosphorous are present in the diet at ratio of 2:1, with calcium concentration ranging from 0.95-1.00% in starter and 0.70-0.75% in finisher phase (NRC, 1994; Aviagen, 2014; Cobb-Vantress, 2016). The chicken's requirements of calcium and

phosphorous are usually covered by supplementing the diet with limestone, dicalcium phosphate, and calcium carbonate. In addition, vitamin and trace minerals are usually provided in form of concentrated premix to cover the bird's requirements for these nutrients while sodium needs are usually met by adding salt (sodium chloride) to the diet (Leeson and Summers, 2001).

The choice of the raw materials to be included in the diet depends upon different aspects. First, ingredients should not be avoided by the legislation in force (e.g. animal proteins and antibiotics as growth promoters in Europe) and their consumption should not represent a threaten for animal health and thereby product safety (e.g. not contaminated with exogenous substances such as toxins). Then, availability and price are important aspects that need to be carefully considered during feed formulation. Consequently, according to the geographical area in which the diet is formulated and manufactured, different blends of raw materials can be used.

Raw materials can be broadly classified as:

- Energy sources
- Protein sources
- Feed additives

1.3.1. Energy sources

1.3.1.1. Cereals

Corn (*Zea mays*) is the main energy source in poultry diets and it is highly available in most of the geographical areas of the world. Its energy content (AME = 3,200-3,300 kcal/kg) is higher if compared to other cereals, while protein content is modest (from 8 to 11% with poor biological value). In particular, corn is poor of some essential amino acids. Different varieties of corn are currently available for poultry producers: yellow corn, white corn, high-lysine corn, and high-oil corn (6-8% oil). In commercial practices, the use of the latter two varieties is not very common and strictly depends on their availability and cost. Finally, particular attention should be given to field

practices and stocking conditions as corn is rather prone to be contaminated by mycotoxins, such as aflatoxins and zearalenone, or molds (Leeson and Summers, 2001).

Sorghum (*Sorghum spp.*) can be considered a valid alternative for corn in poultry diets (Leeson and Summers, 2001) presenting a similar energy content coupled with a slightly higher protein level (9.5%). Furthermore, depending on the geographical area, the price of sorghum could be lower than corn, making its dietary inclusion advantageous from an economic point of view. The major concern regarding the use of sorghum in poultry diets is represented by its relatively high content of tannins, anti-nutritional compounds which can limit feed intake and protein digestibility (around 10%) (Leeson and Summers, 2001). However, different varieties of sorghum with a low tannin content (i.e. white sorghum) are currently available for poultry feed formulation. In addition, starch and protein fractions are intimately connected, potentially leading to a further reduction of their digestibility especially when sorghum is not subjected to heat processing. Finally, sorghum is characterized by a poor content of carotenes and xanthophylls which may impact on the visual appearance of poultry products (Leeson and Summers, 2001).

Wheat (*Tritiucm aestivum*) can be used for poultry feed formulation when economically feasible. Indeed, most of the available wheat is usually intended for human consumption and hence its market price could be quite high. Its energy content is slightly lower than corn while protein level is higher (10-13% according to the variety). Wheat also has a relatively high concentration of non-starch polysaccharides, such as arabinoxilans and pentosans, which have a detrimental effect on productive performance mainly by increasing digesta viscosity and limiting nutrient digestibility (Choct and Annison, 1992). For this reason, the inclusion rate of wheat into diets for growing broilers should not exceed 30% (Leeson and Summers, 2001). However, the dietary supplementation of exogenous enzymes (i.e. xylanase) can partially overcome this limitation (Annison and Choct, 1991; Bedford, 1995; Choct, 2006). Finally, a limited amount of wheat could be useful for improving pellet durability. Wheat by-products result from flour manufacturing, in which wheat passes through a series of grinders of decreasing sieve size producing middlings

(Leeson and Summers, 2001). Wheat bran is obtained from the outer seed coat and hence its fiber content is markedly higher which make its use in poultry feeding, apart from specific applications, quite limited. Wheat bran also shows low bulk density and low metabolizable energy content (Leeson and Summers, 2001).

Barley (*Hordeum sativum*) contains approximately 75% of the energy content of corn while fiber content is 3 times higher (Schiavone, 2008). Barley also has a remarkably high concentration of complex carbohydrates such as β-glucans. Therefore, its utilization in poultry feeding is less indicated than corn, even though the dietary use of β-glucanase can represent a strategy to limit some of the negative aspects reported above (Bedford, 1995; Choct, 2006).

Other cereals such as oats, rye, triticale, and rice can be used for poultry feed manufacturing. Although each of them has interesting peculiarities, it could be stated that their actual use is very limited. For instance, the use of oats in poultry diets is extremely unusual due to its elevated fiber content even though the biological value of its protein is rather high. Oats could be used to limit the energy content of the diet, which represents an important aspect for laying hens and breeders. Similarly, rye utilization is very limited due to the high concentration of pentosans which increase digesta viscosity and the production of sticky feces (Schiavone, 2008). Finally, the main limitation related to the use of triticale and rice is represented by their price, which is usually markedly higher in most of the markets.

1.3.1.2. Fats and oils

Fats and oils are the feed ingredients with the highest energy density, at least twice than carbohydrates and proteins (NRC, 1994). Therefore, even moderate inclusions of these compounds can induce significant changes in the energy content of the diet (Leeson and Summers, 2001). Different fats and oils are currently used in feed formulation to meet the high energy demand of modern fast-growing broiler genotypes. In addition, fats and oils can improve feed palatability and absorption of lipophilic vitamins and carotenoids, as well as reducing dustiness and aiding the

lubrication of feed mill equipment (Ravindran et al., 2016). Ravindran et al. (2016) reported that, despite their wide use, feed grade fats represent the least understood feed ingredient.

From a chemical point of view, triacylglycerols or triglycerides are the major components of lipids. Triglycerides are constituted by a molecule of glycerol esterified with three fatty acids, which degree of unsaturation significantly influence the physical appearance of the lipid source. Fats, mainly animal by-products, are characterized by a high amount of saturated fatty acids and hence its melting point is usually higher than 40°C. On the contrary, a higher percentage of unsaturated fatty acids can be found in vegetable oils and therefore they usually are in a liquid form (melting point < 0°C) (Schiavone, 2008).Therefore, according to these differences, the term "fats" is generally used for lipids showing a solid aspect at room temperature, while "oils" is used for those presenting in a liquid form (Enser, 1984). Furthermore, due to the different fatty acids composition, oils are more prone to oxidative and rancidity processes compared to fats. Another concern related to use of fats in poultry nutrition is represented by the level of free fatty acids, as they can be more subjected to peroxidation processes. Particularly, free fatty acids represent a huge problem if fat is predominately saturated and administered to young birds (Leeson and Summers, 2001).

In commercial practice, a broad range of fats and oils is available for the poultry feed industry including vegetable oils (e.g. soybean, corn, and palm oil), rendering and processing by-products (e.g. beef tallow, lard, poultry fat), acidulated soapstocks (i.e. by-products of vegetable oil refining), restaurant greases (e.g. recovered frying oils, also reported as yellow grease), and hydrogenated fats (i.e. fats and oils converted into saturated fatty acids by the addition of hydrogen atoms). These products usually show a very high variability in terms of composition and the cost is one of the main drivers for their utilization (Ravindran et al., 2016). Being no legislative aspects to comply with, the use of animal or vegetable fats completely depends upon the company choice. Currently, the use of vegetable oils is much more frequent than that of animal fats mainly to accomplish the consumer's willingness to buy products obtained by animals fed only-vegetable

diets. Despite this, tallow and poultry fat are still used although their dietary administration in young birds is not recommended as these animals have a poor ability to digest saturated fatty acids. Indeed, Leeson and Summers (2001) suggested that the use of pure tallow in chicken diets must be severely restricted until 15-17 d of life.

1.3.2. Protein sources and amino acid

In poultry feed formulation, protein sources represent the second greater component after energy-yielding raw materials and probably the most expensive one (Beski et al., 2015). From a biochemical perspective, proteins can be considered as polymers of α-amino acids linked together by means of peptide bonds (Beski et al., 2015). Important aspects to consider regarding protein sources are the capacity to supply adequate amounts of essential amino acids, the digestibility and biological value of the proteins, as well as the level of anti-nutritional compounds (Scanes et al., 2004). Protein sources can be roughly divided in vegetable and animal protein sources with the former covering most of the dietary protein requirements in poultry (Beski et al., 2015).

Vegetable protein sources are usually obtained from seeds by extracting the oil fraction using either mechanical or chemical methods. According to these two methods, expellers or meal can be respectively obtained (Schiavone, 2008). Soybean meal represents the most important protein source in poultry feed manufacturing and has become the worldwide standard against the other protein sources are compared (Leeson and Summers, 2001). Its protein content ranges from 43-44% to 48-50%, depending on the quantity of hulls removed and oil extracted (Beski et al., 2015). During processing, soybean is dehulled (approximately 4% by weight), cracked, and conditioned at 70°C. The hot cracked beans are then flaked (about 0.25 mm) to facilitate oil extraction by means of a solvent, usually hexane, which should be subsequently removed from the meal. Despite the high cost, soybean meal is preferred than the other protein sources due to its well-balanced amino acid profile, with special regard to some essential ones (e.g. lysine and tryptophan), which enables it to balance most cereal-based diets (Ravindran, 2013). As most of the plant protein

sources, soybean contains different anti-nutritional factors such as the trypsin inhibitor and hemagglutinins (lectins). However, these compounds are inactivated by the heat treatment applied during soybean processing (Leeson and Summers, 2001). In general, besides this aspect, the thermal process also has the purpose of increasing the nutritional value of plant proteins (Beski et al., 2015).

Other important vegetable protein sources available for the poultry industry are canola meal (high quality feed-stuff for poultry, genetic selection has reduced goitrogens and uric acid content), corn gluten (CP = 40-60%, poor in lysine but rich in carotenoids and xanthophyll), sunflower meal (CP = 28 to 45%, depending on the quantity of hulls removed), flax meal (high cost, source of ω-3 fatty acids), and alfalfa meal (CP = 18-20%, mainly used for organic production as source of xanthophyll). Although all these products have a high protein content, the presence of anti-nutritional and toxic factors, as well as their unbalanced amino acid profile, strongly limit their use in poultry feeding. Indeed, vegetable proteins are generally unbalanced or poor of specific essential and non-essential amino acids (Siegert et al., 2016). This reduces their biological value as they are not able to provide the limiting amino acids required for a proper growth of broiler chickens (Beski et al., 2015).

Animal protein sources are mainly represented by animal by-products, defined as parts of slaughtered animals which do not directly contribute to human nutrition (Hazarika, 1994). These protein sources are usually obtained from rendering and slaughtering operations as well as from meat, milk, and fish processing and packaging (Denton et al., 2005). In general, animal by-products are characterized by a high protein content (up to 85-90% of CP on a dry basis), good digestibility and balanced amino acid profile while had no fiber or anti-nutritional factors (Konwar and Barman, 2005). Blood, fish, meat, and bone meals, as well as spray-dried plasma and hydrolyzed feathers meal, can be likely considered the most important animal protein sources (Beski et al., 2015). In Europe, the use of animal protein sources for livestock nutrition has been abolished with the only exception of fish meal administration to non-ruminant animals (European Commission, 2001). In addition, the increasing interest of consumers towards products obtained from animals fed only-

vegetable diets has dampened the use of animal proteins even in some extra-EU countries (Schiavone, 2008).

As previously reported, some vegetable protein sources are generally not well-balanced and poor of specific essential amino acids. This limitation forces nutritionists to include animal proteins or synthetic amino acids in poultry feed to meet the animal's requirements of specific amino acids (Beski et al., 2015). Indeed, animal proteins usually show a more balanced amino acid profile for maximizing body growth and development. On the other hand, synthetic amino acids can directly cover the lack of specific amino acids in the diet. The dietary supplementation of synthetic amino acids represents the only available solution when the use of animal protein sources is not allowed by the legislation in force (i.e. European Union). A detailed description of the use of synthetic amino acids will be provided in the specific chapters.

1.3.3. Feed additives

The European Union's ban of antibiotics as growth promoters have strengthened the interest towards alternative solutions that might provide beneficial effects on productive performance and health status of livestock. Furthermore, the increasing public awareness towards antibiotic-resistant bacteria, as well as the consumers demand for animal products from antibiotic-free production systems, have determined a significant reduction in the use of antibiotics as growth promoters even in extra-EU countries, such as the USA (Van Boeckel et al., 2015). However, the growing demand for meat products in middle-income countries, and the consequent shift from small-scale to large-scale farms, could strengthened the use of antimicrobials in developing countries (Van Boeckel et al., 2015).

The beneficial effect of sub-therapeutic dosages of antibiotics on productive aspects was mainly mediated by a positive modulation of the gut microflora, which enhances a better exploitation of the dietary nutrients while avoiding harmful bacteria development and subclinical infections (Dibner and Richards, 2005). According to the EU Regulation 1831/2003, feed additives

can be defined as substances, micro-organisms, or preparations other than feed materials and premixtures, intentionally added to the feed or water to favorably affect the characteristics of feed, animal products, as well as animal production, performance and welfare (particularly by affecting the gastrointestinal microflora), digestibility of feeding-stuffs, and the environmental consequences of animal production. Furthermore, feed additives can be considered compounds able to satisfy the nutritional needs of animals, to have a coccidiostatic or histomonostatic effect, or to be able to positively affect the colour of ornamental fishes and birds. Therefore, feed additives can be classified as:

- *Technological additives*:

  any substance added to the feed for a technological purpose, such as preservatives, emulsifiers, antioxidants, binders, etc.

- *Sensory additives*:

  any substance, such as colorants and flavouring compounds, the addition of which improves or changes the organoleptic properties of the feed, or the visual characteristics of the food derived from animals.

- *Nutritional additives*:

  E.g. vitamins, pro-vitamins, and chemically well-defined substances having similar effect; trace elements; amino acids, their salts and analogues; urea and its derivatives.

- *Zootechnical additives*:

  any additive used to favorably affect the performance of animals in good health or the environmental impact of animal productions (e.g. digestibility enhancers, gut flora stabilizers, others).

- *Coccidiostats and histomonostats*.

1.3.3.1. Focus on the dietary utilization of a lysophospholipids-based emulsifier in broiler chickens

An experiment aimed evaluating productive performance, nutrient digestibility, and carcass quality traits of broiler chickens fed diets supplemented with a lysophospholipids-based exogenous emulsifier was carried out and the manuscript written during the PhD timeframe (Zampiga et al., 2016). Given the considerable amount of lipids in current commercial broiler diets, the use of exogenous emulsifiers may support bile salts in both emulsion and micelle formation process, determining a potential positive effect on lipids digestibility and overall productive performance. Lysophospholipids are mono-acyl derivatives of phospholipids (obtained by enzymatic conversion of soy lecithin) resulting from the action of phospholipase A1 or A2, which hydrolyse the ester bond at sn-1 and sn-2 position respectively (Joshi et al., 2006). Presenting a single fatty acid, these compounds are characterized by a higher hydrophilic–lipophilic balance and thus a better oil–water emulsification capacity than the corresponding phospholipids (Schwarzer & Adams, 1996). Lysophospholipids show a lower critical micelle concentration than bile salts and lecithin (Zubay, 1983) and form smaller micelles compared to phospholipids (Mine et al. 1993). On the other hand, lysophospholipids are mentioned to improve gut permeability to macromolecules like proteins and dextrans (Tagesson et al., 1985), to regulate the activity of several enzymes (Shier et al. 1976; Tagesson et al. 1985), to influence the formation of protein channels (Lundbaek & Andersen, 1994) and to induces epithelial cells hypertrophy in broiler duodenum (Khonyoung et al. 2015). 1,755 one-day-old male Ross 308 chicks were randomly divided into three experimental groups of 9 replications each: control group (CON) fed a corn–soybean basal diet, and two groups fed CON diet supplemented with constant (1 kg/ton) or variable (1–1.5 kg/ton) level of emulsifier (CONST and VARI, respectively). At slaughter (42 d), birds receiving the emulsifier had a statistically significant ($P<0.05$) lower feed conversion rate compared to the control. Body weight and daily weight gain were only slightly influenced by lysophospholipids supplementation, while mortality and feed intake resulted similar among the groups. No statistically significant effect of the emulsifier was

observed on nutrient digestibility as well as slaughtering yields, skin pigmentation, and incidence of foot pad dermatitis. The use of two different doses of emulsifier led to the same results for all the considered productive parameters and for nutrient digestibility indicating that the lower dose could be the more suitable solution for feed formulation. Therefore, from the results obtained in this experiment, the use of a lysophospholipids-based emulsifier could represent a potential solution to improve feed efficiency in broiler chicken (Zampiga et al., 2016).

1.4. Importance of amino acid nutrition in broiler chickens

1.4.1. General aspects

Protein represents one of the greatest costs in poultry feed manufacturing. Therefore, maximizing the efficiency of protein and amino acid utilization is of vital importance for the poultry producers. Amino acids also play an extraordinary role in physiological and metabolic aspects of the animal as elegantly reviewed by Wu (2014). Beyond their role as building blocks for protein, they can regulate gene expression, cell signaling, food intake and nutrient metabolism, as well as antioxidant mechanisms and hormone synthesis (Wu, 2014). In addition, an inefficient utilization of dietary protein may increase the amount of nitrogen excreted with the feces with a tremendous negative impact on the environmental sustainability of the poultry industry (Nahm, 2002; Ritz et al., 2004). Therefore, it is strictly necessary to formulate diets with amino acids levels able to guarantee a proper animal growth, health, and well-being, maximizing lean deposition with minimal nitrogen excess and excretion in order to limit environmental impact and pollution (Han and Lee, 2000).

Traditionally, amino acids can be classified as nutritionally essential or nonessential. Nutritionally essential amino acids are those whose carbon skeleton cannot be synthesized de novo or those amino acid which can be synthesized but not in a sufficient amount to fulfill the animal requirement (Wu, 2014). Therefore, essential amino acids must be supplied with the diet to sustain proper growth and physiological functions (Wu, 2014). Arginine, cysteine, glycine, histidine,

isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, and valine are considered essential amino acids for poultry (Leeson and Summers, 2001).

As previously stated, the lack of specific amino acids in vegetable protein sources can be overcome by the use of synthetic crystalline amino acids, whose dietary inclusion allows to fulfill the requirement of limiting amino acids while achieving an optimum balance among them. Providing the dietary amino acids in a pattern to match the requirements of an animal has known as the "ideal protein concept". This concept began to develop when Mitchell (1964) discussed the idea of an "ideal protein" or a "perfect amino acid balance" considering that the chemical score (Mitchell and Block, 1946), which use the egg protein as ideal standard, showed not to be so accurate due to the high concentration of certain amino acids in the egg protein. In chickens, researches in this field were underpinned in the late 50s when Scott's group at the University of Illinois started developing a purified crystalline amino acid diet for chicks (Glista et al., 1951; Klain et al., 1960) and designed an ideal pattern of dietary essential amino acids according to the amino acid composition of the chick's carcass (Fisher and Scott, 1954). Based on the extensive researches conducted in 60s and 70s, several versions of the "chick amino acid requirement standard" for the first three weeks of life were defined: the Dean and Scott Standard (Dean and Scott, 1965), the Huston and Scott Reference Standard (Huston and Scott, 1968), the modified Sasse and Baker Reference Standard (Sasse and Baker, 1973), and finally the Baker and Han's Ideal Chick Protein (Baker and Han, 1994). In 1994, the National Research Council (NRC, 1994) defined their own nutritional requirements for broilers and a few years later Baker updated the ideal protein for chickens from 0 to 56 d of life (Baker, 1997). Recently, taking advantage from the important knowledge regarding nutritional and metabolic aspects of the chickens, Wu (2014) proposed the Texas A&M University's optimal ratios of digestible amino acids in chicken diet which also include recommended levels for non-essential amino acids. Additional information regarding recommended dietary concentrations of amino acids are also reported in the nutrition specifications provided by the breeding companies (Aviagen, 2014; Cobb-Vantress, 2018).

The dietary supplementation of synthetic amino acids may allow the reduction of the crude protein levels in the diet (i.e. "protein sparing" effect) without compromising animal performance while having positive outcomes on feeding costs, nutrient digestibility, and nitrogen excretion (Han and Lee, 2000). On the other hand, imbalances among dietary amino acids showing chemical or structural similarity could cause amino acids antagonism, resulting in impaired growth rate and feed intake as well as abnormal behavior (Wu, 2014). In corn-soybean diet, which represents the worldwide standard for broilers, the order of limiting amino acids was reported to be 1) methionine, 2) threonine, 3) lysine, 4) valine, 5) arginine and 6) tryptophan (Fernandez et al., 1994). These amino acids are usually included in commercial diets in form of synthetic crystalline amino acids obtained by bacterial fermentation. At the beginning of new century, Han and Lee (2000) reported that lysine-HCl and DL-methionine were widely used by the poultry industry, while other crystalline amino acids (L-threonine and L-tryptophan) just started to be available for the producers. Nowadays, taking advantage from the important progresses in fermentation technologies, several crystalline amino acids are available in the market at a reasonable price. Among them, crystalline L-arginine has been recently approved as feed additive in Europe (European Food Safety Authority, 2016; 2017; 2018) resulting in a sensible reduction of its price. Beyond this aspect, arginine is receiving increasing attention due to its important roles in different metabolic, pathophysiological and immunological aspects of poultry which will be discussed in detail the next chapter.

1.4.2. Arginine: an underestimated essential amino acid?

Arginine is a basic amino acid and it is considered essential for chickens as they lack a functional urea cycle (Fouad et al., 2012). Indeed, despite to mammals which can use the enzymes of the urea cycle to synthesis L-arginine from ornithine, ammonia, and the amino-nitrogen of aspartate (Khajali and Wideman, 2010), chickens (uricotelic organisms) lack an important enzyme such as phosphate synthase I (EC 6.3.5.5.) and have a limited activity of hepatic arginase (EC 3.5.3.1., 2) and ornithine transcarbamoylase (OTC, EC 4.4.4.17). Therefore, a proper amount of

arginine should be provided with the diet. Among livestock, chickens showed the highest requirement of arginine (Ball et al., 2007). Dietary arginine is absorbed in the small intestine of chickens through sodium-dependent and –independent mechanisms, with the latter showing a greater effectiveness (Brake and Balnave, 1995; Rueda et al., 2003). As most of the arginase activity is located in the kidney (Tamir and Ratner, 1963), a substantial amount of dietary arginine may pass through the brush border and then enter the blood circulation with only limited degradation (Wu et al., 2014).

Arginine is involved in complex metabolic, immunological and physiological aspects of chickens (Khajali and Wideman, 2010; Fouad et al., 2012) being a substrate for the biosynthesis of several molecules including nitric oxide, creatine, ornithine, proline, glutamine, agmatine, glutamate, and polyamines (Fouad et al., 2012). In addition, arginine is thought to act as secretagogue involved in growth hormone (GH) and insulin-like growth factor (IGF) secretion (Fernandes and Murakami, 2010).

Arginine is also involved in immunological aspects of poultry, either directly or indirectly through its metabolites. In particular, most of these effects seem to be mediated by nitric oxide (NO), a free radical molecule obtained by the enzymatic conversion of arginine into citrulline (Fernandes and Murakami, 2010, Khajali and Wideman, 2010). Activated macrophages produce high quantity of NO and reactive oxygen species such as superoxide. In turn, these molecules promote the formation of reactive nitrogen species (e.g. nitrite, nitrate, peroxynitrite) that exert cytostatic and cytotoxic effects to specific cells and pathogens (Tomas-Cobos et al., 2008). Several studies showed that L-arginine might represent a limiting substrate for the production of NO by avian macrophages (Taylor et al., 1992; Wideman et al., 1995; Kidd et al., 2001, Ruiz-Feria et al., 2001; Villamor et al., 2002). Furthermore, positive effects of arginine have been reported on the weight of lymphoid organs (Kwak et al., 1999), percentage and absolute number of heterophils and heterophil/lymphocyte ratios in blood (Lee et al., 2002), percentage of CD8+ and CD3+ cells (Abdukalykova et al., 2008), and primary antibodies levels (Deng et al., 2005). Recently, beneficial

effects of arginine have been reported on the intestinal mucosal disruption in coccidiosis-challenged chickens (Tan et al., 2014a), on the reduction of systemic inflammation and the overexpression of pro-inflammatory cytokines in chickens challenged with lipopolysaccharide (Tan et al., 2014b), as well as in alleviating the immunosuppression caused by the infectious bursal disease vaccine (Tan et al., 2015).

The pivotal role of arginine and its metabolite NO in attenuating the onset of pulmonary hypertension syndrome (ascites syndrome) was firstly reported by Wideman et al. (1995). Indeed, the dietary administration of high dosages of arginine can increase plasma concentration of NO, reduce pulmonary arteriole muscularization and facilitate flow-dependent vasodilation, resulting in a lower mortality associated with ascites in broilers (Wideman et al., 1995; 1996; Tan et al., 2005; 2006). The effects of the dietary supplementation of arginine on productive aspects of broiler chickens are somehow inconsistent and discordant. This discrepancy could be likely due to the huge variability in bird's genotype, age, and sex, composition of the basal diet, number and length of feeding phases and inclusion rate of arginine in published studies. The results reported in literature will be discussed in detail in the discussion section of the RESEARCH WORK #1.

1.4.3. Antagonism between dietary arginine and lysine: classical aspects and current perspectives

The nutritional antagonism between dietary arginine and lysine was firstly studied and reported in the 50's and 60's (Anderson and Combs, 1952; Anderson and Dobson, 1959; Snetsinger and Scott, 1961; Jones, 1964; O'Dell and Savage, 1966; Boorman and Fisher, 1966; Dean and Scott, 1968). These works mainly evaluated the effects of the administration of ingredients with a high concentration of lysine [e.g. casein, arginine to lysine (Arg:Lys) ratio = 0.45] or the dietary supplementation of lysine-HCl (Balnave and Brake, 2002). The existence of a specific relationship between dietary lysine and arginine was clearly demonstrated by D'Mello and Lewis (1970). In this work, the Authors observed that the growth depression induced by the addition of lysine in diets

limiting in either methionine, histidine, tryptophan, or threonine could be reversed by supplementing the diets with arginine and not with the originally limiting amino acid. Based on these observations, D'Mello and Lewis (1970) stated that "the interaction between lysine and arginine cannot be shown not to be specific". This antagonism was thought to be associated either with a limited availability of arginine from specific feed ingredients in which lysine concentration was excessively high (Jones, 1964) or with an increased utilization of arginine for the excretion of the nitrogen surplus through the uric acid cycle (Snetsinger and Scott, 1961). Another aspect that was considered to explain the lysine-arginine antagonism was that both are basic amino acids and they may compete for the absorption in the gastrointestinal tract (Balnave and Brake, 2002). However, Jones et al. (1967) negated this hypothesis by showing that lysine did not affect digestion and absorption of arginine. However, lysine excess was associated with the inhibition of renal reabsorption of arginine while stimulating renal arginase activity (Boorman et al., 1968; Austic and Nesheim, 1970).

It is evident that shifts from the optimal Arg:Lys ratio could negatively affect productive traits as well as metabolic and physiological aspects. This effect is more evident with an excess of lysine (low Arg:Lys ratio) rather than an excess of arginine (high Arg:Lys ratio) (Balnave and Brake, 2002). Several authoritative sources suggested optimal Arg:Lys ratios in broiler diets. According to the NRC (1994), the optimal Arg:Lys ratio should be 1.14 to 3 weeks, 1.10 from 3 to 6 weeks, and 1.18 from 6 to 8 weeks. On the other hand, Baker (1997) indicated lower Arg:Lys ratio than the NRC (1.05, 1.08 and 1.08 from 0-3, 3-6 and 6-8 weeks of broiler age). Balnave and Brake (2002) suggested that, based on referenced literature, the optimum Arg:Lys ratio should range from 0.90 to 1.18. More recent reports suggested a ratio of 1.05, 1.08 and 1.08 from 0 to 21 d, 21 to 42 d, and 42 to 56 d, respectively (Wu, 2014). Similarly, other current nutrition specifications (Aviagen, 2014; Cobb-Vantress, 2018) indicate lower Arg:Lys ratios than those recommended by the NRC. In addition, the amino acids composition of the whole-body protein of 10-d-old chicks showed that arginine content was 111% in respect to lysine (Wu, 2014).

It should be considered that the over-time reduction of the dietary Arg:Lys ratio is mainly due to the increase in dietary lysine concentration rather than a reduction of the arginine levels. Indeed, as showed by Dozier III et al. (2008), lysine concentration in commercial broiler diets tended to increase from 2001 to 2005. Similarly, current dietary recommendations (Aviagen, 2014; Cobb-Vantress, 2018) indicate higher lysine concentrations than those reported by the NRC (1994). This increase was mainly driven by the high lysine requirement of modern fast-growing broiler chickens selected for an increased feed efficiency and breast meat yield. Indeed, the importance of dietary lysine on growth performance, breast meat yield, and meat quality has been well documented and established (Hickling et al., 1990; Moran and Bilgili, 1990; Bilgili et al., 1992; Leclercq, 1998; Berri et al., 2008). However, when the dietary concentration of a specific amino acid is subjected to variation, the concentration of the other amino acids should be modified accordingly to maintain the ideal amino acid profile. Indeed, as suggested by Kidd et al. (1997), increasing the dietary concentration of lysine without considering other important amino acids such as threonine or arginine may lead to a marginal deficiency of those.

In commercial diets, especially when the use of animal by-products is not allowed either by the legislation in force (e.g. European countries) or by voluntary decision (e.g. vegetable-only diet), the Arg:Lys levels are usually lower than the requirements defined by the NRC (1994). However, considering the important functions of arginine, it should be questioned whether the status quo of dietary Arg:Lys ratios are sufficient to meet the modern broiler requirement in arginine. Deficiencies or excesses of arginine may have a strong adverse effect on productive efficiency with negative effects on animal health, welfare and productivity as well as on the economic and environmental sustainability of the poultry industry.

There is also a paucity of scientific information regarding the effects of different Arg:Lys ratios in broiler diets on meat quality attributes and occurrence of breast muscle myopathies. As previously reported, L-arginine can be converted stoichiometrically into citrulline and NO by means of the enzyme nitric oxide synthase (Fernandes and Murakami, 2010; Khajali and Wideman, 2010).

NO is a potent vasodilator molecule (Khajali and Wideman, 2010), which could enhance blood flow to the muscle improving oxygen supply as well as the removal of harmful catabolites while alleviating the hypoxic condition usually observed in breasts affected by severe woody breast (WB) or white striping (WS) myopathies. Very recently, Bodle et al. (2018) showed that WB but not WS average score was significantly reduced by increasing the level of dietary arginine.

Therefore, to address these concerns, a trial was carried out to investigate the effects of the dietary arginine supplementation on growth performance, with special regards to FE, breast meat quality, incidence and severity of breast muscle myopathies and foot pad dermatitis, as well as plasma and muscle metabolomics profile in modern fast-growing broilers. The results of this study are shown in the RESEARCH WORK #1 chapter.

In this research, an NMR approach was applied to evaluate the molecular responses to the different concentration of dietary amino acids. The application of innovative analytical techniques identified as omics technologies showed a great potential in investigating the global variations of genes, proteins, and metabolites expression levels in body fluids or tissues in response to dietary treatments (Baeza et al., 2015; Zampiga et al., 2018). In the next chapter, an overview of the potential applications of omics technologies in nutritional studies was provided.

1.5. Application of *–omics* technologies for the evaluation of the molecular responses to nutritional treatments

Nutrition can be considered one of the most important environmental factors affecting genome expression. Indeed, nutrients should not be merely considered as a provider of nutritive principles but also a source of various molecules which can be sensed by the organism and influence genome expression (Garcìa-Canas et al., 2012). Therefore, a possible application of omics technologies in nutritional studies might be the identification of the molecular mechanisms laying behind the phenotypic responses to the dietary administration of different kind of compounds and additives.

Considering macronutrients, dietary amino acids play a central role in protein metabolism (e.g. protein synthesis, proteolysis and amino acid oxidation). Besides this aspect, amino acids can also act as regulators of different metabolic pathways related to muscle development and mRNA translation into proteins (Tesseraud et al., 2011). It is well established that the dietary supplementation of lysine can improve growth performance and breast yield in broilers (Kidd et al., 1998; Sterling et al., 2006; Berri et al., 2008), but also meat quality traits such as water holding capacity and pH (Berri et al., 2008). The dietary supplementation of lysine in lysine-deficient diets stimulated protein synthesis in skeletal muscle, whereas its dietary deprivation increased the fractional rate of protein breakdown (proteolysis) in *Pectoralis major* muscle of growing chickens (Tesseraud et al., 1996; 2001). Furthermore, also daily variations in dietary lysine content (sequential feeding) have been associated with an altered expression of genes related to proteolysis in breast muscle of chickens (Tesseraud et al., 2009).

Dietary methionine levels can deeply affect productive performance and breast meat yield in broilers (Corzo et al., 2006; Zhai et al., 2012; Wen et al., 2014a,b). It has been reported that dietary methionine concentrations altered the expression of myogenic genes (myogenic factor 5, myocyte enhancer factor 2B and myostatin) (Wen et al., 2014a), as well as that of proteins mainly related to citrate cycle, calcium signaling, actin cytoskeleton and clathrin-mediated endocytosis signaling, in chicken breast muscle (Zhai et al., 2012). A previous work showed that peptides belonging to three proteins (pyruvate kinase, myosin alkali light chain-1, and ribosomal-protein-L-29) were exclusively detected in breast muscle of chickens fed a methionine-deficient diet (Corzo et al., 2006). On the other hand, a higher plasma concentration of uric acid and triglycerides was observed in response to the dietary supplementation of methionine (Wen et al., 2014a). Wen et al. (2014b) also reported that increasing the dietary methionine levels could be a valuable strategy to support productive performance and breast yield of chickens with a low hatching weight. As stated by the Authors, these improvements could be likely attributable to alterations in insulin-like growth factor-

I synthesis and expression of genes involved in the target of rapamycin/eIF4E-binding protein 1 and forkhead box O4/atrogin-1 pathway (Wen et al., 2014b).

Several studies have been conducted to evaluate the effects of the dietary supplementation of arginine on both productive and molecular aspects. Fouad et al. (2013) reported that dietary arginine can modulate lipid metabolism as indicated by the reduced abdominal fat content as well as the lower plasma triglyceride and total cholesterol concentration in broilers fed arginine-supplemented diets. At the transcriptional level, arginine increased the expression of carnitine palmitoyl transferase1 (CPT1) and 3-hydroxyacyl-CoA dehydrogenase (3HADH) in the heart, while reduced that of fatty acid synthase (FAS) in the liver (Fouad et al., 2013). As previously reported, arginine also showed positive effects on gut mucosa health and integrity in broilers subjected to coccidia challenge (Tan et al., 2014a), as well as on attenuating the inflammatory response elicited by lipopolysaccharide treatment (Tan et al., 2014b) and on the immunosuppression induced by infectious bursal disease virus challenge (Tan et al., 2015).

Considering vitamins, Vignale et al. (2015) observed that the dietary replacement of cholecalciferol (vitamin D_3) with 25-hydroxycholecalciferol [25(OH)D_3], a vitamin D metabolite available for commercial poultry use, increased breast meat yield and fractional synthesis rate of breast muscle proteins. Chickens fed 25(OH)D_3 showed higher expression of vitamin D receptor, a DNA-binding transcription factor that mediates the action of vitamin D, and a greater activation of the mTOR/S6 kinase pathway, highlighting the important role played by this pathway in mediating the effects of 25(OH)D_3 on chicken muscle proliferation and development. These *in-vivo* results were corroborated by the *in-vitro* functional study performed on quail myoblast cells (QM7 cells) in which an increased expression of vitamin D receptor, as well as a greater translocation of it into cell nucleus, has been observed when cells were treated with 25(OH)D_3. Nonetheless, 25(OH)D_3 induced cell proliferation in a dose-dependent fashion and its effect was suppressed by blocking the mTOR pathway with rapamycin (Vignale et al., 2015).

Within the category “zootechnical additives”, a broad number of alternative compounds such as immunomodulators, probiotics, prebiotics, enzymes, organic acids, vegetable extracts and phytogenic compounds can be included. Great efforts have been reserved at evaluating the phenotypic responses of the animal to the dietary supplementation of these kinds of additives as well as to delineate the molecular mechanisms elicited by their utilization. Some examples are reported hereunder. The administration of phytase in broiler diet is reported to have a direct effect on organic phosphorus (phytate) and mineral digestibility, but also an indirect effect on zootechnical performance and muscle development mainly through the release of myo-inositol (Selle and Ravindran, 2007; Lee and Bedford, 2016). Schmeisser et al. (2017) reported that the administration of a 6-microbial phytase in a moderately phosphorous-deficient diet determined significant changes in the expression of genes involved in muscle development through calmodulin/calcineurin and insulin-like growth factor pathways. The activation of these pathways may have enhanced breast muscle development and increased its weight, even though no significant difference has been reported in terms of breast yield. Interestingly, birds receiving the dietary supplementation of dicalcium phosphate instead of phytase reported similar breast weight and yield compared to the phytase-supplemented group even though none of the previous pathways resulted significantly enriched. Therefore, the Authors suggested that the muscle growth observed in these birds was not probably due to the same molecular mechanism (Schmeisser et al., 2017). As previously reported, the dietary administration of lysophospholipids-based emulsifiers has shown a positive effect on feed conversion rate in broiler chickens (Zampiga et al., 2016). A microarray analysis performed on the jejunal epithelium of birds receiving a lysolecithin emulsifier showed an upregulation of genes for collagen, extracellular matrix, and integrins, suggesting that the positive effects of the emulsifier on productive performance might be achieved through changes in the intestinal epithelium (Brautigan et al., 2017). Moreover, Khonyoung et al. (2015) identified a higher expression of cluster of differentiation 36, an integral membrane protein involved in fat absorption, in jejunum of broilers fed diet supplemented with lysolecithin.

Prebiotics, such as yeast cell wall-derived compounds, are receiving even more attention due to their beneficial effects on growth performance, FE and gut health (Patterson and Burkholder, 2003; Hajati and Rezaei, 2010; Spring et al., 2015). However, the molecular mechanism behind their effects has not been totally elucidated. Xiao et al. (2012) applied a genome-wide transcriptional approach to investigate the effects of feeding mannan-oligosaccharides (MOS)-supplemented diets on jejunal gene expression of broiler chickens. Albeit they did not find any significant effect on productive performance, the transcriptomic analysis highlighted major expression of genes involved in protein synthesis, immune processes and antioxidant status in birds receiving MOS. Moreover, several signaling pathways related to mitochondrial functions showed a potential involvement in mediating the effects of dietary MOS (Xiao et al., 2012). In another study, the beneficial effects of MOS have been associated with a reduced gut cells turnover and hence an increased energy preservation for growth, as supported by the downregulation of genes involved in protein synthesis, protein metabolism, cellular assembly and organization, as well as the lower expression of genes of the mTOR pathway, in response to the MOS-supplementation (Brennan et al., 2013). In addition, transcriptomic analysis evidenced common biological functions, such as antiviral and antimicrobial response, between birds receiving prebiotic- or bacitracin-supplemented diets, indicating that MOS could actively stimulate the intestinal innate immune system (Brennan et al., 2013).

The dietary use of probiotics has been reported to be beneficial for chicken health and productivity (Patterson and Burkholder, 2003; Kabir, 2009; Cox and Dalloul, 2015). Luo et al. (2013) showed that the dietary supplementation of *Enterococcus faecium* had only a slightly positive effect on FCR while stimulating the development of immune organs, number of intestinal microvilli, and diversity of gut microflora. A proteomic approach carried out on the intestinal mucosa of the birds receiving the probiotic identified a total of 42 proteins showing differential expression of which 60% could be associated with cytoskeleton and immune system. According to the Authors, the probiotic may have enhanced FE through improving the absorptive area in the

intestine while limiting the energy expenditure for immune system activation. It has also been shown that the dietary supplementation of *E. faecium* can improve breast and legs yield, as well as water-holding capacity of meat, while determined low abdominal fat deposition (Zheng et al., 2014). A proteomic analysis performed on the breast muscle allowed the identification of 22 differentially expressed proteins mainly involved in carbohydrate and energy metabolism, as well as in cytoskeleton and molecular chaperones, which might have contributed to the improvements observed in carcass and meat quality. Recently, the dietary administration of *E. faecium* was associated with significant changes also in the liver proteome, indicating a potential effect in enhancing nutrient metabolism and partitioning as well as in decreasing the inflammatory response (Zheng et al., 2016). Other interesting insights recently obtained in the field of broiler nutrition through the application of omics technologies regarded the effects of heat stress on gene expression and nutrients transporters in the jejunum (Sun et al., 2015), the evaluation of the dietary supplementation of branched-chain amino acids on the expression of hepatic fatty acids metabolism-related genes (Bai et al., 2015) and the modulation of the intestinal phosphate transporters expression in response to the administration of phosphorous and phytase in the diet (Huber et al., 2015).

Overall, these studies confirmed the potential of omics technologies in investigating the molecular mechanisms elicited by dietary treatments, which could allow a better understanding of the responses observed at the phenotypic level. Besides these aspects, omics technologies might be also useful for assessing the molecular aspects associated with the intermediary metabolisms in different tissues of broiler chickens able to influence overall FE.

1.6. Molecular aspects of feed efficiency in broiler chickens: effect of intermediary metabolisms

FE is a vital economic trait for the poultry industry as feeding represents the greatest cost in raising meat-type chickens. From a homeostatic perspective, FE could be defined as the net result

between energy intake (i.e. feed intake and its complex regulatory mechanisms) and energy expenditure (affected by the maintenance metabolism, anabolic processes, tissue-specific intermediary metabolisms, and environmental effects) (Figure 1.1). The selection methods applied in broilers to improve FE and breast yield were mostly performed without taking in consideration the potential changes exerted on the molecular mechanisms and on the physiological/metabolic features of the animal (Dridi et al., 2015), resulting in several undesirable side-effects such as a hyperphagic feeding behavior, obesity, muscle disorders, and leg problems. Therefore, to continuously improving FE, it is fundamental to understand the molecular aspects in different tissues of broilers which may exert a huge effect on the phenotypic expression of this trait. This is achievable by a proper application of new cutting-edge omics technologies which allow to evaluate the global variations of gene, protein or metabolite expression levels adding important insights on the molecular mechanisms associated with FE. The effect of the intermediary metabolisms in the main tissues of broilers on overall FE was reviewed and reported in the next paragraphs.

1.6.1. Muscle

1.6.1.1. Mitochondria dynamics and bioenergetics processes

In chickens, muscle is the main site for thermogenesis since they lack the brown adipose tissue. Being one of the main metabolic organs, the bioenergetics processes within the muscle can deeply influence FE in broilers. As mitochondria are responsible for producing around 90% of the energy pool for cells, studies have been conducted to evaluate whether the expression of different FE phenotypes would be associated with differences or inefficiencies in muscle mitochondria structure and functionality. A first confirmation of this hypothesis was obtained by Bottje et al. (2002) when a potential link between muscle mitochondria functionality and the phenotypic expression of FE was established in a broiler breeder line. The birds, belonging to the same genetic line, were held in thermoneutral environment in individual cages, fed the same diet, and individually phenotyped for FE, and therefore any behavioral, environmental or dietary effect was

excluded from the FE equation (Bottje and Kong, 2013). At the gene level, differences in the expression of genes involved in mitochondria biogenesis [peroxisome proliferator-activated receptor-γ (*PPAR-γ*), PPAR-γ coactivator-1α (*PGC-1α*) and inducible nitric oxide synthase (*iNOS*)] and energy metabolism [avian adenine nucleotide translocator (*avANT*), cytochrome oxidase III (*COX III*), and avian uncoupling protein (*avUPC*)] were observed in breast muscle of birds showing either high or low FE (Ojano-Dirain et al., 2007). Regarding the physiological aspects, the activity of mitochondria complexes I, II, III, and IV has been reported to be higher in breast muscle of high FE birds compared to low ones (Iqbal et al., 2004). Previously, Bottje et al. (2002) reported that the activity of complexes I and II was greater in breast and leg mitochondria of high FE birds. Recently, the upregulation of genes associated with electron transport chain (ETC) complex I (Kong et al., 2011), as well as a greater predicted activity of complexes I, III, IV and V (Kong et al., 2016) in breast muscle of high FE birds, seem to confirm an overall increased activity of mitochondrial complexes in the high FE phenotype. To address whether these differences in respiratory chain complexes activity might be due to an altered expression of mitochondria proteins, post-translational modifications, or oxidative damages, different proteo-genomics approaches were performed. At the protein level, mitochondrial ETC complexes should not be considered as single entities but rather the assemblies of multiprotein subunits, which expression is controlled by both nuclear and mitochondrial DNA (Iqbal et al., 2004). Although the activity of the different complexes appeared higher in most of the reported studies, no significant difference has been observed in complex I protein expression, as well as in the expression of 70S subunit of complex II or α-ATPase (complex V) in breast muscle of birds with different FE phenotype. Nonetheless, cytochrome b, cytochrome c1, core I (complex III) and cytochrome c oxidase subunit II (complex IV) showed higher expression in low FE mitochondria. Considering other chicken tissues over than muscle, only two mitochondrial proteins (cytochrome c1 and cytochrome c oxidase subunit II) exhibited differential expression between high or low FE birds in at least 4 out of 5 examined tissues (breast muscle, heart, duodenum, liver, and lymphocytes), suggesting the existence of tissue-

specific regulatory mechanisms (e.g., post-translational modifications or different cell turnover) (Iqbal et al., 2004; Iqbal et al., 2005; Ojano-Dirain et al., 2004; Lassiter et al., 2006; Tinsley et al., 2010). On the other hand, Kong et al. (2016), using a shotgun proteomic approach, showed a higher mitochondrial proteins expression in breast muscle of high FE birds belonging to the same broiler breeder line and identified "mitochondrial function" and "oxidative phosphorylation" as first and fifth top expressed pathways, respectively. Moreover, it has been reported that the activation of upstream regulators such as progesterone and triiodothyronine would be associated with the increased expression of mitochondrial proteins in the high FE phenotype (Kong et al., 2016). A common feature among the previously mentioned studies was the significantly higher level of oxidized mitochondrial proteins in the tissues of low FE chickens, as indicated by the increased amount of protein carbonyls. Therefore, as suggested by Bottje et al. (2006), the lower respiratory complexes activity observed in low FE mitochondria might be due to the increased level of oxidized proteins rather than a reduced expression of ETC protein subunits, as some of them showed similar or even higher expression in low FE mitochondria.

In conclusion, Bottje and Kong (2013) indicated that at least 2 physiological processes would have contributed to mitochondrial inefficiency and hence to the overall expression of a low FE phenotype. The first physiological process was site-specific defects in ETC that may have increased reactive oxygen species (ROS) production. In turn, the higher levels of ROS were identified to be responsible for the greater amount oxidized proteins in the low FE phenotype (Bottje et al., 2006; Bottje and Carstens, 2009). An increased oxidation of mitochondrial proteins could have played a detrimental effect on FE since energy might have been directed towards reparation and synthesis of mitochondrial proteins rather than for anabolic processes. The second aspect associated with inefficiency was proton leak, which is a movement of protons across the inner mitochondrial membrane at other sites rather than through ATP synthase. Proton leak is fundamental for maintaining homeostasis by reducing mitochondrial ROS production, even though it represents an energetic wasteful process and accounts up to 50% of basal oxygen consumption

rate in mitochondria (Bottje and Kong, 2013). Bottje et al. (2009) reported that proton leak rates in the low FE phenotype were higher, or at least similar, to those observed in the high FE one. Finally, ROS, acting as secondary messengers, may have influenced the expression of genes and proteins involved in mitochondria functionality, activity, or development (Bottje and Kong, 2013).

1.6.1.2. Protein synthesis and cellular anabolic processes

On the same broiler breeder line, breast muscle global mRNA expression was assessed using microarray-based approaches (Kong et al., 2011; Bottje et al., 2012). High FE birds were characterized by an upregulation of genes either involved in anabolic processes (protein packaging and scaffolding activity, purine and pyrimidine biosynthesis, prevention or delay of apoptosis and modulation of gene transcription), or related to major signal transduction and cascade mechanisms pathways [protein kinase-A (PKA), c-Jun NH(2)-terminal protein kinase (Jnk), retinoic acid and retinoid X receptor (RAR-RXR)] or in sensing the energy status and regulating energy production in the cell [adenosine monophosphate AMP-activated protein kinase (AMPK) and protein kinase AMP-activated non-catalytic subunit gamma 2 (PRKAγ2)]. At the same time, high FE birds showed downregulation of genes associated with cytoskeletal organization, as well as architecture and integrity-related genes, major histocompatibility complex cell recognition, stress-related heat shock proteins and several platelet derived growth factors genes. A global overview of the cellular processes which might have contributed to the phenotypic expression of FE has been summarized by Bottje and Kong (2013). Recent findings also suggested a potential role of insulin receptor, insulin-like growth factor 1 receptor, nuclear factor erythroid 2-like 2 (Kong et al., 2016), progesterone (Kong et al., 2016; Bottje et al., 2017a), as well as mechanistic target of rapamycin (mTOR) and protein degradation pathways (Bottje et al., 2014), in the phenotypic expression of FE in broiler chickens. On the other hand, rapamycin independent companion of target of rapamycin (RICTOR), mitogen-activated protein kinase 4 (MAP4K4), and serum response factor were predicted to be downregulated in muscle of high FE chickens (Kong et al., 2016). Combining gene

and protein expression analysis, Bottje et al. (2017b) also reported an enhanced mitochondrial and cytosolic ribosomal construction, protein translation, proteasomes and autophagy, in breast muscle of high FE birds. On the other hand, consistently with previous findings, Kong et al. (2016) highlighted that several upstream regulators involved in the activation of cytoarchitecture-related genes were inhibited in the high FE phenotype. Overall, broiler chickens showing a high FE phenotype seem to achieve a greater efficiency through reducing the energy expenditure for maintaining cytoskeletal architecture and function, as well as for substituting damaged proteins, while directing energy towards anabolic-related processes that may enhance overall cellular efficiency (Kong et al., 2016). However, considering the less organized cytoskeletal architecture observed in high FE birds, it would be interesting to evaluate whether the selective pressure applied to improve FE may have negatively contributed to the increased incidence and severity of muscle myopathies recently observed in fast-growing broiler genotypes.

Recently, the biological basis of the differences between high and low FE chickens was investigated by Zhou et al. (2015a) through mRNA-seq and pathways analysis. Despite previously reported studies, which were focused on a broiler breeder line, the research of Zhou et al. (2015a) was carried out on breast muscle of male chickens obtained by crossing three commercial pure lines. The RNA-seq analysis identified a total of 1,059 differentially expressed genes between high and low FE chickens. High FE birds had a greater expression of genes related to muscle development, hypertrophy, and remodeling, coupled with a decreased expression of protein degradation and atrophy-related genes. Moreover, transcriptional factors involved in muscle development resulted upregulated in these birds. These results, associated with the predicted activation of GH and insulin-like growth factor-I/phosphatidylinositol 3-kinase/protein kinase B (IGFs/PI3K/Akt) signaling pathways, might explain the higher breast yield observed in high FE birds. Other important findings were the upregulation of genes related to inflammatory response and macrophage infiltration, as well as an increased expression of glutathione s-transferase superfamily genes which encode for antioxidant proteins. Moreover, the activation of hypoxia-inducible

transcription factor-1α would suggest that a hypoxic condition may occur in breast muscle of high FE birds, which might be ascribed either to the increased inflammatory condition, to the excessive muscle remodeling, or to the higher production of ROS (Zhou et al., 2015a). It is interesting to note that most of the biological features observed by Zhou et al. (2015a) in the breast of high FE birds can overlap those reported in breast muscle of birds affected by wooden breast myopathy. Even though Zhou et al. (2015a) reported no clinical symptoms of sickness or muscle damages, the similarity in gene expression profile may indicate common biological patterns and thereby a possible relationship between FE and wooden breast occurrence.

1.6.2. Adipose tissue and liver

Adipose tissue plays a central role in energy homeostasis being a metabolically active organ with endocrine and regulatory functions. On the same chicken population of Zhou et al. (2015a), another RNA-seq analysis was conducted to investigate the gene expression profile in abdominal fat (Zhou et al., 2015b). Low FE chickens showed higher lipid accumulation, which was likely determined by the upregulation of genes involved in lipid synthesis, as well as downregulation of genes enhancing triglyceride hydrolysis and cholesterol transport from adipose tissue. Moreover, the predicted activation of sterol regulatory element binding proteins, as well as the inhibition of insulin-induced gene 1, was consistent with the higher cholesterol accumulation observed in low FE birds (Zhou et al., 2015b).

On the other hand, adipose tissue also has a secretory function. Leptin, for instance, is a peptide hormone secreted by the adipose tissue which is involved in the regulation of feed intake and energy metabolism in both mammals and avian species. In chickens, leptin is also expressed in the liver and it is regulated by the nutritional state of the birds (Dridi et al., 2005). As in mammals, leptin is recognized as “satiety hormone” in chickens as well, since it reduces feed intake while increases energy expenditure through the interaction with its receptors localized both in brain neurons and in other peripheral tissues (Richards, 2003). Understanding the role of different

molecules such as leptin in both central and peripheral tissues of the chickens is fundamental to increase our knowledge regarding the molecular basis of FE.

1.6.3. Brain

Feeding behavior and body energy homeostasis are intimately connected with the brain (Kuenzel et al., 1999; Richards, 2003; Richards and Proszkowiec-Weglarz, 2007; Bungo et al., 2011), in particular with the infundibular nucleus of the hypothalamus (Bungo et al., 2011). Here, the hypothalamic melanocortin system contains two different populations of neurons which can modulate feed intake through the secretion of various neuropeptides. Briefly, a reduction of feed consumption is mediated by the α-melanocyte stimulating hormone, released by the proopiomelanocortin neurons (POMC), and the cocaine- and amphetamine-regulated transcript (CART). On the other hand, neuropeptide Y (NPY) and agouti-related protein can stimulate appetite and increase feed intake by repressing the melanocortin anorexigenic effect (Bungo et al., 2011). Differences in the expression levels of these neuropeptides and some feeding-related genes have been reported in the hypothalamus of chickens (Sintubin et al., 2014) and quails (Blankenship et al., 2016) divergently selected for RFI and FE, respectively. However, other factors such as leptin (Dridi et al., 2005) and several gut hormones (Honda et al., 2017) can affect central feed intake regulation and hence energy homeostasis in chickens.

1.6.3.1. Focus on Neuropeptide Y (NPY)

This research topic was developed during my 5-month research period at the Center of Excellence for Poultry Science – University of Arkansas. This study was carried out to characterize the expression of neuropeptide Y in chicken muscle and assess its effects on mitochondria dynamics.

NPY is an orexigenic 36-amino acid peptide mainly expressed in the hypothalamus of chickens (Kuenzel et al., 1987). While the distribution of NPY and its receptors in mammals is well

documented and defined, there is a paucity of scientific information regarding these aspects in avian (non-mammalian vertebrates) species. Indeed, NPY expression was only recently reported in other chicken tissues beside the brain, such as liver and skeletal muscle (Gao et al., 2017). In addition, although the role of hypothalamic NPY on the regulation of feeding behavior is well known (Saneyasu et al., 2011; Newmyer et al., 2013), its effect on physiological and metabolic features in peripheral tissues is still not well defined. Therefore, due to the important functions of NPY on overall energy homeostasis and hence on feed efficiency in chickens, a study was conducted to investigate whether NPY and its receptors are expressed in the skeletal muscle, the main site for thermogenesis in poultry species, and then to investigate its potential role in regulating the expression of mitochondrial biogenesis-, function-, and dynamics-related genes.

For the characterization, 6 birds showing the same age, gender and genetic line, fed the same diet and raised in the same environmental conditions, were humanely euthanized to collect breast and leg muscle, and hypothalamus. Quail leg muscle and quail myoblast (QM7) cells were also used for the characterization. Total mRNA was extracted (Trizol method) according to the manufacturer's recommendations, and its integrity and quality were evaluated using 1% agarose gel electrophoresis. RNA concentration and purity were assessed for each sample by Take 3 Micro-Volume Plate using Synergy HT multi-mode micro plate reader (BioTek,Winooski, VT). Then, 1 μg of RNA was reverse transcribed using qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD) and the expression of NPY and its receptors was assessed with conventional PCR.

To understand the physiological role of NPY in chicken muscle, QM7 cells were treated either with 0 (CON), 1 (NPY1) or 100 (NPY100) nM of recombinant NPY. Total mRNA was extracted from treated QM7 cells as described above, and RT-qPCR analysis performed to determine the relative expression of NPY and its receptors as well as that of mitochondrial biogenesis-, function-, and dynamics-related genes. Briefly, real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) was performed using 5 μL of 10X diluted cDNA, 0.5 μM

of each forward and reverse primer, and SYBR Green Master Mix (ThermoFisher Scientific, Rockford, IL) for a total 25 µL reaction. The qPCR cycling conditions were the same reported by Lassiter et al. (2014) and 18S gene was used as housekeeping gene. The $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) was used to determine the relative expression of target genes considering the untreated group as calibrator.

From this study emerged that NPY is expressed in both chicken breast and leg muscles, quail leg muscle, and in QM7 cells, as shown in Figure 1.6.1. The mRNA expression of NPY and its receptors in QM7 cells was reduced by the NPY1 treatment while was increased with the NPY100 (Figure 1.6.2). In addition, the treatment with recombinant NPY altered the mRNA expression of mitochondrial biogenesis-, function-, and dynamics-related genes in QM7 cells (Figure 1.6.3).

Figure 1.6.1 Characterization of NPY and its receptors in chicken and quail tissues (hypothalamus and muscles) through conventional PCR.

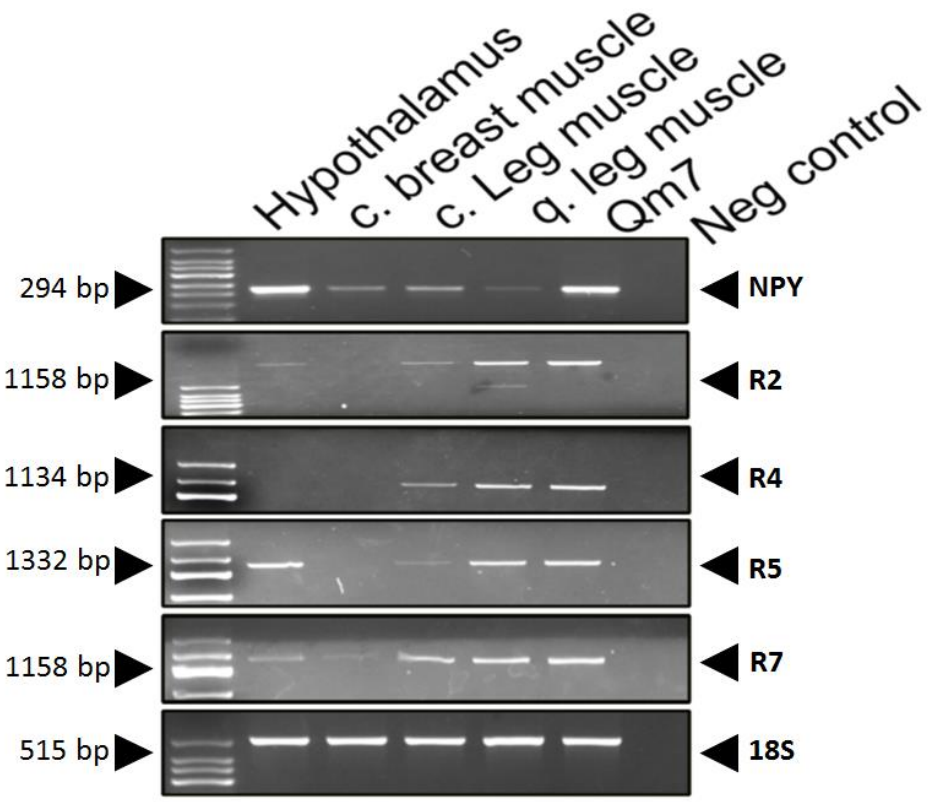


Figure 1.6.2 mRNA expression of NPY and its receptors in QM7 cells treated with recombinant NPY.

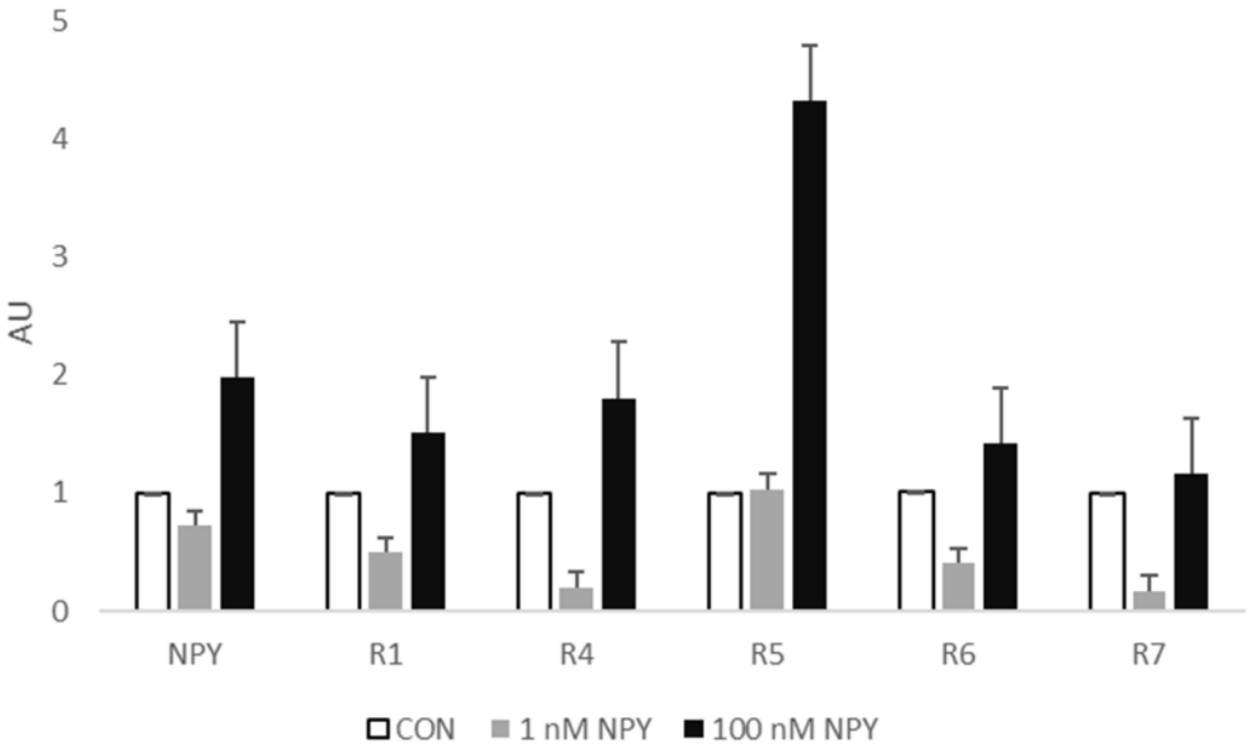
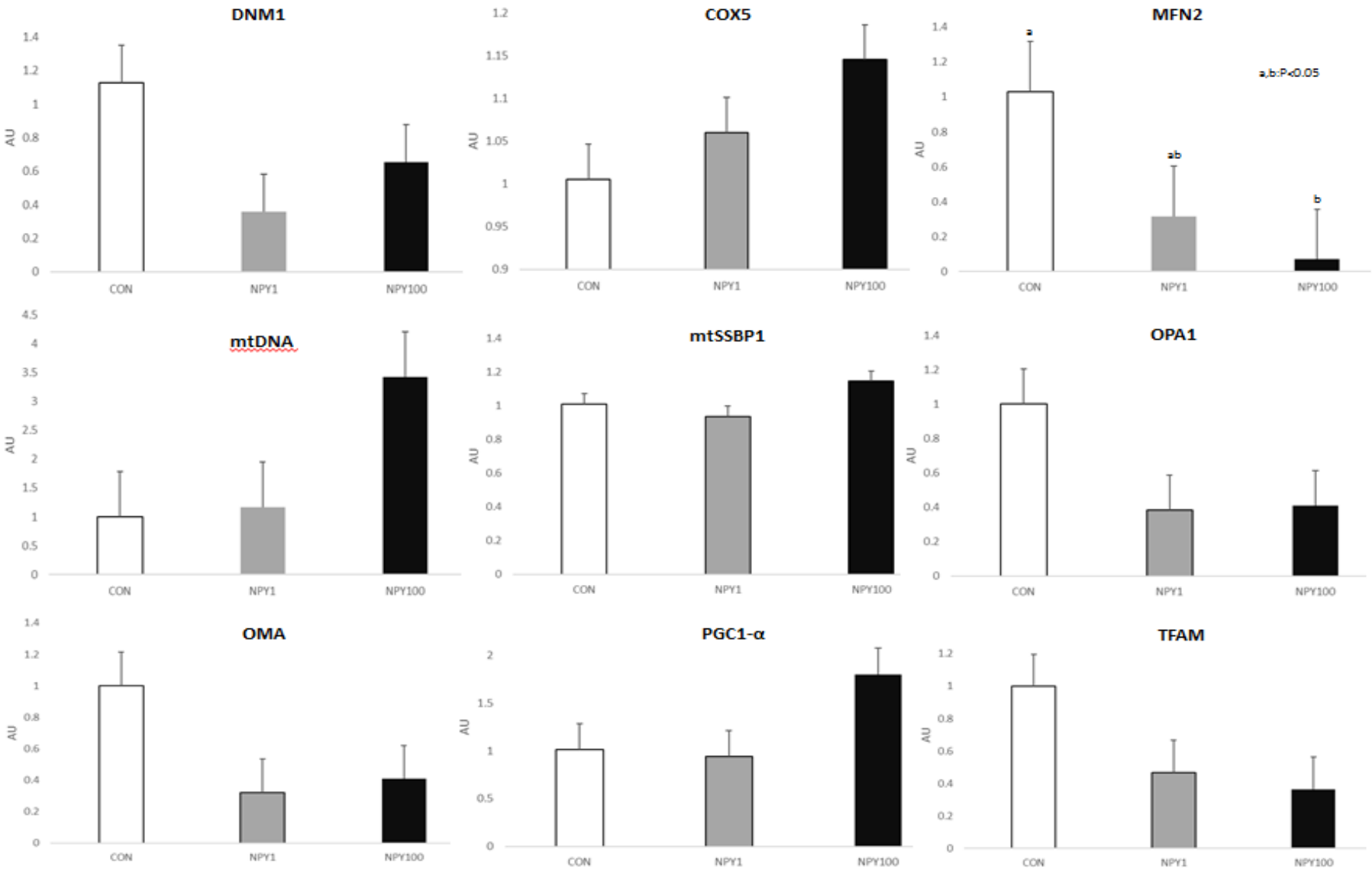


Figure 1.6.3 mRNA expression of mitochondrial biogenesis-, function-, and dynamics-related genes in QM7 cells treated with recombinant NPY.



In addition, an *in vivo* study was performed. 10-d-old chicks were intraperitoneally injected with different dosages of NPY (0, 60, 120, 240 nM NPY). The results (data not showed) indicated that NPY treatments stimulated feed but not water consumption. Similarly, the treatments determined significant changes in NPY expression in blood as well as in the expression of NPY, NPY receptors, and mitochondria genes, in breast and leg muscle. Furthermore, we observed significant changes in the expression of NPY and related receptors according to the feeding state of the birds (fasted vs. fed). Ongoing analysis on mitochondria bioenergetics processes are defining the role of NPY on metabolic aspects of muscle mitochondria. However, due to the deadline anticipation for submitting the PhD thesis (occurred for the first time in this academic year), the results of the bioenergetics analysis, currently ongoing, have not been included in this dissertation.

Taken together, these results suggest that:

- NPY is expressed in chicken breast and leg muscle,
- NPY regulates its own system,
- NPY may play a pivotal role in mitochondria function and dynamics.

Therefore, due to its important functions on feed intake and mitochondria activity in muscle tissue, NPY could be considered a crucial factor involved in energy homeostasis processes and thereby in FE in broiler chickens.

1.6.4. Gut

The gut is one of the most important tissues able to influence the expression of different FE phenotypes due to its pivotal function in nutrient digestion and absorption, as well as for its immunological and regulatory roles (Scanes and Pierzchala-Koziec, 2014).

Ojano-Dirain et al. (2007) found a higher level of oxidized proteins in duodenal mucosa homogenate and duodenal mitochondria of low FE birds. On the other hand, higher mRNA expression of *PPAR-γ* and *iNOS* was observed in the duodenum of high FE birds, whereas no

significant difference was reported for *PGC-1α*, *avANT* and *COXIII* (Ojano-Dirain et al., 2007). Lee et al. (2015) analyzed the transcriptomic profile in duodenum of chickens divergently selected for RFI. The Authors observed that the selection process improved FE by reducing feed intake without significant changes in body weight gain. The molecular mechanism behind this improvement has been associated with the upregulation of genes involved in the reduction of appetite and increased cellular oxidative stress, prolonged cell cycle, DNA damage and apoptosis, as well as greater oxidation of dietary fats and efficient fatty acids transport from the intestine. Moreover, a differential expression of genes involved in the avian target of rapamycin (avTOR) signaling pathway has been observed in liver and small intestine of meat-type chickens divergently selected for RFI, confirming a potential involvement of avTOR/PI3K pathway in determining FE in chickens (Lee and Aggrey, 2016).

Recently, the development of new omics technologies and platforms has strengthened the possibility to investigate the gut microbiota and its metabolic activities in farm animals (Deusch et al., 2015). Several papers reported differences in the intestinal microbiota between chickens showing different FCR (Stanley et al., 2012; 2016; Mignon-Grasteau et al., 2015). Stanley et al. (2012) observed no significant difference in jejunum microbiota composition between birds showing high or low FCR as this tract was almost exclusively populated by members of the genus *Lactobacillus*. On the contrary, caecum microbiota showed higher diversity and 24 unclassified bacterial species were found to be differentially expressed between high and low performing birds. In a recent study, three families, Lachnospiraceae, Ruminococcaceae, and Erysipelotrichaceae, have been associated with the phenotypic expression of high FE (Stanley et al., 2016). In these birds, higher abundance of *Ruminococcus*, *Faecalibacterium*, *Clostridium*, and two unknown genera from the Lachnospiraceae family was also observed. Even if some strains of *Lactobacillus* are aimed to improve broilers performance, Stanley et al. (2016) identified others which have an undesirable outcome on the overall productive efficiency mainly through a stimulation of feed consumption. In

another study, Mignon-Grasteau et al. (2015) reported that birds selected for a low FCR showed lower cecal counts of *Lactobacillus*, *L. salivarius* and *E. coli* compared to the high ones. These variations in bacterial groups affected also the equilibrium between bacteria in the gut. Indeed, low FCR birds exhibited less *L. salivarius* and more *L. crispatus* to *Lactobacillus* ratio, as well as a higher ratio of clostridia to *Lactobacillus* and to *E. coli*. Albeit it has been calculated on a limited number of animals, the genetic heritability of microbiota was rather low, even if appreciable heritability coefficients (between 0.16 and 0.24) were observed for the ratios of *L. crispatus*, *C. leptum* and *C. coccoides* to *E. coli*. Finally, the Authors identified 14 Quantitative Trait Loci (QTL) which can affect the composition of the microbiota, even if they resulted significant only on a chromosome-wide scale. Interestingly, the only QTL close to genome-wide significance (QTL for *C. leptum* on chromosome 6) was located in a region containing genes involved in inflammatory response and intestinal motility (Mignon-Grasteau et al., 2015). However, as emerged in three different trials performed by Stanley et al. (2016), the microbiota associated with the phenotypic expression of FE resulted characterized by a great variability, indicating that other efforts should be done to identify probiotic bacteria and microbiota composition able to provide positive effects on FE.

The gut mucosa plays a critical role in bird's physiology since it acts as a physical barrier which must be permeable to nutrients, electrolytes, and water, while at the same time should avoid bacteria and antigens translocation to the underneath tissues. In addition, the small intestine also has important immunological, endocrine, and regulatory functions (Scanes and Pierzchala-Koziec, 2014) which can deeply affect health status (Sugiharto, 2016), as well as feeding behavior and overall energy homeostasis in the chickens (Honda et al., 2017). Therefore, the metabolic dynamics occurring in the small intestine of broiler chickens deserve much more attention when investigating molecular traits related to FE. A similar approach was applied in the RESEARCH WORK #2 as shown in the next chapter.

2. AIM OF THE RESEARCH

In the present dissertation, two research works aimed at evaluating nutritional and physiological aspects involved on overall productivity of broiler chickens, with special regard to feed efficiency applying both traditional and innovative approaches, have been included. Due to the vastness of these research fields, special attention was paid to specific topics such as protein and amino acid nutrition (RESEARCH WORK #1) as well as metabolic and physiological features of the small intestine (RESEARCH WORK #2) in fast-growing broilers, respectively.

Due to the progressive increase of lysine concentration in broiler diets, it may be questionable whether the dietary ratios between specific amino acids and lysine, currently recommended and adopted in commercial practices, are sufficient to meet the amino acid requirements of modern fast-growing broilers. In particular, this might be the case of arginine which received only limited attention in the past years despite its important roles in poultry as described in the previous chapters. To address this concern, the RESEARCH WORK #1 was aimed at evaluating the effects of the dietary supplementation of crystalline L-arginine to increase the arginine to lysine ratio beyond the ones currently suggested on growth performance, with particular focus on feed efficiency, breast meat quality, incidence and severity of breast muscle myopathies and foot pad dermatitis, as well as plasma and muscle metabolomics profile in modern fast-growing broilers.

Regarding productive efficiency, another aspect to be considered is that, despite similar selection criteria have been likely adopted for all the fast-growing chicken lines currently available for the poultry industry, differences in growth performance, feed efficiency and meat quality have been observed among commercial hybrids. However, the biological determinants of these differences have not been totally elucidated yet. For instance, a detailed investigation of the metabolic dynamics occurring in a key organ such as the small intestine, which is involved in nutrient digestion and absorption but also in important immunological, endocrine, and regulatory aspects, might be useful to explain the differences in productive efficiency among different fast-

growing broiler lines. Therefore, the RESEARCH WORK #2 was focused on the characterization of productive traits, with special regard to feed efficiency, and intestinal transcriptomic profile through microarray analysis in two fast-growing chicken hybrids raised in the same environmental conditions and fed the same diet.

3. RESEARCH WORK #1: Effect of dietary arginine to lysine ratios on productive performance, plasma and muscle metabolomics profile, and meat quality in fast-growing broiler chickens.

3.1. Background and aim

The selective processes applied to fast-growing broiler chicken lines have changed the metabolic and physiological features of these animals, and consequently their nutritional requirements of specific dietary compounds. In addition, the European ban of animal proteins as feeding-stuffs contributed to the limited provision of certain essential amino acids, including arginine. Arginine is considered an essential amino acid for the chickens and it is involved in crucial physiological, metabolic and immunological processes as described in the previous chapters (Fernandes and Murakami, 2010; Khajali and Wideman, 2010; Fouad et al., 2012). The repartitioning of arginine in the different body apparatus and organs to perform its functions may impair overall efficiency if inadequate amounts of this amino acid are provided with the diet. Hence, it should be questioned whether the currently adopted dietary arginine to lysine (Arg:Lys) ratios are sufficient to meet the modern broiler requirement in arginine without compromising feed efficiency and growth rate. The present study aimed, therefore, at evaluating the effects of the dietary supplementation of crystalline L-arginine in a commercial broiler diet on growth performance, with special regards to feed efficiency, plasma and muscle metabolomics profile, breast meat quality, incidence and severity of breast muscle myopathies and foot pad dermatitis, in modern fast-growing broilers.

3.2. Materials and Methods

3.2.1. Animals and housing

A total of 1,170 one-d-old Ross 308 male chicks, obtained from the same breeder flock and incubated in the same environmental conditions, was vaccinated (coccidiosis, Infectious Bronchitis Virus, Marek's disease virus, Newcastle and Gumboro disease) and allotted to an environmental

controlled poultry house. Chicks were divided in 18 pens of 6 m^2 each (9 replications/group, 65 birds/replication, 11 birds/m^2) and chopped straw (2 kg/m^2) was used as litter material. Replications were distributed in randomized blocks inside the poultry house in order to limit any environmental effect. Stocking density was defined according to the legislation in force (maximum 33 kg/m^2) (European Commission, 2007). Two circular pan feeders able to guarantee at last 2 cm of front space/bird and 10 nipples were provided for each pen. A photoperiod of 23L:1D of artificial light was adopted in the first 7 d and in the last 3 d of trial, while 18L:6D was used for the remaining days (European Commission, 2007). The environmental temperature was settled according to the age of the birds following the management guide provided by the breeding company. Birds were handled, raised, and processed according to the European legislation (European Commission, 2007; 2009; 2010). The experiment was approved by the Ethical Committee of the University of Bologna (ID: 928/2018).

3.2.2. Experimental diets

The same commercial corn-wheat-soybean basal diet (Table 3.1) was used to produce both the experimental diets. The basal diet was formulated to meet or slightly exceed the Ross 308 nutrition recommendations (Aviagen, 2014) and maintaining the ideal amino acid profile. The feeding program was composed of 4 phases: starter (0-12 d), grower I (13-22 d), grower II (23-33 d) and finisher (34-43 d). The CON group received the basal diet without any arginine supplementation (digestible Arg:Lys ratio = 1.05, 1.05, 1.06, 1.07 in starter, grower I, grower II and finisher phase, respectively). The ARG diet was obtained by supplementing on-top the basal diet with 1.20, 1.15, 1.10 and 0.95 g/kg of crystalline L-arginine (purity 99%, Barentz, Hoofddorp, The Netherlands) in starter, grower I, grower II and finisher feeding phase, respectively. Samples were obtained from both the experimental diets to evaluate proximate composition. Moisture and ash content were determined in duplicate according to the Association of Official Analytical Chemists

procedure (AOAC, 1990). Crude protein content was assessed by the standard Kjeldahl copper catalyst method as reported in AOAC (1990). Crude fat was determined using the Soxhlet method (AOAC, 1990), which allowed to extract the ethyl-ether soluble substances contained in the sample. The amino acid concentration of the experimental diets was analyzed by AMINOLab® (Evonik Industries, Hanau, Germany). Digestible amino acid values were calculated by multiplying digestibility coefficients (Ajinomoto Heartland, 2015) to the analyzed total amino acid content of each ingredient. In the ARG diet, the crude protein concentration was 23.4, 22.7, 20.3 and 18.2% in starter, grower I, grower II and finisher feeding phase, respectively. The calculated digestible lysine and arginine concentration was 1.25, 1.15, 1.05, and 0.93%, and 1.44, 1.32, 1.22, and 1.10%, corresponding to digestible Arg:Lys ratios of 1.15, 1.15, 1.16 and 1.17, respectively. The values concerning the CON diet are reported in Table 3.1. Both the diets were administered in a mash form and feed and water provided for *ad libitum* consumption.

3.2.3. Productive performance and slaughtering measurements

Number and weight of the birds were recorded on a pen basis at housing (0 d), at each diet switch (12, 22, 33 d) and at slaughter (43 d). Feed intake was recorded at the end of each feeding phase (12, 22, 33, 43 d). Mortality was recorded on a daily basis and dead birds were weighed, necropsied, and recorded to calculate the mortality percentage and to correct the productive performance results. Body weight (BW), daily weight gain (DWG), daily feed intake (DFI), feed conversion ratio (FCR) and cumulative FCR were determined for each feeding phase and for the overall rearing period. At 43 d, all birds were processed in a commercial plant and slaughtered according to the legislation in force using water-bath electrical stunning (200-220 mA, 1,500 Hz). Birds and carcasses belonging to the different experimental groups were clearly identified and kept separated throughout the processing phases. For each experimental group, all birds were mechanically processed and eviscerated to obtain carcass yield on a group basis by removing blood,

feathers, head, neck, viscera, abdominal fat, and feet. The overall carcass weight of each group was recorded after air-chilling and carcass yield calculated as percentage of body liveweight. Similarly, skinless and deboned breast was mechanically obtained from the carcass and yield calculated on a group basis as percentage of carcass weight. The incidence and severity of foot pad dermatitis (FPD) were macroscopically evaluated on all birds (1 foot/bird) using the 3-point scale evaluation system proposed by Ekstrand et al. (1997) [score 0 = no lesions; score 1 = mild lesions (<0.8 cm); score 2 = severe lesions (>0.8 cm)].

3.2.4. Blood and breast muscle collection

At slaughter (43 d), 9 birds/group (1 bird/replication) selected with similar BW and clearly labelled were subjected to blood withdrawal. Blood was obtained from the wing vein, collected into 4 mL lithium-heparin vials and immediately centrifuged (4,000 × *g* for 15 min) to obtain plasma, which was transferred into 1.5 mL labeled vials and stored at -80°C until metabolomic analysis. From the same 9 birds/group, a sample of *Pectoralis major* muscle was obtained, put into a 1.5 mL vial, immediately frozen under liquid nitrogen and then kept at -80°C until metabolomic analysis. The samples were consistently obtained from the same area of the breast muscle showing no macroscopic defects.

3.2.5. Incidence of breast meat abnormalities

The incidence and severity of white striping (WS), wooden breast (WB) and spaghetti meat abnormalities (SM) were evaluated on 150 randomly collected breasts/group approximately 24 h after processing. For each defect, a 3 point-scale evaluation system (NOR: normal; MOD: moderate; SEV: severe) was used to classify the magnitude of the myopathy. All the scorings were performed by the same operator in the same environmental conditions. For WS, the classification criteria was the dimension of white striation (Kuttapan et al., 2012), whereas the hardness at

palpation was used for the WB defect (Sihvo et al., 2014). Finally, the proneness to show muscle deconstruction in response to an external stimulus (finger pinching), as described by Sirri et al. (2016), was used to score the breasts according to the SM defect.

3.2.6. Meat quality attributes

Twelve breasts/group not showing macroscopic defects (e.g. visual signs of muscle myopathies, hemorrhages, or lesions), and obtained from carcasses with BW similar to the average BW of each group, were collected and used to assess meat quality attributes and proximate composition. Breast muscle pH was determined 48 h post-mortem using a modification of the iodoacetate method (Jeacocke, 1977) as previously reported (Sirri et al., 2017). The system color profile (CIE, 1978) of breast muscle was obtained by a reflectance colorimeter (Minolta Chroma Meter CR-300, Minolta Italia S.p.A., Milan, Italy) using illuminant source C. The results were reported as lightness (L^*), redness (a^*), and yellowness (b^*) and represent the average of 3 independent measurements performed on the medial surface of the fillet (bone side) in an area showing no evident color defects. In addition, a parallelepiped meat cut (8 cm × 4 cm × 3 cm) weighing about 80 g was excised from the cranial part of each fillet and used to determine drip (of refrigerated storage) and cooking losses (in a water bath at 80°C for 45 min) using the same procedures described in our previous study (Sirri et al., 2017). A second parallelepiped meat cut (8 cm × 4 cm × 2 cm) weighing about 60 g was excised from the middle part of each fillet and was individually labeled and tumbled with a 15% (wt/wt) brine solution containing sodium tripolyphosphate (2.3%) and sodium chloride (7.6%) and subsequently cooked in a water bath at 80°C for 25 min. Marinade uptake and cooking losses were calculated for each sample.

Proximate analysis was performed on breast meat samples to assess moisture, crude protein, total fat and ash content. Moisture and ash were obtained in duplicate according to the procedure described by the AOAC (1990). Total fat and crude protein content was determined using the

chloroform:methanol extraction procedure reported by Folch et al. (1957) and the standard Kjeldahl copper catalyst method (AOAC, 1990), respectively.

3.2.7. Plasma and muscle metabolomics analysis

Plasma samples were prepared for proton NMR (^{1}H-NMR) analysis by centrifuging 650 µL of each sample for 15 min at 15,000 r/min (18,630 x *g*) and 4°C. 500 µL of supernatant were added to 100 µL of a D_2O solution of 2,2,3,3-D4-3-(trimethylsilyl)-propionic- acid sodium salt 10 mmol/L, used as NMR chemical-shift reference, buffered at pH 7.00 by means of 1 mol/L phosphate buffer. Finally, each sample was centrifuged again at the above conditions.

Meat samples were prepared for NMR analysis by adding 0.5 g of meat to 3 mL distilled water and by homogenizing the mixture for 2 min by means of a high-speed disperser (IKA, USA). One mL of the obtained sample was centrifuged for 15 min at 15,000 r/min (18,630 x *g*) and 4°C. To remove fat from samples, 700 µL of supernatant were added to 800 µL $CHCl_3$, vortex mixed for 3 min and centrifuged again at the above conditions. 500 µL of supernatant were added to 200 µL of a D_2O solution of 2,2,3,3-D4-3-(trimethylsilyl)-propionic acid sodium salt 10 mmol/L, used as NMR chemical-shift reference, buffered at pH 7.00±0.02 by means of 1 mol/L phosphate buffer. 10 µL of NaN_3 2 mmol/L were also added to avoid microbial proliferation. Finally, each sample was centrifuged again at the above conditions.

^{1}H-NMR spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz. Following Ventrella et al. (2016), the signals from broad resonances originating from large molecules were suppressed by a CPMG-filter composed by 400 echoes with a τ of 400 µs and a 180° pulse of 24 µs, for a total filter of 330 ms. The water residual signal was suppressed by means of presaturation. This was done by employing the cpmgpr1d sequence, part of the standard pulse sequence library. Each spectrum was acquired by summing up 256 transients using 32,000 data points over a 7184 Hz spectral window, with an

acquisition time of 2.28s. In order to apply NMR as a quantitative technique (Barbara et al., 2016), the recycle delay was set to 5s, keeping into consideration the relaxation time of the protons under investigation. ^{1}H-NMR spectra were baseline-adjusted by means of the peak detection according to the "rolling ball" principle (Kneen and Annegarn, 1996) implemented in the baseline R package (Liland et al., 2010). In order to make the points pertaining to the baseline randomly spread around zero, a linear correction was then applied to each spectrum. Differences in water and fibers content among samples were taken into consideration by probabilistic quotient normalization (Dieterle et al., 2006) applied to the entire spectra array. The signals were assigned by comparing their chemical shift and multiplicity with the Human Metabolome Database (Wishart et al., 2007) and Chenomx software library (Chenomx Inc., Canada, ver. 10). This was done by taking advantage of the "autofit" utility of Chenomx software (ver. 8.3).

3.2.8. Statistical analysis

Once assessed that the effect of the block as well as the interaction between block and dietary treatments were not significant, block effect was not considered in the analysis and productive performance data were analyzed applying the Student T-test (SAS Institute, 1988) considering the dietary supplementation of L-arginine as independent variable. Pen was considered as the experimental unit for productive performance data. Prior to analysis, mortality data were submitted to arcsine transformation. Similarly, meat quality attributes were analyzed by means of the Student T-test (SAS Institute, 1988), considering the bird as experimental unit. The occurrence of FPD and breast meat abnormalities was analyzed using the Chi-square test considering the bird as experimental unit. Differences were considered statistically significant when P-value was lower 0.05.

Regarding metabolomics, molecules whose concentration varied in relation to the dietary supplementation of L-arginine were compared by means of Wilcoxon test in agreement with

previous investigations (Ventrella et al., 2016; Foschi et al., 2018). For this purpose, a significance limit P-value of 0.05 was accepted. To highlight the underlying trends characterizing the samples, principal component analysis model in its robust version (rPCA) was built on the molecules concentrations, centered and scaled to unity variance, according to Hubert et al. (2015). For each rPCA model, the scoreplot, that is the projection of the samples in the PC space tailored to highlight the underlying structure of the data, was calculated. Besides, the correlation plot was obtained by relating the concentration of each variable to the components of the rPCA model, therefore tailored to highlight the most important molecules in determining the trends highlighted by the scoreplot.

3.3. Results

3.3.1. Productive performance and slaughtering measurements

The productive performance results are reported in Table 3.2. Both the experimental groups showed similar body weight at the beginning of the trial. After 12 d, ARG group showed a lower FCR (1.352 vs. 1.401, for ARG and CON, respectively; $P<0.05$) whereas BW, DWG, and DFI remained unaffected between the groups. At 22 d, cumulative FCR was significantly lower in ARG compared to the control group (1.398 vs. 1.420, $P<0.01$). The dietary supplementation did not elicit any significant effect on other productive traits. After 33 d, ARG-fed birds exhibited higher BW (1,884 vs. 1,829 g, $P<0.05$) and lower cumulative FCR (1.494 vs. 1.524, $P<0.05$) compared to the CON-fed group. Furthermore, DWG tended to be higher and FCR tended to be lower in ARG compared to CON group (93.1 vs. 89.3 g/bird/d, and 1.571 vs. 1.610, respectively for ARG and CON; $P= 0.08$). In the finisher feeding phase (34-43d), no significant difference was observed between the experimental groups. In the overall period of trial (0-43 d), the dietary supplementation of arginine tended to improve FCR (1.646 vs. 1.675, respectively for ARG and CON; $P= 0.09$), while it had only limited effect on BW, DWG and DFI. Mortality rate was not significantly affected by the dietary treatment in each feeding phase as well as in the overall period of trial.

At processing, eviscerated carcass yield was 71.3 and 70.9% for ARG and CON, respectively. Skinless breast yield, expressed as percentage of carcass weight, was 30.4 and 29.3% for ARG and CON, respectively. As shown in Figure 3.1, no significant effect of dietary arginine supplementation was detected on the incidence and severity of FPD.

3.3.2. Incidence of breast muscle myopathies and meat quality

The dietary supplementation of arginine did not affect the incidence and severity of WS, WB and SM (Table 3.3). The results of the evaluation of the breast meat quality attributes are shown in Table 3.4. Breast meat pH, color, drip and cooking losses, as well as marinade uptake and purging loss, showed no significant change in response to the dietary treatment. Considering the proximate composition of breast meat, the dietary supplementation of arginine had no significant effect on moisture, crude protein, total fat, as well as ash content (Table 3.4).

3.3.3. Plasma and breast muscle metabolome

^{1}H-NMR spectra were registered on plasma samples and 62 molecules quantified. Six molecules, listed in Table 3.5, exhibited significant variation in their plasma concentration in response to the dietary supplementation of arginine. ARG birds showed significantly higher plasma arginine and leucine concentrations, whereas plasma acetoacetate, adenosine, glutamate and proline were more abundant in CON birds. To obtain an overview about the molecules undergoing the greatest differences between the groups, the 6 molecules of Table 3.5 were employed as a basis for a rPCA model shown in Figure 3.2.

In parallel to what was done on plasma, ^{1}H-NMR spectra were obtained from breast muscle samples. From a total of 37 quantified molecules, 4 showed a significantly different concentration between CON and ARG group. Breast muscle from ARG group exhibited higher levels of arginine

and acetate and lower levels of acetone and inosine (Table 3.6). The rPCA model obtained using the 4 molecules of Table 3.6 is shown in Figure 3.3.

3.4. Discussion

In the present study, broilers were fed either a basal diet (CON group), formulated to meet or slightly exceed the current recommendations (Aviagen, 2014) and widely used in commercial practice, or the same basal diet supplemented with crystalline L-arginine (ARG group) to increase the digestible Arg:Lys ratio. Considering other published studies aimed at evaluating the effects of the dietary supplementation of arginine in broilers, huge differences regarding bird's genotype, age, and gender, composition of the basal diet, number and length of feeding phases, and inclusion rate of arginine, were observed and therefore care should be used in comparing results from different studies (Brake and Balnave, 1995; Kidd et al., 2001; Corzo and Kidd, 2003; Corzo et al., 2003; Rueda et al., 2003; Escobar et al., 2005; Jiao et al., 2010; Fouad et al., 2013; Ebrahimi et al., 2014; Laika and Jahanian, 2017; Christensen et al., 2015; Jahanian and Khalifeh-Gholi, 2018; Xu et al., 2018).

As for productive aspects, the dietary supplementation of arginine at the level tested in this study improved cumulative FCR at 12, 22, and 33 d and tended to improve it in the overall period of trial (0-42 d). ARG birds exhibited improved FCR in each feeding phase, even though significant differences between the groups were detected only in the starter phase (0-12 d). Corzo and Kidd (2003) stated that the dietary supplementation of arginine might exert positive effects during the starter phase by counteracting the early microbial challenges and aiding the immune system development. Similarly to our findings, Jahanian and Khalifeh-Gholi (2018) reported that broilers fed a diet with an arginine level of 100% of NRC recommendation (total Arg:Lys = 1.14 and 1.10 in starter and grower phase, respectively) exhibited lower FCR at 21 d, as well as from 1 to 42 d, if compared to broilers receiving an arginine-deficient diet (90% NRC, Arg:Lys = 1.02 and 0.99 in

starter and grower phase, respectively). It has been reported that feed efficiency was affected at 10, 24 and 46 d of age by increasing the level of digestible arginine from 100% of Ross recommendations to 153, 168 and 183% (Ebrahimi et al., 2014). On the other hand, no significant difference was observed in terms of FCR in broilers fed diets with arginine levels either to meet (100%) or exceed (105 and 110%) the NRC recommendations (Laika and Jahanian, 2017). Similarly, the administration of graded levels of arginine (0.45, 0.90, 1.35 and 1.90%) in an arginine-deficient diet (total Arg:Lys = 0.67 and 0.69 in starter and grower, respectively) did not exert any significant effect on FCR from 0 to 21 d and from 21 to 42 d of broilers age, even if a quadratic response was observed in the overall period of trial (Xu et al., 2018). When increasing the total dietary Arg:Lys ratio from 1.17 to 2.10 between 21 and 42 d, Fouad et al. (2013) observed no significant alteration in feed to gain ratios of broilers.

In the present study, broilers receiving the arginine-supplemented diet also showed a significantly higher BW at 33 d while both groups reached the same live-weight at slaughter. It has been reported that the dietary supplementation of arginine from 21 to 42 d (total Arg:Lys ratio = 1.17, 1.40, 1.63 and 2.10) had no effect on BW at processing (Fouad et al., 2013). Xu et al. (2018) observed a quadratic improvement in BW both at 21 and 42 d of age in response to the dietary supplementation of arginine, with the birds fed either a arginine-deficient diet (total Arg:Lys ratio = 0.67 and 0.69 in starter and grower, respectively) or the highest level of arginine supplementation (total Arg:Lys ratio = 2.07 and 2.53 in starter and grower, respectively) showing lower BW compared to the others. Moreover, DFI was similar between CON and ARG group, indicating that the dietary supplementation of arginine did not exert any effect on feeding behavior of broiler chickens at different age. This observation is in accordance with other previous studies (Corzo and Kidd, 2003; Fouad et al., 2013; Jahanian and Khalifeh-Gholi, 2018; Ebrahimi et al., 2014; Laika and Jahanian, 2017; Xu et al., 2018). However, Corzo et al. (2003) reported a significant effect of progressive amounts of dietary arginine from 42 to 56 d of broiler age on feed consumption.

Furthermore, mortality rate was similar between the two experimental groups, which is in line with previous findings (Kidd et al., 2001; Corzo and Kidd, 2003; Corzo et al., 2003; Fouad et al., 2013). Based on the results obtained in the present study, the Arg:Lys ratios currently adopted at least in Countries where the animal protein sources are not allowed in feed formulation (i.e. European Union) appears not adequate to exploit the productive potential of modern fast-growing broiler chickens.

No significant difference was observed between the groups concerning the incidence and severity of WS, WB and SM. It has been previously reported that the administration of diets with an Arg:Lys ratio of 0.95 and 1.25 exerted no significant effect on the occurrence of WS and WB in 53-d-old broilers (Christensen et al., 2015). Bodle et al. (2018) recently reported that increasing the digestible Arg:Lys ratio to from approximately 111-113% to 120-125% reduced the severity of WB while had no effects on WS.

Quality attributes and proximate composition of breast meat were not significantly affected by the arginine supplementation. It has been reported that the dietary supplementation of 153% of digestible arginine in a control diet significantly increased crude protein and dry matter content in breast meat, whereas ash and fat content were significantly improved by supplementing 183% and 168% of digestible arginine, respectively (Ebrahimi et al., 2014). On the other hand, Fouad et al. (2013) observed no significant alteration in the intramuscular fat content of breast muscle of broilers fed diets with different concentrations of arginine (total Arg:Lys ratio = 1.17, 1.40, 1.64 and 2.10) from 21 to 42 d. Considering breast meat quality traits, administering diets with arginine levels from 80 to 140% of NRC recommendation increased L* value and cooking loss, while showed no effects on a* and b* value and drip loss (Jiao et al., 2010). The dietary supplementation of 0.80, 0.95, 1.10 and 1.25% of L-arginine from 42 to 56 d significantly affected lightness (L*) and yellowness (b*) of breast fillets (Corzo et al., 2003). Finally, also the incidence and severity of foot pad dermatitis exhibited no significant difference in response to the dietary treatment.

Concerning metabolomics, the rPCA models showed differential levels of plasma and muscle metabolites between groups (Figure 3.2 and 3.3, respectively) indicating a clear separation of them according to the dietary supplementation of arginine. In fact, increasing the level of dietary arginine significantly enhanced the plasma concentration of arginine and leucine while reduced that of proline, glutamate, acetoacetate and adenosine. In addition, ARG birds exhibited higher levels of breast muscle arginine and acetate, whereas the concentration of acetone and inosine was reduced. According to these findings, the dietary supplementation of L-arginine was able to increase its concentration in both plasma and *Pectoralis major* muscle, indicating that arginine can be effectively absorbed by the intestinal epithelium and can enter the systemic circulation reaching peripheral tissues such as breast muscle. As previously reported, dietary arginine is absorbed in the small intestine using both sodium-dependent and –independent mechanisms with the latter showing a greater effectiveness. As most of the arginase activity is located in the kidney, a substantial amount of dietary arginine may have passed the brush border and then entered the systemic circulation with only limited degradation. Once in the muscle, arginine could stimulate protein synthesis and cell proliferation (Fouad et al., 2010). Moreover, plasma concentration of leucine appeared higher in birds receiving the arginine-supplemented diet. Higher plasma levels of leucine have been associated with a greater protein synthesis in skeletal muscle of pigs (Escobar et al., 2005). Similarly, Baeza et al. (2015) reported a positive correlation between *Pectoralis major* weight and plasma histidine concentration, which was numerically higher in ARG birds (4.85×10^{-2} vs. 3.97×10^{-2} mmol/L, respectively for ARG and CON group, $P<0.1$; data not shown). Taken together, these results indicate that the dietary arginine supplementation may improve anabolic processes within breast muscle probably via protein synthesis enhancement and this merit further in-depth investigations.

Furthermore, the dietary supplementation of arginine appears to modulate energy and protein metabolism. Two ketone bodies, acetoacetate and acetone, showed lower concentrations in

ARG plasma and breast muscle, respectively. Ketone bodies can be recruited from blood circulation by peripheral tissues, including breast muscle, and catabolized to produce energy. Therefore, these findings may indicate an increased utilization of ketone bodies in peripheral tissues in response to the dietary supplementation of arginine. Fouad et al. (2010) reported that dietary arginine supplementation can modulate body fat deposition in chickens. Indeed, Fouad et al. (2013) associated the lower abdominal fat deposition in response to the dietary supplementation of arginine to both the increased expression of genes involved in fatty acid β-oxidation and to the reduced expression of fatty acid synthase gene in heart and liver, respectively. A potential effect of the dietary supplementation of arginine on energy and fat metabolism has been previously reported also in meat-type ducks (Wu et al., 2011).

Glutamate and proline, both resulting from arginine metabolism (Fernandes and Murakami, 2010; Fouad et al., 2010), also showed lower concentration in plasma of ARG birds. In mammals, glutamate has been reported to be associated with several physiological aspects such as cell proliferation, biosynthesis of neurotransmitters and other amino acids, immune functionality, acid-base balance and gene expression (Newsholme et al., 2003). Proline is involved in important biological functions related cellular metabolism, including the regulation of gene expression and cell differentiation, scavenging oxidants, protein synthesis and structure, cell signaling and bioenergetics (Wu et al., 2011). However, in particular metabolic conditions (e.g., nutritional or metabolic stress), glutamate can participate to gluconeogenesis in kidney (Newsholme et al., 2003) or enter the citric acid cycle (Krebs cycle) (Scanes, 2015). Similarly, proline metabolism can generate electrons which can enter the mitochondrial electron transport chain to produce ATP (Phang et al., 2008; 2010). Otherwise, proline can be also degraded to produce α-ketoglutarate, an intermediate of the citric acid (Krebs) cycle (Scanes, 2015). Therefore, it may be hypothesized that the lower concentration of glutamate and proline in plasma of ARG birds may be due to an

increased recruitment and utilization of these amino acids in peripheral tissues, possibly the skeletal muscle, to provide energy substrates for the cell.

Inosine represents a metabolite of ATP degradation which can be converted to hypoxanthine and then released into blood circulation (McConell et al., 2005; Bishop, 2010). Plasma concentration of hypoxanthine was higher in birds received the dietary supplementation of arginine compared to CON (6.80 $\times 10^{-3}$ vs. 3.92 $\times 10^{-3}$ mmol/L, $P<0.1$; data not shown) suggesting that muscle ATP could have been catabolized to provide energy within the cell. Although the molecular mechanism is still unknown, the increased concentration of acetate in breast muscle suggests that the muscle acetate-mevalonate pathway is activated to promote muscle cell development through steroids and/or triterpenoids.

Finally, the adenosine concentration was also reduced in plasma of birds fed the supplemented diet. In mammals, adenosine could be released in plasma by endothelial cells and myocytes in response to ischemia, hypoxia, or oxidative stress (Eltzschig et al., 2006; Hack et al., 2006). L-arginine has also been shown to have marked antioxidant properties (Wallner et al., 2001). Therefore, the reduction of plasma adenosine might be related to the potential effect of arginine on oxidative status and hypoxic condition likely occurring in breast muscle of fast-growing broiler chickens.

A global hypothesis of the molecular responses to the dietary supplementation of arginine is reported in Figure 3.4. Overall, arginine supplementation could stimulate anabolic processes within the muscles and improve FE. The increased energy depletion, as suggested by the lower value of inosine in breast muscle and the increased concentration of hypoxanthine in plasma, appears consistent with this hypothesis. In turn, skeletal muscle cells may have stimulated the recruitment of several plasma metabolites (e.g. acetoacetate, glutamate, proline) which can be used to restore the energy pool through energy producing pathways (e.g. Krebs cycle).

In conclusion, based on our experimental conditions, the Arg:Lys ratios currently adopted at least in Countries where the animal protein sources are not allowed in feed formulation (i.e. European Union) appear to be inadequate to exploit the maximum productive potential of modern fast-growing broilers, with particular regard to FE. The Arg:Lys ratios tested herein had positive effects on FE without showing any negative effect on meat quality attributes, foot pad condition and incidence of breast meat abnormalities. Furthermore, plasma and muscle metabolome showed significant alterations in response to the arginine supplementation. According to this analysis, the improvements observed in FE in the supplemented group might be likely ascribed to a potential modulatory effect of arginine on energy and protein metabolism and hence on the overall energy homeostasis in broiler chickens. In addition, the present study confirms the usefulness of NMR-based approaches in investigating the molecular responses to different dietary treatments in avian species. Further studies are warranted to investigate the effects of graded Arg:Lys ratios on productive aspects and meat quality attributes in broiler chickens. In addition, other mechanistic studies are necessary to define and delineate the role of arginine on energy and protein metabolism in breast muscle as well as in other tissues, such as liver and adipose tissue.

Table 3.1 Composition of the basal diet in each feeding phase.

Items	0-12 d	13-22 d	23-33 d	34-43 d
Ingredients, g/100g				
Corn	33.4	36.7	19.2	15.0
White corn	0.00	0.00	15.0	18.1
Wheat	20.0	20.0	25.0	30.0
Vegetable oil	2.45	2.68	3.61	3.97
Soybean meal 48%	18.2	20.2	14.2	9.33
Full-fat soybean	10.0	10.0	15.0	15.0
Concentrated SBM	5.00	0.00	0.00	0.00
Corn gluten	2.00	2.00	0.00	0.00
Pea	3.00	3.00	3.00	3.00
Sunflower	2.00	2.00	2.00	3.00
Lysine sulphate	0.54	0.53	0.46	0.43
DL-Methionine	0.29	0.00	0.00	0.00
Methionine hydroxy analogue	0.00	0.32	0.33	0.26
L-Threonine	0.12	0.11	0.10	0.08
Choline chloride	0.10	0.10	0.05	0.00
Calcium carbonate	0.53	0.52	0.60	0.69
Dicalcium phosphate	1.29	0.80	0.47	0.21
Sodium chloride	0.29	0.30	0.23	0.21
Sodium bicarbonate	0.05	0.05	0.15	0.25
Premix vit.-min.[1]	0.54	0.46	0.38	0.30
Phytase	0.05	0.05	0.05	0.05
Xylanase	0.05	0.05	0.05	0.05
Emulsifier	0.08	0.08	0.08	0.08
Proximate composition				
AME, MJ/kg	13.0	13.2	13.7	13.9
Dry matter*, %	88.8	88.2	88.5	88.5
Crude protein*, %	23.2	22.8	19.8	18.2
Total lipid*, %	6.25	6.51	8.29	8.64
Crude fiber, %	2.96	2.92	2.99	3.08
Ash*, %	5.24	4.60	4.29	4.03
Ca (total), %	0.77	0.62	0.55	0.50
P (total), %	0.61	0.51	0.44	0.38
Dig. Lysine*, %	1.25	1.15	1.05	0.94
Dig. Arginine*, %	1.32	1.21	1.11	1.00
Dig. Met.+Cys*, %	0.93	0.85	0.79	0.70
Dig. Threonine*, %	0.81	0.75	0.68	0.61
Dig. Valine*, %	0.94	0.87	0.79	0.72
Dig. Isoleucine*, %	0.84	0.77	0.70	0.63
Dig. Arg:Lys	1.06	1.05	1.06	1.07
Dig. Lys:AME, g/MJ	0.96	0.87	0.77	0.68

[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.
*Analysed values. Amino acid concentration of the experimental diets was analyzed by AMINOLab® (Evonik Industries, Hanau, Germany) and results reported outside the brackets. Digestible amino acid (dig.) values were calculated by multiplying digestibility coefficients (Ajinomoto Heartland, 2015) to the analyzed total amino acid content of each ingredient.

Table 3.2 Productive performance of broiler chickens fed a commercial basal diet (CON, digestible Arg:Lys = 1.05, 1.05, 1.06, 1.07 in starter, grower I, grower II and finisher feeding phase, respectively) or the same basal diet supplemented with L-arginine (ARG, digestible Arg:Lys = 1.15, 1.15, 1.16, 1.17, respectively).

Variables	CON	ARG	SEM	*P*-value
n.	9	9		
0-12 d				
Chick body weight, g	37.1	36.9	0.09	0.27
Body weight, g	288.5	293.0	2.47	0.38
Daily weight gain, g/bird/d*	21.0	21.3	0.20	0.39
Daily feed intake, g/bird/d*	29.3	28.8	0.19	0.21
Feed conversion rate (0-12 d)*	1.401	1.352	0.01	0.02
Mortality, %	0.00	0.17	0.01	0.33
13-22 d				
Body weight, g/bird	846.7	856.1	6.08	0.46
Daily weight gain, g/bird/d*	55.8	56.3	0.43	0.56
Daily feed intake, g/bird/d*	79.7	79.8	0.60	0.92
Feed conversion rate (13-22 d)*	1.429	1.419	0.01	0.37
Cumulative feed conversion rate (0-22 d)*	1.420	1.398	0.01	<0.01
Mortality, %	0.34	0.52	0.02	0.69
23-33 d				
Body weight, g/bird	1,829	1,884	12.8	0.03
Daily weight gain, g/bird/d*	89.3	93.1	1.11	0.08
Daily feed intake, g/bird/d*	143.6	145.5	1.02	0.39
Feed conversion rate (23-33 d)*	1.610	1.571	0.01	0.09
Cumulative feed conversion rate (0-33 d)*	1.524	1.494	0.01	0.02
Mortality, %	0.17	0.35	0.01	0.55
34-43 d				
Body weight, g/bird	2,864	2,920	26.0	0.30
Daily weight gain, g/bird/d*	101.6	102.3	1.62	0.83
Daily feed intake, g/bird/d*	197.1	196.9	1.41	0.94
Feed conversion rate (34-43 d)*	1.949	1.926	0.02	0.62
Mortality, %	1.37	1.71	0.02	0.62
0-43 d				
Body weight, g/bird	2,864	2,920	26.0	0.30
Daily weight gain, g/bird/d*	65.7	67.0	0.60	0.30
Daily feed intake, g/bird/d*	109.1	109.2	0.54	0.94
Feed conversion rate (0-43)*	1.675	1.646	0.01	0.09
Mortality, %	1.88	2.74	0.02	0.21

* corrected for mortality; a, b: $P < 0.05$; A, B: $P < 0.01$.

Table 3.3 Incidence and severity of white striping, wooden breast and spaghetti meat defect in breast muscle of broiler chickens fed a commercial basal diet (CON, digestible Arg:Lys = 1.05, 1.05, 1.06, 1.07 in starter, grower I, grower II and finisher feeding phase, respectively) or the same basal diet supplemented with L-arginine (ARG, digestible Arg:Lys = 1.15, 1.15, 1.16, 1.17, respectively).

Variables	CON	ARG
n.	150	150
White striping		
0 (no lesions), %	17	7
1 (mild lesions), %	52	53
2 (severe lesions), %	31	39
Chi-square	0.08	
Wooden breast		
0 (no lesions), %	43	44
1 (mild lesions), %	38	37
2 (severe lesions), %	19	19
Chi-square	0.99	
Spaghetti meat		
0 (no lesions), %	65	60
1 (mild lesions), %	29	33
2 (severe lesions), %	6	7
Chi-square	0.77	

Table 3.4 Meat quality attributes and proximate composition of *Pectoralis major* muscle belonging to broiler chickens fed a commercial basal diet (CON, digestible Arg:Lys = 1.05, 1.05, 1.06, 1.07 in starter, grower I, grower II and finisher feeding phase, respectively) or the same basal diet supplemented with L-arginine (ARG, digestible Arg:Lys = 1.15, 1.15, 1.16, 1.17, respectively).

Variables	CON	ARG	SEM	*P*-value
n.	12	12		
Meat quality attributes				
pH 48 h post-mortem	5.81	5.76	0.05	0.44
Lightness (L*)	59.5	60.4	0.94	0.29
Redness (a*)	2.07	2.07	0.25	0.75
Yellowness (b*)	5.85	6.46	0.44	0.23
Drip loss, %	1.97	1.81	0.18	0.47
Cooking loss – raw meat, %	15.4	15.4	1.02	0.98
Marinade uptake, %	10.6	9.9	0.95	0.61
Cooking loss – marinated meat, %	12.5	12.9	0.70	0.58
Proximate composition				
Moisture, %	76.4	76.8	0.49	0.20
Crude protein, %	21.7	21.8	0.43	0.91
Total fat, %	1.71	1.61	0.19	0.59
Ash, %	1.40	1.34	0.17	0.75

Table 3.5 Relative concentration of differentially expressed plasma metabolites in broiler chickens received a commercial basal diet (CON, digestible Arg:Lys = 1.05, 1.05, 1.06, 1.07 in starter, grower I, grower II and finisher feeding phase, respectively) or the same basal diet supplemented with L-arginine (ARG, digestible Arg:Lys = 1.15, 1.15, 1.16, 1.17, respectively).

Metabolite[a]	CON	ARG	Trend	*P*-value
n.	*9*	*9*		
Arginine, mmol/L	$4.30\times10^{-3}\pm6.86\text{x}10^{-5}$	$5.64\times10^{-3}\pm1.08\times10^{-4}$	↑	0.004
Leucine, mmol/L	$1.79\times10^{-1}\pm2.41\times10^{-3}$	$2.11\times10^{-1}\pm3.93\times10^{-3}$	↑	0.01
Acetoacetate, mmol/L	$5.94\times10^{-2}\pm1.60\times10^{-3}$	$4.35\times10^{-2}\pm1.08\times10^{-3}$	↓	0.02
Glutamate, mmol/L	$7.31\times10^{-2}\pm6.31\times10^{-4}$	$6.56\times10^{-2}\pm4.96\times10^{-4}$	↓	0.01
Adenosine, mmol/L	$1.38\times10^{-3}\pm2.39\times10^{-4}$	$7.84\times10^{-5}\pm3.92\times10^{-5}$	↓	0.04
Proline, mmol/L	$1.23\times10^{-1}\pm2.18\times10^{-3}$	$1.05\times10^{-1}\pm2.06\times10^{-3}$	↓	0.04

[a] Results are reported as mean ± SEM.

Table 3.6. Relative concentration of differentially expressed metabolites in breast muscle of broiler chickens fed a commercial basal diet (CON, digestible Arg:Lys = 1.05, 1.05, 1.06, 1.07 in starter, grower I, grower II and finisher feeding phase, respectively) or the same basal diet supplemented with L-arginine (ARG, digestible Arg:Lys = 1.15, 1.15, 1.16, 1.17, respectively).

Metabolite[a]	CON	ARG	Trend	*P*-value
n.	*9*	*9*		
Arginine, mmol/L	$3.35\times10^{-4}\pm5.59\times10^{-6}$	$3.98\times10^{-4}\pm3.78\times10^{-6}$	↑	0.008
Acetate, mmol/L	$2.48\times10^{-4}\pm1.29\times10^{-6}$	$2.67\times10^{-4}\pm2.16\times10^{-6}$	↑	0.02
Inosine, mmol/L	$4.88\times10^{-4}\pm8.97\times10^{-6}$	$4.06\times10^{-4}\pm3.90\times10^{-6}$	↓	0.008
Acetone, mmol/L	$2.35\times10^{-5}\pm1.71\times10^{-6}$	$1.10\times10^{-5}\pm5.86\times10^{-7}$	↓	0.03

[a] Results are reported as mean ± SEM.

Figure 3.1 Incidence of foot pad dermatitis in broiler chickens fed a commercial basal diet (CON; n = 574; digestible Arg:Lys = 1.05, 1.05, 1.06, 1.07 in starter, grower I, grower II and finisher feeding phase, respectively) or the same basal diet supplemented with L-arginine (ARG; n = 569; digestible Arg:Lys = 1.15, 1.15, 1.16, 1.17, respectively) [0 = no lesions, 1 = mild lesions (<0.8 cm), and 2 = severe lesions (>0.8 cm)].

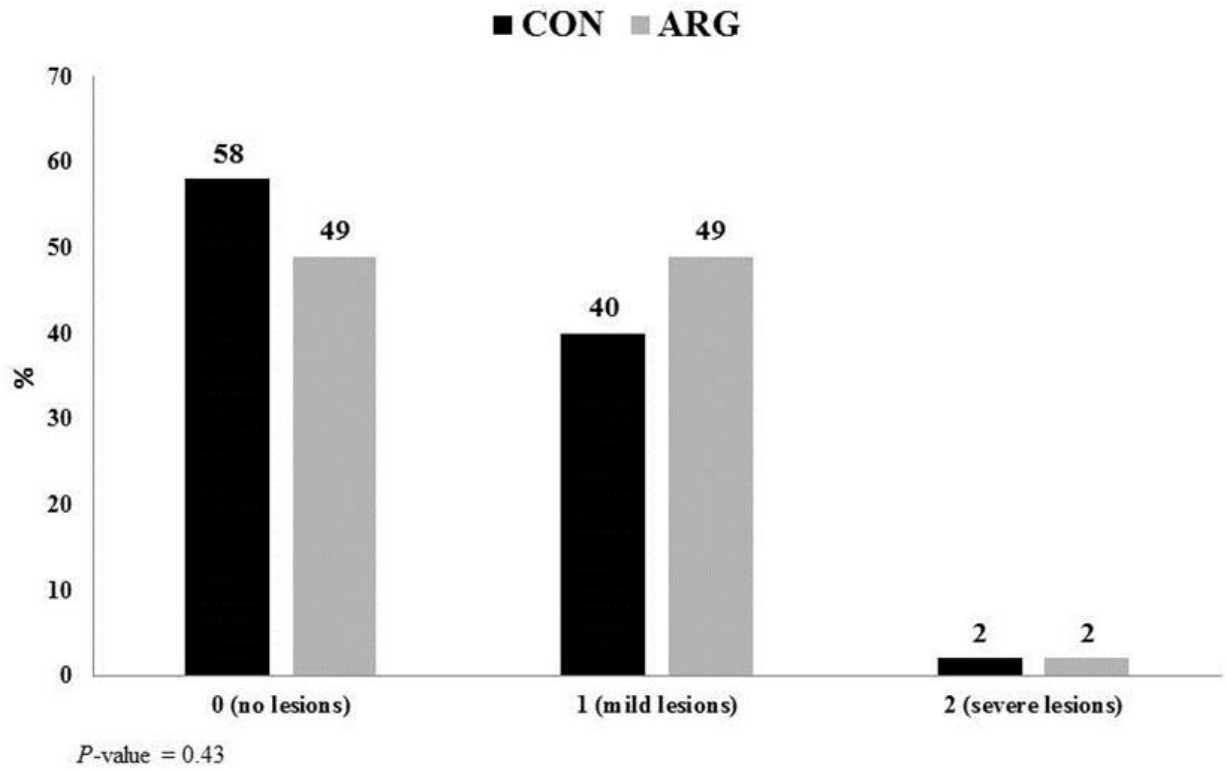
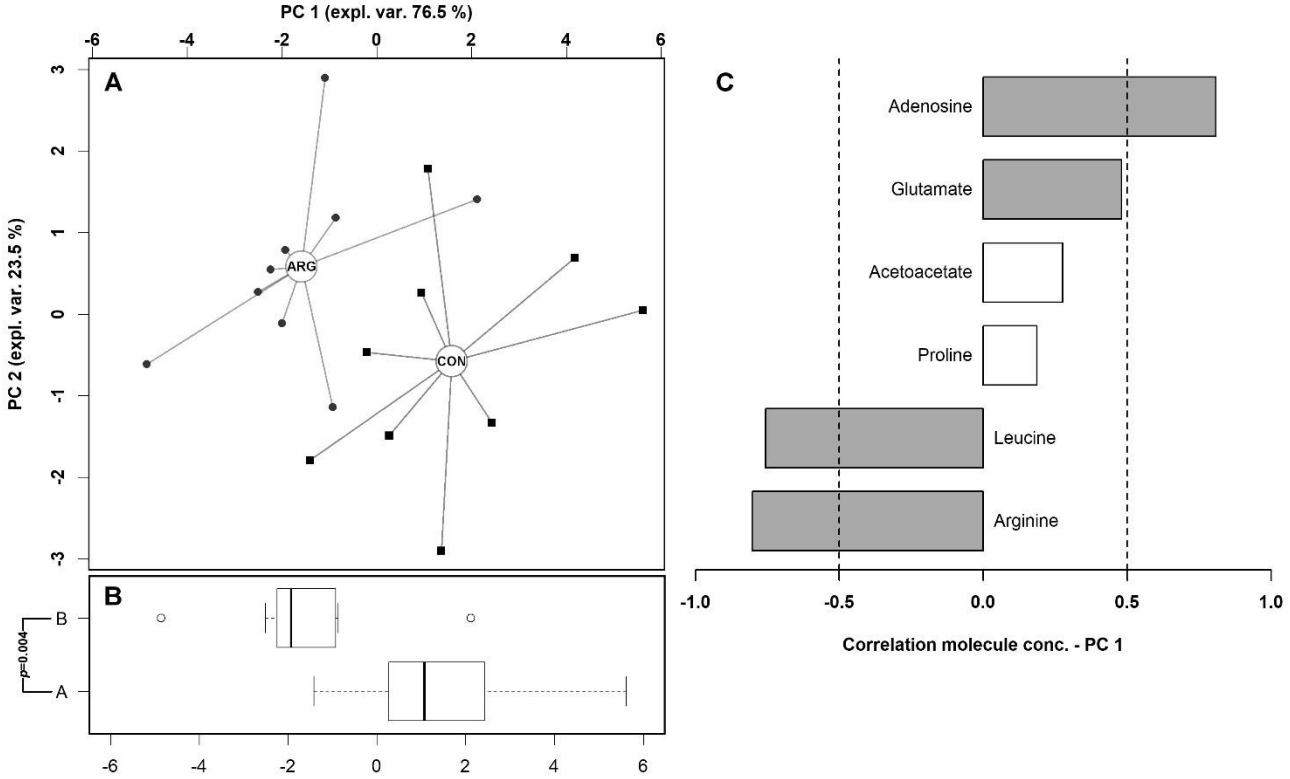
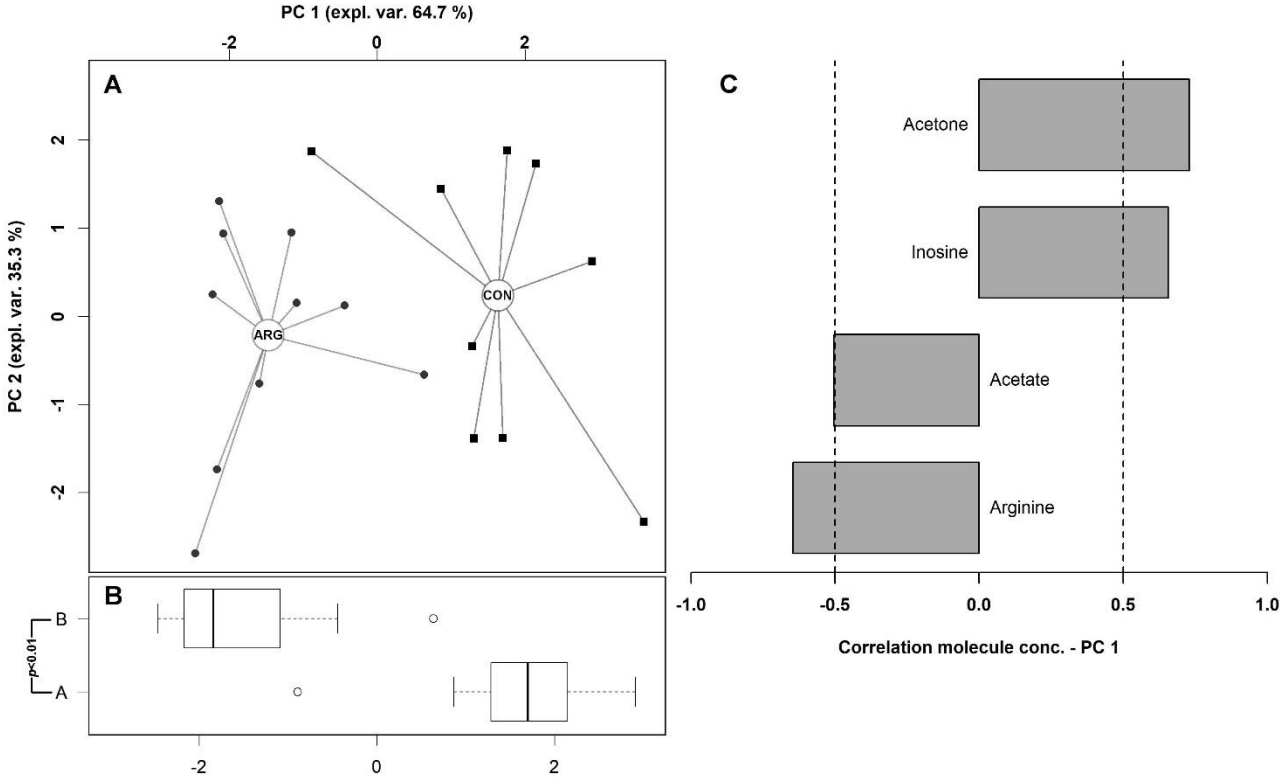


Figure 3.2 Robust Principal Component Analysis (rPCA) on plasma metabolites showing differential expression between CON (n = 9; digestible Arg:Lys = 1.05, 1.05, 1.06, 1.07 in starter, grower I, grower II and finisher feeding phase, respectively) and ARG group (n = 9; digestible Arg:Lys = 1.15, 1.15, 1.16, 1.17, respectively).



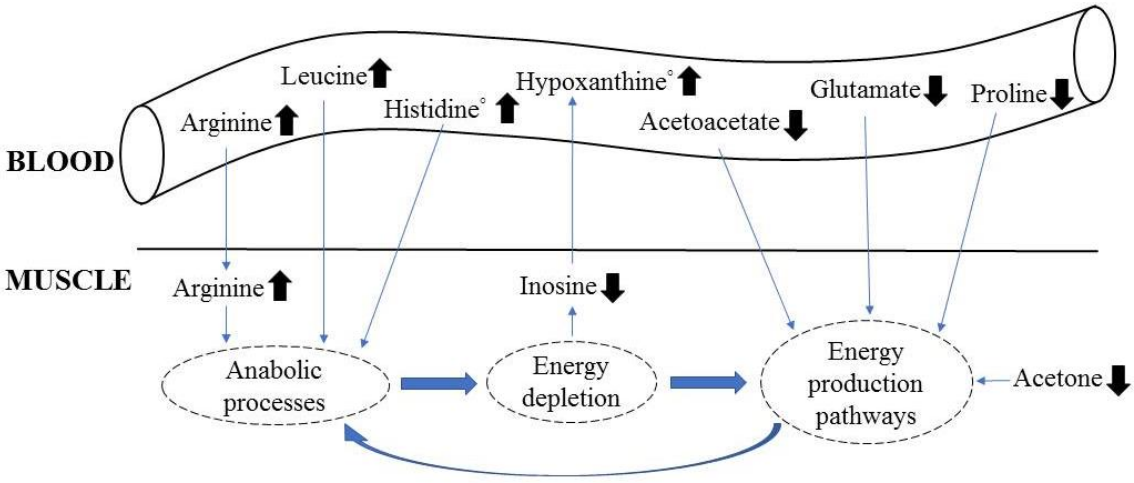
A) In the scoreplot, samples from chicken fed with different diets are represented with squares and circles respectively. The wide, empty circles represent the median of the samples.
B) Boxplot summarizing the position of the subjects along PC 1.
C) Loadingplot (C) reports the correlation between the concentration of each substance and its importance over PC 1. Highly significant correlations (p<0.05) are highlighted with gray bars.

Figure 3.3 Robust Principal Component Analysis (rPCA) on breast muscle metabolites showing differential expression between CON (n = 9; digestible Arg:Lys = 1.05, 1.05, 1.06, 1.07 in starter, grower I, grower II and finisher feeding phase, respectively) and ARG group (n = 9; digestible Arg:Lys = 1.15, 1.15, 1.16, 1.17, respectively).



A) In the scoreplot, samples from chicken fed with different diets are represented with squares and circles respectively. The wide, empty circles represent the median of the samples.
B) Boxplot summarizing the position of the subjects along PC 1.
C) Loadingplot (C) reports the correlation between the concentration of each substance and its importance over PC 1. Highly significant correlations (p<0.05) are highlighted with gray bars.

Figure 3.4 Hypothetical molecular responses to the dietary supplementation of L-arginine in broiler chickens.



Significantly upregulated; Significantly downregulated; °: *P*-value<0.1.

4. RESEARCH WORK #2: Comparison of growth performance and ileum transcriptomic profile in two modern fast-growing chicken hybrids.

4.1. Background and aim

The intestinal mucosa can be considered as a physical barrier between the molecules located in the gut lumen and the underneath tissues. Gut mucosa plays a crucial role in bird's physiology since it must be permeable to nutrients, electrolytes, and water, while at the same time should avoid bacteria and antigens translocation to the lamina propria. In addition, the small intestine also has important immunological, endocrine, and regulatory functions (Scanes and Pierzchala-Koziec, 2014) which can deeply affect health status (Sugiharto, 2016) as well as feeding behavior and overall energy homeostasis in chickens (Honda et al., 2017). A better knowledge of the metabolic dynamics in a key organ such as the small intestine might be useful to explain the phenotypical differences observed in growth performance and feed efficiency among different fast-growing chicken lines. Currently, microarray analysis offers the possibility to investigate the expression level of thousands of genes simultaneously, allowing to extrapolate important information regarding the biological pathways expressed in the examined tissues (Cogburn et al., 2003; 2007). In addition, useful information regarding bird's nutritional and physiological needs can be obtained, allowing in the future to formulate tailored diets with undoubtedly positive implications on productive efficiency, animal health and welfare, and environmental sustainability. Therefore, this study was undertaken to characterize productive traits and intestinal transcriptomic profile of two fast-growing chicken hybrids currently available for the poultry industry**.**

4.2. Materials and Methods

4.2.1. Animals, housing and diet

A total of 1,170 one-day-old female chicks belonging to 2 modern fast-growing hybrids (585 for each genotype, HA and HB respectively) both currently available for the poultry industry was obtained by a commercial hatchery. The broiler chickens lines tested in this study belong to 2

different breeding companies and are not genetically related. For each genotype, eggs were obtained by the same breeder flock and incubated in the same environmental conditions following the procedures commonly used in the hatchery. All the chicks were vaccinated against coccidiosis, infectious bronchitis virus, Marek's disease virus, Newcastle and Gumboro disease. The chicks, kept separated according to the genotype, were transferred to an environmental controlled poultry house and divided in 18 pens of 6 m^2 each (9 replications/group, 65 birds/replication, 11 birds/m^2). Stocking density was defined according to the European legislation in force (European Commission, 2007) to simulate the environmental conditions usually adopted in commercial practices. Inside the poultry house, pens were distributed in randomized blocks in order to minimize any environmental effect. Each pen was equipped with 2 circular pan feeders able to guarantee at least 2 cm of front space/bird and 10 nipples, while the floor was covered with chopped straw (2 kg/m^2). According to the legislation in force (European Commission, 2007), birds received 23L:1D of artificial light from 0 to 7 d and in the last 3 days before slaughter, whereas a photoperiod of 18L:6D was adopted in the remaining days. Both the experimental groups received the same commercial corn-wheat-soybean basal diet (Table 4.1) formulated according to the current recommendations. The feeding program was composed of 4 phases: starter (0-9 d), grower I (10-21 d), grower II (22-34 d) and finisher (35-43 d). All the diet switches were made uniformly for both the experimental groups. Water and feed were provided for *ad libitum* consumption. All the operations related to handling, raising and processing were in accordance with the European legislation (European Commission, 2007; 2009; 2010). The Ethical Committee of the University of Bologna approved the experimental protocol (ID: 928/2018).

4.2.2. Evaluation of productive performance

The number and weight of the birds present in each pen were recorded at housing (0 d), at each diet switch (9, 21, 35 d) and before slaughter (43 d). Similarly, feed was weighed at the beginning

(0, 10, 22, 35 d) and at the end of each feeding phase (9, 21, 34, 43 d), respectively. Number, age, and weight of birds that died during the trial were recorded to calculate mortality percentage. According to these measurements, body weight (BW), daily weight gain (DWG), feed intake (FI), daily feed intake (DFI) and feed conversion rate (FCR) were calculated on a pen basis and corrected for mortality.

At the end of the trial (43 d), all the birds were slaughtered separately per group in a commercial processing plant. Eviscerated carcass yield after air-chilling, as well as skinless breast, legs, and wings yields, were recorded on all the slaughtered broilers and reported on a group basis. During slaughtering operations, a foot from each bird was collected and the severity of foot pad dermatitis (FPD) was macroscopically evaluated using a 3-point scale evaluation system: 0 = no lesions; 1 = mild lesions (<0.8 cm); 2 = severe lesions (>0.8 cm) (Ekstrand et al., 1997).

4.2.3. Tissue Collection

At the processing plant (43 d), ileum was collected from 1 bird/replication selected with similar BW and clearly labelled. The ileum was longitudinally opened, and the internal surface washed with phosphate buffer saline (PBS) pH 7.4. Then, the mucosa was gently scraped with a microscope slide, put into sterile vials, immediately frozen in liquid nitrogen and preserved at -80°C until further processing.

4.2.4. RNA extraction and microarray analysis

Total mRNA was extracted from ileum mucosa homogenate using the TAKARA® Fast Pure Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Purity and concentration of the total extracted RNA were assessed by Nanodrop ND 1000 (Nanodrop Technologies, Wilmington, Delaware, USA) whereas RNA integrity was evaluated through Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). The analysis of whole transcript expression was performed by an outsource company (Cogentech Microarray Unit, Milan,

Italy) using Affimetrix© Chicken Gene Chip 1.1 ST array strips (Affymetrix, Santa Clara, California, USA). Hybridized arrays were scanned on a GeneAtlas imaging station (Affymetrix, Santa Clara, California, USA). Performance quality tests of the arrays, including the labeling, hybridization, scanning and background signals from a Robust Multichip Analysis were carried out on the CEL files using Affymetrix Expression ConsoleTM.

4.2.5. Statistical analysis

Productive performance data were analyzed by one-way ANOVA using the GLM procedure of SAS (SAS Institute, 1988) considering the genotype of the birds as the independent variable. Student Newman–Keuls test was used as post-hoc test to separate the means. Prior to analysis, mortality data were submitted to arcsine transformation. Frequency distribution of foot-pad lesions was analyzed using the Chi-square test. Differences were considered statistically significant when nominal P-value was lower than 0.05.

As for transcriptomics, an exploratory functional analysis was conducted on the processed gene expression values through the Gene Set Enrichment Analysis software using the C5.V6 catalog of gene sets (based on Gene Ontology (GO)) (http://software.broadinstitute.org/gsea/msigdb/index.jsp). The normalized enrichment score (NES) was calculated for each gene set and p-values of the NES were obtained from a gene set-based permutation procedure. Gene sets were considered significantly enriched when both NES p-value and False Discovery Rate q-value were lower than 0.05. Significantly enriched gene sets were then further classified according to their main biological function.

4.3. Results

4.3.1. Productive performance

The productive performance of both genotypes is shown in Table 4.2. At hatch, HA chicks were heavier than HB ones (43.8 vs. 40.1 g, respectively; $P<0.01$). After 9 days of the trial, HA birds

showed higher BW (228 vs. 217 g, respectively, for HA and HB; P<0.05) and lower FCR (1.352 vs. 1.419, respectively for HA and HB; P<0.05) compared to the counterpart. Moreover, DWG was higher in HA group (20.5 vs. 19.6 g/bird/d, respectively for HA and HB; P= 0.06). No significant difference between the groups was observed for DFI and mortality. At 21 d, HA birds exhibited lower BW (772 vs. 807 g, respectively for HA and HB; P<0.05), DWG (45.3 vs. 49.2 g/bird/d, respectively, for HA and HB; P<0.01) and DFI (70.6 vs. 74.9 g/bird/d, respectively for HA and HB; P<0.01). However, the two genotypes showed similar FCR and mortality from 10 to 21 d of trial. After 34 d, HA broilers showed lower BW than HB ones (1,857 vs. 1,930 g, respectively; P<0.05). From 22 to 34 d of trial, DWG and DFI tended to be lower in HA birds (83.4 vs. 86.2 g/bird/d, respectively for HA and HB; P= 0.07; and 139.6 vs. 144.4 g/bird/d, respectively; P= 0.07). In this feeding phase, neither FCR nor mortality exhibited significant differences between the two chicken genotypes. Considering the finisher feeding phase (35-43 d), HA birds reported lower BW (2,607 vs. 2,734 g, for HA and HB, respectively; P<0.01), DWG (83.3 vs. 91.9 g/bird/d, respectively; P<0.01) and DFI (182.4 vs. 196.5 g/bird/d, respectively; P<0.01), whereas similar values of FCR and mortality were observed. In the overall period of trial (0-43d), HA broilers showed lower DWG (59.6 vs. 62.6 g/bird/d, respectively; P<0.01) and DFI (104.7 vs. 110.3 g/bird/d, respectively; P<0.01). However, no significant differences between the groups were noticed for FCR and mortality. The two broiler chicken hybrids reported similar carcass yield (70.8 vs. 71.2%, respectively for HA and HB), as well as breast (31.3 vs. 30.8%), legs (42.2 vs. 43.9%) and wings (18.8 vs. 18.5%) yields.

The results of the evaluation of FPD occurrence are reported in Figure 4.1. The HA group showed a lower percentage of birds with no lesions (class 0) (48 vs. 75%, respectively for HA and HB; P<0.001), while exhibited a higher percentage of those with moderate lesions (class 1) (50 vs. 24%, respectively for HA and HB; P<0.001). However, both the genotypes tested in this trial

showed a very low percentage of birds with severe lesions (class 2) (2 and 1%, respectively for HA and HB).

4.3.2. Intestinal transcriptomic profile

A total of 114 and 179 gene sets resulted significantly enriched in the ileum mucosa of HA and HB broilers, respectively (Supporting Information Table 1 and 2, respectively). In the HA group (Table 4.3), a high percentage of biological gene sets involved in cellular energy metabolism and mitochondria structure and functionality was observed (43 and 23% of the total, respectively). Other gene sets that were significantly enriched in the ileum mucosa of HA birds were related to ribosome structure and protein synthesis (11%), cell structure and integrity (8%), as well as antioxidant and detox mechanisms (6%). On the other hand, a significant enrichment in gene sets related to immune system activation (28% of the total) was observed in the ileum mucosa of HB birds (Table 4.3). Moreover, an increased expression of gene sets involved in signal transduction and cell signaling (20%), DNA remodeling and replication – chromatin/histone modification (15%), cell activation, migration and adhesion (12%), inflammation (7%) and bone remodeling (4%) was detected in the HB group.

4.4. Discussion

In this trial, the growth pattern of two different fast-growing chicken hybrids (HA and HB), both currently available for the poultry industry, was characterized. In addition, ileum transcriptomic profile and gene set analysis were also investigated in an effort to better understand the physiological dynamics occurring in the small intestine of different chicken genotypes raised in the same environmental conditions and fed the same basal diet, which was formulated according to the current indications and widely used in commercial practices.

Considering the productive performance in the overall period of trial, HB broilers reported higher BW, DWG, and DFI compared to HA ones, whereas no significant difference was observed

in terms of FCR and mortality. However, the two genotypes showed different growth patterns throughout the study, as previously observed by other Authors comparing different fast-growing broiler lines (Marcato et al., 2008; Hristakieva at al., 2014). At hatch, HA chicks were heavier than HB ones. It is widely known that the hatching weight of the chicks can be influenced by the age of the breeder hens and the environmental conditions applied during the incubation process. Since in the present trial eggs were obtained from breeders' stock showing the same age and they were subjected to the same incubation conditions, the different body weight of the chicks might be due to other factors such as a different availability of yolk nutrients or a different embryonic development and metabolism during incubation. The chicken genotypes tested in this study have been subjected to different selection processes, which may have exerted differences in embryo development and metabolism and possibly in its incubation requirements. Previously, Suarez et al. (1997) reported a significant effect of the broiler breeder genotype on the hatch weight of the chicks. Similarly, differences in hatchling weight were observed when Cobb 500 and Ross 308 lines were compared (Hristakieva et al., 2014). On the contrary, Tona et al. (2010) reported no significant difference in the weight of day-old chicks belonging to different fast-growing broiler lines.

After 9 d of trial, HA birds maintained higher BW than the HB ones likely through a better exploitation of the dietary nutrients, as suggested by the lower value of FCR observed in this group during the starter phase. Overall, these data suggest a greater precocity of HA chicks compared to the counterpart. On the other hand, HB birds achieved higher BW at 21, 34 and 43 d of trial indicating that this genotype is characterized by a later development compared to the counterpart. This improvement could be mainly attributable to the higher feed intake observed in the HB group during these feeding phases and in the overall period of the trial. Apart from the starter phase, in which HA birds showed a better FCR, the two genotypes exhibited comparable feed efficiency throughout the trial, confirming that the higher body weight reached by HB birds was mainly due to their higher voluntary feed intake rather than a better exploitation of the dietary nutrients. The

mechanism affecting feed intake regulation in chickens is extremely complex since different physiological and environmental factors are involved (Richards and Proszkowiec-Weglarz, 2007). It can be hypothesized that the different selective processes applied to the two genotypes may have exerted changes in their feeding behavior. However, other scientific insights such as the assessment of the expression of feeding-related neuropeptides, are necessary to confirm this hypothesis.

Footpad dermatitis is a contact dermatitis affecting broilers and turkeys, and it has very important effects on animal welfare and health, food safety, and economic aspects (Shepherd and Fairchild, 2010). The incidence and severity of these necrotic lesions can be considered reliable indicators of chicken welfare and management conditions (Meluzzi and Sirri, 2009). The differences among various chicken strains in the proneness to develop FPD have been previously reported (Kestin et al., 2001; Bilgili et al., 2006). In this study, HB birds showed a lower incidence of FPD despite their higher body weight, which is considered a predisposing factor in the onset of this condition. Overall, these results indicated that the HB genotype seems characterized by a greater resistance of the foot pads and a lower proneness to develop FPD.

Significant differences between the two genotypes were observed also in terms of gene and biological pathways expressed in the ileum mucosa. HA birds showed a greater expression of gene sets related to cellular energy production, mitochondria structure and functionality, as well as cell structure and integrity, ribosome structure and assembly, protein synthesis, and antioxidant capacity. Taken together, these results seem to indicate an overall healthy condition of the intestinal mucosa, as also suggested by the upregulation of some cell structure and integrity pathways in the HA birds (e.g. GO_APICAL_JUNCTION_COMPLEX, GO_BRUSH_BORDER, GO_CADHERIN_BINDING). Maintaining a proper epithelial integrity and functionality is a vital process for the animals, even though it represents a notable energy expenditure especially in the small intestine where cells turnover and renewal are extremely fast. In the chickens, the gastrointestinal tract accounts up to 8% of the energy metabolized even though it represents only

1.5% of the body weight (Spratt et al., 1990). The upregulation of pathways related to mitochondria structure (e.g. GO_RESPIRATORY_CHAIN; GO_INNER_MITOCHONDRIAL_MEMBRANE_PROTEIN_COMPLEX; GO_MITOCHONDRIAL_ENVELOPE; GO_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_ASSEMBLY) and functionality (e.g. GO_OXIDATIVE_PHOSPHORYLATION; GO_CELLULAR_RESPIRATION; GO_AEROBIC_RESPIRATION; GO_ELECTRON_CARRIER_ACTIVITY; GO_ENERGY_COUPLED_PROTON_TRANSPORT_DOWN_ELECTROCHEMICAL_GRADIENT) may indicate a greater presence and activity of mitochondria in the intestinal mucosa of HA birds. Overall, the upregulation of pathways related to mitochondria, as well as that of pathways related to cellular energy metabolism (e.g. GO_ATP_BIOSYNTHETIC_PROCESS; GO_NUCLEOSIDE_TRIPHOSPHATE_METABOLIC_PROCESS; GO_FATTY_ACID_BETA_OXIDATION; GO_ENERGY_DERIVATION_BY_OXIDATION_OF_ORGANIC_COMPOUNDS; GO_GENERATION_OF_PRECURSOR_METABOLITES_AND_ENERGY), may suggest an increased energy production in the epithelial cells of HA birds, which can be used for maintaining a proper epithelial structure and integrity. Similarly, the upregulation of protein synthesis processes, as well as ribosome structure and assembly (e.g. GO_RIBOSOME; GO_CYTOSOLIC_RIBOSOME; GO_PROTEIN_LOCALIZATION_TO_ENDOPLASMIC_RETICULUM) might be consistent with the phenotypic expression of an overall greater functionality of the epithelium. In HA group, also some antioxidant and detox mechanism related-pathways showed significant enrichment (e.g. GO_GLUTATHIONE_METABOLIC_PROCESS; GO_GLUTATHIONE_TRANSFERASE_ACTIVITY), suggesting a greater antioxidant capacity in the small intestine of these birds, which can be required to counteract the oxidative stress occurring

with the increased activation of mitochondria and, in general, with the metabolic processes within the cell.

A different transcriptomic profile has been observed in the ileum mucosa of HB birds. Indeed, according to the functional analysis, it appeared that HB birds may have experienced an inflammatory condition in the small intestine, as also indicated by the upregulation of GO_INFLAMMATORY_RESPONSE pathway. As shown in Table 4.3, approximately 30% of the significantly enriched pathways were involved in the activation of the immune system (e.g. GO_IMMUNE_SYSTEM_DEVELOPMENT; GO_IMMUNE_RESPONSE; GO_ACTIVATION_OF_IMMUNE_RESPONSE; GO_ADAPTIVE_IMMUNE_RESPONSE; GO_POSITIVE_REGULATION_OF_IMMUNE_RESPONSE). Within this group, a noticeable upregulation of cytokine-related pathways (e.g. GO_POSITIVE_REGULATION_OF_INTERLEUKIN_2_PRODUCTION; GO_CYTOKINE_BINDING; GO_CYTOKINE_RECEPTOR_ACTIVITY; GO_REGULATION_OF_TUMOR_NECROSIS_FACTOR_SUPERFAMILY_CYTOKINE_PRODUCTION (FDR q-value= 0.05)) was observed. Furthermore, HB birds reported an increased activation of pathways related to leukocyte (e.g. GO_POSITIVE_REGULATION_OF_LEUKOCYTE_PROLIFERATION; GO_LEUKOCYTE_ACTIVATION; GO_LEUKOCYTE_DIFFERENTIATION), B cells (e.g. GO_B_CELL_ACTIVATION_INVOLVED_IN_IMMUNE_RESPONSE; GO_POSITIVE_REGULATION_OF_B_CELL_PROLIFERATION; GO_POSITIVE_REGULATION_OF_B_CELL_ACTIVATION) and immunoglobulins (e.g. GO_IMMUNOGLOBULIN_PRODUCTION; GO_SOMATIC_DIVERSIFICATION_OF_IMMUNOGLOBULINS). Moreover, the upregulation of pathways mainly involved in cell activation, migration and adhesion, such as GO_CELL_CHEMOTAXIS, GO_LEUKOCYTE_CHEMOTAXIS,

GO_LEUKOCYTE_MIGRATION, may suggest an increased afflux of immune cells toward the ileum mucosa in response to an inflammatory event. The enrichment of other gene sets involved in angiogenesis (e.g. GO_SPROUTING_ANGIOGENESIS, GO_POSITIVE_REGULATION_OF_VASCULATURE_DEVELOPMENT), as well as the upregulation of GO_RESPONSE_TO_HEAT, GO_NEGATIVE_REGULATION_OF_BLOOD_CIRCULATION and GO_WOUND_HEALING gene sets, appears consistent with an inflammatory condition as well. An increased expression of gene clusters involved in signal transduction and cell signaling was also observed. Within this group, a higher activation of phosphatidylinositol-3-kinases (PI3Ks) (e.g. GO_POSITIVE_REGULATION_OF_PHOSPHATIDYLINOSITOL_3_KINASE_SIGNALING; GO_PHOSPHATIDYLINOSITOL_3_KINASE_ACTIVITY) and mitogen-activated protein (MAP) kinase (e.g. GO_POSITIVE_REGULATION_OF_MAP_KINASE_ACTIVITY; GO_POSITIVE_REGULATION_OF_MAPK_CASCADE) gene sets was detected. In mammals, MAP kinase signaling cascades transduce different extracellular signals that regulate cellular responses. These cascade mechanisms can be activated by tumor necrosis factor α, leading to an increased expression of several inflammatory cytokines (Sabio and Davis, 2014). Similarly, a potential role of PI3-Ks in intestinal inflammation (Weaver and Ward, 2001; Cahill et al., 2012) and cell migration (Cain and Ridley, 2009) has been hypothesized in human.

The increased expression of gene sets involved in DNA remodeling and replication (e.g. GO_DNA_REPLICATION; GO_DNA_HELICASE_ACTIVITY; GO_DNA_BIOSYNTHETIC_PROCESS) and chromatin/histone modification (e.g. GO_HISTONE_DEMETHYLASE_ACTIVITY; GO_HISTONE_METHYLTRANSFERASE_ACTIVITY; GO_REGULATION_OF_CHROMATIN_ORGANIZATION) might represent a potential cellular response to the increased signaling status elicited by the inflammation and the immune system

activation. Finally, the upregulation of bone remodeling-related pathways is not clear. However, it would be possible that gene sets related to inflammation and immune system activation may share some genes with pathways involved in bone metabolism, resulting in an indirect upregulation of the latter biological pathway.

Overall, the activation of the immune system and the apparent gut inflammation observed in HB birds pave the way to some reflections, both from a nutritional and a physiological point of view. The upregulation of these gene sets seems not due to clinical diseases as birds showed high productive performance, excellent health status and low mortality through the trial. Therefore, other factors should be considered to understand the reasons behind the inflammatory condition and the activation of the immune system. A possible explanation could lie in the high feed intake observed in the HB birds. The ingested feed can determine oxidative injury and intestinal inflammation involving the epithelium and immune/inflammatory cells (Bhattacharyya et al., 2014). Therefore, feed ingestion should be balanced with an efficient digestive capacity in order to exploit the dietary nutrients provided with the diet and allow the maximum growth and efficiency, but also to maintain a proper gut health and functionality. As previously reported, the different selective processes applied to the two genotypes may have exerted changes in their feeding behavior. In order to maximize growth rate, broiler chickens have been selected for decades for increased feed intake (Tallentire et al., 2018), resulting in hyperphagic, heavy, and obese animals (Piekarski et al., 2015; Piekarski-Welsher et al., 2016). Recent evidence supported that modern meat-type chickens tend to consume feed to maximize gut fill (Ferket and Gernat, 2006; Classen, 2016). Therefore, it can be hypothesized that the selective process applied to HB birds may have increased their voluntary feed ingestion capacity probably beyond their digestive functionality, leading to an increased oxidative stress and inflammation in the gut. A similar scenario has been recently defined by Kogut et al. (2018) as “metabolic inflammation”, which represents a chronic low-grade inflammation triggered

by the excessive nutrient intake and the metabolic surplus which may occur in modern fast-growing broiler lines.

Moreover, the undigested or poorly digested feed can be used as a substrate for the growth of harmful bacteria. Potential changes in the intestinal microbiota may dysregulate cross-talk among bacteria, intestinal epithelium, and mucosal immune system, resulting in local inflammatory conditions (Kaiser and Balic, 2015). However, further insights are necessary to evaluate whether the gut microbes may have played any roles in triggering gut inflammation and immune system activation and also if the latter factors would have limited the growth potential of HB broilers. In addition, inflammation and immune system activation may have also exerted changes in the metabolic requirement of specific nutrients which should be integrated with the diet to sustain animal health and productivity.

In conclusion, the two fast-growing broiler chicken genotypes tested in this trial exhibited different growth patterns, with HA birds showing a greater precocity and HB characterized by a later development mainly sustained by an increased feed intake. The transcriptomic analysis revealed that HA broilers might be characterized by a healthier condition of the intestinal mucosa likely supported by an increased mitochondria functionality and antioxidant capacity. On the other hand, HB chickens reported a potential inflammatory condition in the gut coupled with a marked activation of the immune system. The factors involved in the onset of this condition, as well as the determinants of the differences observed in feeding behavior of the two genotypes, are not clear and other scientific insights are necessary to better understand these aspects.

Table 4.1 Composition of the basal diet in each feeding phase.

	Starter 0-9 d	Grower I 10-21 d	Grower II 22-34 d	Finisher 35-43 d
Ingredients, g/100g				
Corn	33.4	36.7	34.2	33.1
Wheat	20.0	20.0	25.0	30.0
Vegetable oil	2.45	2.68	3.61	3.97
Soybean meal 48%	18.2	20.2	14.2	9.33
Full-fat soybean	10.0	10.0	15.0	15.0
High-protein soybean meal	5.00	0.00	0.00	0.00
Sunflower	2.00	2.00	2.00	3.00
Pea	3.00	3.00	3.00	3.00
Corn gluten	2.00	2.00	0.00	0.00
Lysine	0.54	0.53	0.46	0.43
DL-Methionine	0.29	0.32	0.33	0.26
L-Threonine	0.12	0.11	0.10	0.08
Choline chloride	0.10	0.10	0.05	0.00
Calcium carbonate	0.53	0.52	0.60	0.69
Dicalcium phosphate	1.29	0.80	0.47	0.21
Sodium chloride	0.29	0.30	0.23	0.21
Sodium bicarbonate	0.05	0.05	0.15	0.25
Premix vit.-min.[1]	0.54	0.46	0.38	0.30
Phytase	0.05	0.05	0.05	0.05
Xylanase	0.05	0.05	0.05	0.05
Emulsifier	0.08	0.08	0.08	0.08
Calculated chemical composition (* analyzed)				
Dry matter*, %	88.8	88.2	88.5	88.5
Crude protein*, %	22.7	21.0	19.1	17.5
Total lipid*, %	6.25	6.51	8.29	8.64
Crude fiber*, %	2.96	2.92	2.99	3.08
Ash, %	5.24	4.60	4.29	4.03
Lysine (total), %	1.42	1.31	1.20	1.07
Met. + Cyst. (total), %	0.99	0.92	0.85	0.76
Arginine (total), %	1.46	1.34	1.25	1.13
Threonine (total), %	0.94	0.87	0.79	0.71
Ca (total), %	0.77	0.62	0.55	0.50
P (total), %	0.61	0.51	0.44	0.38
AME, kcal/kg	3,100	3,150	3,275	3,325

[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.

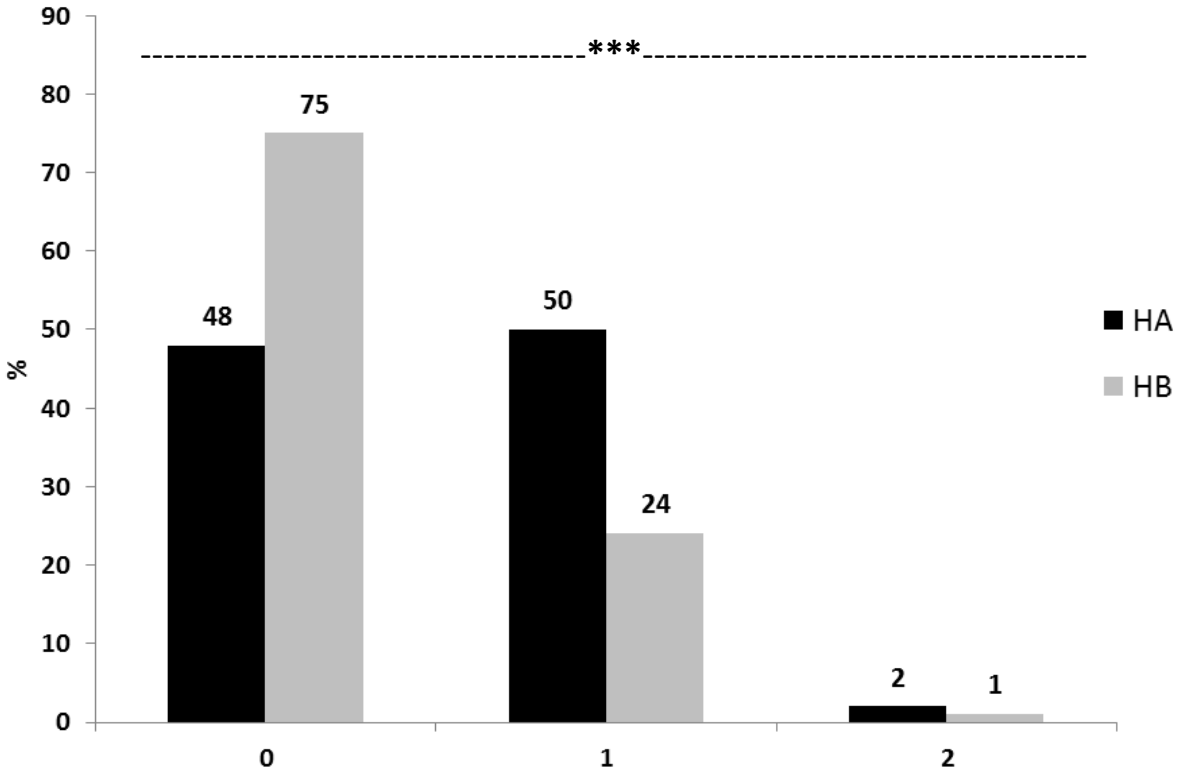
Table 4.2 Productive performance of broiler chickens of both the genotypes (HA and HB) in each feeding phase and in the overall period of trial.

	HA	HB	SEM	P-value
n.	*9*	*9*		
0-9 d				
Chick body weight (g)	43.8	40.1	0.15	<0.01
Body weight (g)	228	217	2.84	0.02
Daily weight gain (g/bird/d)†	20.5	19.6	0.32	0.06
Daily feed intake (g/bird/d)†	27.6	27.7	0.37	0.84
Feed conversion ratio†	1.352	1.419	0.02	0.04
Mortality (%)	0.17	0.00	0.01	0.33
10-21 d				
Body weight (g/bird)	772	807	9.11	0.02
Daily weight gain (g/bird/d)†	45.3	49.2	0.68	<0.01
Daily feed intake (g/bird/d)†	70.6	74.9	0.86	<0.01
Feed conversion ratio†	1.558	1.523	0.02	0.12
Mortality (%)	0.35	0.35	0.02	-
22-34 d				
Body weight (g/bird)	1,857	1,930	21.1	0.02
Daily weight gain (g/bird/d)†	83.4	86.2	1.07	0.07
Daily feed intake (g/bird/d)†	139.6	144.4	1.67	0.06
Feed conversion ratio†	1.676	1.675	0.02	0.96
Mortality (%)	0.17	0.17	0.01	-
35-43 d				
Body weight (g/bird)	2,607	2,734	17.9	<0.01
Daily weight gain (g/bird/d)†	83.3	91.9	1.72	<0.01
Daily feed intake (g/bird/d)†	182.4	196.5	2.46	<0.01
Feed conversion ratio†	2.200	2.144	0.06	0.49
Mortality (%)	0.00	0.00	0.01	-
0-43 d				
Body weight (g/bird)	2,607	2,734	17.9	<0.01
Daily weight gain (g/bird/d)†	59.6	62.6	0.42	<0.01
Daily feed intake (g/bird/d)†	104.7	110.3	0.99	<0.01
Feed conversion ratio†	1.772	1.776	0.01	0.85
Mortality (%)	0.69	0.52	0.02	0.83

† corrected for mortality

Means within a row not sharing a common superscript are significantly different (A,B: $P< 0.01$; a,b: $P< 0.05$).

Figure 4.1 Incidence of foot pad dermatitis in HA and HB broiler chickens classified as 0 = no lesions, 1 = mild lesions (<0.8 cm), and 2 = severe lesions (>0.8 cm).



***: P< 0.001

Table 4.3 Classification of significantly enriched gene sets (NES *p*-value and False Discovery Rate *q*-value<0.05) in ileum mucosa of HA and HB broiler chickens.

Main biological functions of enriched gene sets	% of total enriched gene sets
HA genotype	
Cellular energy metabolism	43%
Mitochondria structure and functionality	23%
Ribosome structure and protein synthesis	11%
Cell structure and integrity	8%
Antioxidant and detox mechanisms	6%
Other	9%
HB genotype	
Immune system activation	28%
Signal transduction and cell signalling	20%
DNA remodelling and replication, chromatin/histone modification	15%
Cell activation, migration and adhesion	12%
Inflammation	7%
Bone remodelling	4%
Other	14%

Supporting Information. Table 1. Complete list of upregulated gene sets in ileum mucosa of HA broiler chickens.

GS follow link to MSigDB	GS DETAILS	ES	NES	NOM p-val	FDR q-val
1	GO_INNER_MITOCHONDRIAL_MEMBRANE_PROTEIN_COMPLEX	-0.70	-3.18	0.000	0.000
2	GO_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_ASSEMBLY	-0.73	-3.05	0.000	0.000
3	GO_RESPIRATORY_CHAIN	-0.73	-3.04	0.000	0.000
4	GO_ELECTRON_TRANSPORT_CHAIN	-0.70	-3.03	0.000	0.000
5	GO_NADH_DEHYDROGENASE_COMPLEX_ASSEMBLY	-0.77	-2.99	0.000	0.000
6	GO_MITOCHONDRIAL_MEMBRANE_PART	-0.60	-2.98	0.000	0.000
7	GO_OXIDATIVE_PHOSPHORYLATION	-0.72	-2.98	0.000	0.000
8	GO_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_I_BIOGENESIS	-0.77	-2.98	0.000	0.000
9	GO_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_I_ASSEMBLY	-0.77	-2.96	0.000	0.000
10	GO_MITOCHONDRIAL_PROTEIN_COMPLEX	-0.62	-2.95	0.000	0.000
11	GO_NADH_DEHYDROGENASE_ACTIVITY	-0.76	-2.77	0.000	0.000
12	GO_ORGANELLAR_RIBOSOME	-0.63	-2.70	0.000	0.000
13	GO_NUCLEOSIDE_TRIPHOSPHATE_METABOLIC_PROCESS	-0.52	-2.67	0.000	0.000
14	GO_CELLULAR_RESPIRATION	-0.56	-2.64	0.000	0.000
15	GO_MITOCHONDRIAL_ELECTRON_TRANSPORT_NADH_TO_UBIQUINONE	-0.72	-2.64	0.000	0.000
16	GO_NADH_DEHYDROGENASE_COMPLEX	-0.71	-2.63	0.000	0.000
17	GO_RIBOSOMAL_SUBUNIT	-0.52	-2.59	0.000	0.000
18	GO_OXIDOREDUCTASE_ACTIVITY_ACTING_ON_NAD_P_H_QUINONE_OR_SIMILAR_COMPOUND_AS_ACCEPTOR	-0.66	-2.57	0.000	0.000
19	GO_ATP_SYNTHESIS_COUPLED_PROTON_TRANSPORT	-0.81	-2.56	0.000	0.000
20	GO_RIBONUCLEOSIDE_TRIPHOSPHATE_BIOSYNTHETIC_PROCESS	-0.68	-2.53	0.000	0.000
21	GO_ENERGY_COUPLED_PROTON_TRANSPORT_DOWN_ELECTROCHEMICAL_GRADIENT	-0.81	-2.49	0.000	0.000

22	GO_RIBOSOME	-0.48	-2.46	0.000	0.000
23	GO_MULTIVESICULAR_BODY_ORGANIZATION	-0.67	-2.45	0.000	0.000
24	GO_ORGANELLE_INNER_MEMBRANE	-0.42	-2.43	0.000	0.000
25	GO_FATTY_ACID_CATABOLIC_PROCESS	-0.57	-2.42	0.000	0.000
26	GO_LARGE_RIBOSOMAL_SUBUNIT	-0.54	-2.40	0.000	0.000
27	GO_NUCLEOSIDE_TRIPHOSPHATE_BIOSYNTHETIC_PROCESS	-0.58	-2.39	0.000	0.000
28	GO_MITOCHONDRIAL_TRANSLATION	-0.51	-2.37	0.000	0.000
29	GO_FATTY_ACID_BETA_OXIDATION	-0.62	-2.35	0.000	0.000
30	GO_MICROBODY_PART	-0.51	-2.35	0.000	0.000
31	GO_PROTON_TRANSPORTING_TWO_SECTOR_ATPASE_COMPLEX	-0.61	-2.35	0.000	0.000
32	GO_TRANSLATIONAL_TERMINATION	-0.53	-2.34	0.000	0.000
33	GO_LIPID_OXIDATION	-0.54	-2.32	0.000	0.000
34	GO_OXIDOREDUCTASE_COMPLEX	-0.51	-2.32	0.000	0.000
35	GO_ATP_BIOSYNTHETIC_PROCESS	-0.70	-2.31	0.000	0.000
36	GO_RESPONSE_TO_XENOBIOTIC_STIMULUS	-0.59	-2.29	0.000	0.000
37	GO_MULTI_ORGANISM_ORGANELLE_ORGANIZATION	-0.71	-2.29	0.000	0.000
38	GO_MULTI_ORGANISM_MEMBRANE_BUDDING	-0.71	-2.29	0.000	0.000
39	GO_PROTON_TRANSPORTING_TWO_SECTOR_ATPASE_COMPLEX_PROTON_TRANSPORTING_DOMAIN	-0.70	-2.29	0.000	0.000
40	GO_MONOCARBOXYLIC_ACID_CATABOLIC_PROCESS	-0.50	-2.28	0.000	0.000
41	GO_VIRAL_BUDDING	-0.71	-2.28	0.000	0.000
42	GO_HYDROGEN_ION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	-0.50	-2.28	0.000	0.000
43	GO_MICROBODY	-0.47	-2.28	0.000	0.000
44	GO_HYDROGEN_TRANSPORT	-0.49	-2.28	0.000	0.000
45	GO_HYDROGEN_ION_TRANSMEMBRANE_TRANSPORT	-0.52	-2.27	0.000	0.000
46	GO_NUCLEOTIDE_SUGAR_BIOSYNTHETIC_PROCESS	-0.74	-2.27	0.000	0.000
47	GO_NUCLEOSIDE_MONOPHOSPHATE_METABOLIC_PROCESS	-0.42	-2.26	0.000	0.000
48	GO_STRUCTURAL_CONSTITUENT_OF_RIBOSOME	-0.44	-2.24	0.000	0.000

49	GO_MICROBODY_LUMEN	-0.58	-2.24	0.000	0.000
50	GO_CYTOSOLIC_LARGE_RIBOSOMAL_SUBUNIT	-0.54	-2.22	0.000	0.001
51	GO_GABA_RECEPTOR_COMPLEX	-0.72	-2.22	0.000	0.001
52	GO_MONOCARBOXYLIC_ACID_BINDING	-0.57	-2.21	0.000	0.001
53	GO_SMALL_RIBOSOMAL_SUBUNIT	-0.53	-2.21	0.000	0.001
54	GO_MITOCHONDRIAL_ENVELOPE	-0.37	-2.20	0.000	0.001
55	GO_FATTY_ACID_BINDING	-0.65	-2.18	0.000	0.001
56	GO_PYRIMIDINE_NUCLEOSIDE_BIOSYNTHETIC_PROCESS	-0.62	-2.18	0.000	0.001
57	GO_NUCLEOTIDE_SUGAR_METABOLIC_PROCESS	-0.60	-2.17	0.000	0.001
58	GO_GLUTATHIONE_METABOLIC_PROCESS	-0.57	-2.16	0.000	0.001
59	GO_ORGANELLAR_SMALL_RIBOSOMAL_SUBUNIT	-0.66	-2.15	0.000	0.001
60	GO_ESCRT_COMPLEX	-0.65	-2.14	0.000	0.001
61	GO_CYTOSOLIC_RIBOSOME	-0.46	-2.14	0.000	0.001
62	GO_ELECTRON_CARRIER_ACTIVITY	-0.46	-2.14	0.000	0.001
63	GO_GABA_RECEPTOR_ACTIVITY	-0.65	-2.13	0.000	0.001
64	GO_FATTY_ACYL_COA_BINDING	-0.60	-2.11	0.003	0.002
65	GO_CELLULAR_LIPID_CATABOLIC_PROCESS	-0.43	-2.11	0.000	0.002
66	GO_ENDOSOME_ORGANIZATION	-0.50	-2.10	0.000	0.002
67	GO_MEMBRANE_BUDDING	-0.45	-2.09	0.000	0.002
68	GO_AEROBIC_RESPIRATION	-0.53	-2.09	0.000	0.002
69	GO_GLYCOSYL_COMPOUND_METABOLIC_PROCESS	-0.38	-2.08	0.000	0.003
70	GO_OXIDOREDUCTASE_ACTIVITY_ACTING_ON_NAD_P_H	-0.45	-2.06	0.000	0.003
71	GO_OXIDOREDUCTASE_ACTIVITY_ACTING_ON_THE_CH_CH_GROUP_OF_DONORS	-0.52	-2.05	0.000	0.003
72	GO_ORGANELLAR_LARGE_RIBOSOMAL_SUBUNIT	-0.60	-2.04	0.000	0.004
73	GO_ENERGY_DERIVATION_BY_OXIDATION_OF_ORGANIC_COMPOUNDS	-0.39	-2.00	0.000	0.006
74	GO_NUCLEOBASE_CONTAINING_SMALL_MOLECULE_INTERCONVERSION	-0.58	-1.98	0.000	0.008
75	GO_PYRIMIDINE_RIBONUCLEOTIDE_METABOLIC_PROCESS	-0.59	-1.96	0.000	0.008
76	GO_SOLUTE_PROTON_SYMPORTER_ACTI	-0.58	-1.96	0.005	0.009

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77	GO_MITOCHONDRIAL_TRANSMEMBRANE_TRANSPORT	-0.50	-1.95	0.000	0.010
78	GO_GLUTATHIONE_TRANSFERASE_ACTIVITY	-0.60	-1.95	0.000	0.010
79	GO_MACROMOLECULAR_COMPLEX_DISASSEMBLY	-0.39	-1.95	0.000	0.010
80	GO_PEROXISOMAL_TRANSPORT	-0.63	-1.94	0.002	0.010
81	GO_TRANSLATIONAL_ELONGATION	-0.41	-1.93	0.000	0.011
82	GO_ORGANELLE_ENVELOPE_LUMEN	-0.46	-1.93	0.000	0.011
83	GO_CELLULAR_PROTEIN_COMPLEX_DISASSEMBLY	-0.40	-1.92	0.000	0.013
84	GO_VIRION_ASSEMBLY	-0.53	-1.91	0.000	0.013
85	GO_NUCLEOBASE_CONTAINING_SMALL_MOLECULE_METABOLIC_PROCESS	-0.33	-1.89	0.000	0.017
86	GO_REGULATION_OF_VIRAL_RELEASE_FROM_HOST_CELL	-0.58	-1.88	0.000	0.017
87	GO_PURINE_CONTAINING_COMPOUND_METABOLIC_PROCESS	-0.33	-1.88	0.000	0.017
88	GO_ESTABLISHMENT_OF_PROTEIN_LOCALIZATION_TO_ENDOPLASMIC_RETICULUM	-0.41	-1.88	0.000	0.017
89	GO_STEROL_BIOSYNTHETIC_PROCESS	-0.49	-1.87	0.003	0.019
90	GO_PROTEIN_LOCALIZATION_TO_ENDOPLASMIC_RETICULUM	-0.39	-1.86	0.000	0.020
91	GO_REGULATION_OF_SPINDLE_ORGANIZATION	-0.60	-1.85	0.005	0.022
92	GO_NUCLEOSIDE_MONOPHOSPHATE_BIOSYNTHETIC_PROCESS	-0.42	-1.84	0.000	0.024
93	GO_ORGANIC_ACID_CATABOLIC_PROCESS	-0.35	-1.83	0.000	0.024
94	GO_PYRIMIDINE_CONTAINING_COMPOUND_BIOSYNTHETIC_PROCESS	-0.48	-1.83	0.005	0.025
95	GO_CARBOXYLIC_ACID_CATABOLIC_PROCESS	-0.35	-1.83	0.000	0.025
96	GO_COFACTOR_BINDING	-0.34	-1.83	0.000	0.025
97	GO_APICAL_JUNCTION_COMPLEX	-0.37	-1.81	0.000	0.029
98	GO_CELLULAR_ALDEHYDE_METABOLIC_PROCESS	-0.42	-1.80	0.003	0.030
99	GO_GENERATION_OF_PRECURSOR_METABOLITES_AND_ENERGY	-0.34	-1.79	0.000	0.034
100	GO_MICROBODY_MEMBRANE	-0.44	-1.79	0.000	0.034

101	GO_INTRINSIC_COMPONENT_OF_MITOCHONDRIAL_INNER_MEMBRANE	-0.59	-1.78	0.007	0.035
102	GO_LIPID_CATABOLIC_PROCESS	-0.33	-1.78	0.000	0.035
103	GO_COENZYME_BINDING	-0.35	-1.77	0.000	0.038
104	GO_MONOVALENT_INORGANIC_CATION_TRANSPORT	-0.31	-1.77	0.000	0.039
105	GO_INTRINSIC_COMPONENT_OF_MITOCHONDRIAL_MEMBRANE	-0.47	-1.76	0.006	0.040
106	GO_MULTI_ORGANISM_MEMBRANE_ORGANIZATION	-0.51	-1.76	0.010	0.039
107	GO_OXIDOREDUCTASE_ACTIVITY_ACTING_ON_CH_OH_GROUP_OF_DONORS	-0.38	-1.76	0.000	0.040
108	GO_BRUSH_BORDER	-0.38	-1.76	0.000	0.040
109	GO_OXIDOREDUCTASE_ACTIVITY_ACTING_ON_THE_CH_OH_GROUP_OF_DONORS_NAD_OR_NADP_AS_ACCEPTOR	-0.39	-1.76	0.003	0.040
110	GO_CADHERIN_BINDING	-0.51	-1.76	0.010	0.040
111	GO_DETECTION_OF_LIGHT_STIMULUS	-0.45	-1.75	0.003	0.041
112	GO_OXIDOREDUCTASE_ACTIVITY	-0.30	-1.75	0.000	0.042
113	GO_GLYCOSYL_COMPOUND_BIOSYNTHETIC_PROCESS	-0.37	-1.73	0.000	0.047
114	GO_FATTY_ACID_METABOLIC_PROCESS	-0.32	-1.73	0.000	0.048

Supporting Information. Table 2. Complete list of upregulated gene sets in ileum mucosa of HB broiler chickens.

GS follow link to MSigDB	GS DETAILS	ES	NES	NOM p-val	FDR q-val
1	GO_B_CELL_RECEPTOR_SIGNALING_PATHWAY	0.77	2.51	0.000	0.000
2	GO_CYTOKINE_RECEPTOR_ACTIVITY	0.58	2.34	0.000	0.000
3	GO_B_CELL_ACTIVATION	0.53	2.27	0.000	0.002
4	GO_HELICASE_ACTIVITY	0.49	2.20	0.000	0.004
5	GO_CYTOKINE_BINDING	0.55	2.20	0.000	0.004
6	GO_REGULATION_OF_BONE_REMODELING	0.62	2.18	0.000	0.004
7	GO_ANTIGEN_RECEPTOR_MEDIATED_SIGNALING_PATHWAY	0.48	2.17	0.000	0.005
8	GO_REGULATION_OF_BONE_RESORPTION	0.64	2.16	0.000	0.004
9	GO_ADAPTIVE_IMMUNE_RESPONSE	0.47	2.15	0.000	0.005
10	GO_RECOMBINATIONAL_REPAIR	0.54	2.14	0.000	0.006
11	GO_B_CELL_DIFFERENTIATION	0.55	2.13	0.000	0.006
12	GO_PROTEIN_TYROSINE_KINASE_BINDING	0.59	2.13	0.000	0.006
13	GO_PRODUCTION_OF_MOLECULAR_MEDIATOR_OF_IMMUNE_RESPONSE	0.58	2.12	0.000	0.006
14	GO_POSITIVE_REGULATION_OF_CELL_ACTIVATION	0.44	2.10	0.000	0.008
15	GO_REGULATION_OF_CELL_ADHESION	0.40	2.10	0.000	0.008
16	GO_LYMPHOCYTE_DIFFERENTIATION	0.45	2.09	0.000	0.008
17	GO_POSITIVE_REGULATION_OF_CELL_ADHESION	0.42	2.09	0.000	0.008
18	GO_LYMPHOCYTE_ACTIVATION	0.43	2.08	0.000	0.008
19	GO_HISTONE_METHYLTRANSFERASE_COMPLEX	0.55	2.07	0.000	0.009
20	GO_IMMUNE_RESPONSE_REGULATING_CELL_SURFACE_RECEPTOR_SIGNALING_PATHWAY	0.43	2.06	0.000	0.011
21	GO_LEUKOCYTE_DIFFERENTIATION	0.43	2.06	0.000	0.010
22	GO_REGULATION_OF_SMOOTHENED_SIGNALING_PATHWAY	0.53	2.04	0.000	0.012
23	GO_POSITIVE_REGULATION_OF_PHOSPHATIDYLINOSITOL_3_KINASE_SIGNALING	0.53	2.04	0.000	0.012

24	GO_HISTONE_DEMETHYLASE_ACTIVITY	0.70	2.03	0.000	0.012
25	GO_REGULATION_OF_TISSUE_REMODELING	0.53	2.03	0.000	0.012
26	GO_REGULATION_OF_CELL_ACTIVATION	0.40	2.00	0.000	0.017
27	GO_CELL_CHEMOTAXIS	0.47	2.00	0.000	0.018
28	GO_POSITIVE_REGULATION_OF_BIOMINERAL_TISSUE_DEVELOPMENT	0.57	1.99	0.000	0.018
29	GO_DNA_HELICASE_ACTIVITY	0.54	1.99	0.000	0.019
30	GO_PURINE_NTP_DEPENDENT_HELICASE_ACTIVITY	0.49	1.99	0.000	0.018
31	GO_IMMUNE_SYSTEM_DEVELOPMENT	0.38	1.98	0.000	0.018
32	GO_REGULATION_OF_CELL_SUBSTRATE_ADHESION	0.43	1.98	0.000	0.018
33	GO_REGULATION_OF_LIPID_KINASE_ACTIVITY	0.54	1.98	0.000	0.018
34	GO_EXTERNAL_SIDE_OF_PLASMA_MEMBRANE	0.43	1.98	0.000	0.018
35	GO_INTEGRIN_MEDIATED_SIGNALING_PATHWAY	0.51	1.97	0.000	0.019
36	GO_IMMUNOGLOBULIN_PRODUCTION	0.57	1.96	0.000	0.021
37	GO_REGULATION_OF_HOMOTYPIC_CELL_CELL_ADHESION	0.41	1.96	0.000	0.021
38	GO_REGULATION_OF_BIOMINERAL_TISSUE_DEVELOPMENT	0.49	1.96	0.000	0.021
39	GO_OLFACTORY_LOBE_DEVELOPMENT	0.61	1.96	0.000	0.021
40	GO_DNA_DEPENDENT_ATPASE_ACTIVITY	0.48	1.95	0.000	0.021
41	GO_LEUKOCYTE_MIGRATION	0.42	1.95	0.000	0.020
42	GO_INFLAMMATORY_RESPONSE	0.40	1.95	0.000	0.020
43	GO_POSITIVE_REGULATION_OF_KINASE_ACTIVITY	0.38	1.95	0.000	0.020
44	GO_REGULATION_OF_CELL_CELL_ADHESION	0.39	1.95	0.000	0.021
45	GO_LEUKOCYTE_ACTIVATION	0.39	1.95	0.000	0.020
46	GO_REGULATION_OF_B_CELL_DIFFERENTIATION	0.68	1.94	0.002	0.021
47	GO_REGULATION_OF_PHOSPHATIDYLINOSITOL_3_KINASE_ACTIVITY	0.56	1.94	0.000	0.020
48	GO_NEGATIVE_REGULATION_OF_CHONDROCYTE_DIFFERENTIATION	0.64	1.94	0.000	0.020
49	GO_REGULATION_OF_MAP_KINASE_ACTIVITY	0.40	1.94	0.000	0.019
50	GO_PCG_PROTEIN_COMPLEX	0.56	1.94	0.000	0.020

51	GO_CELLULAR_DEFENSE_RESPONSE	0.60	1.94	0.000	0.020
52	GO_NEGATIVE_REGULATION_OF_IMMUNE_EFFECTOR_PROCESS	0.50	1.94	0.000	0.021
53	GO_IMMUNE_RESPONSE	0.36	1.93	0.000	0.021
54	GO_NEGATIVE_REGULATION_OF_CHROMATIN_MODIFICATION	0.55	1.93	0.002	0.022
55	GO_CORECEPTOR_ACTIVITY	0.56	1.93	0.000	0.022
56	GO_PROTEIN_DEALKYLATION	0.63	1.92	0.002	0.022
57	GO_REGULATION_OF_CELL_MATRIX_ADHESION	0.47	1.92	0.000	0.022
58	GO_PROTEIN_DEMETHYLATION	0.63	1.92	0.000	0.023
59	GO_CILIUM_MORPHOGENESIS	0.41	1.92	0.000	0.023
60	GO_POSITIVE_REGULATION_OF_VASCULATURE_DEVELOPMENT	0.44	1.92	0.000	0.022
61	GO_POSITIVE_REGULATION_OF_CELL_CELL_ADHESION	0.41	1.92	0.000	0.022
62	GO_ACTIVATION_OF_MAPKK_ACTIVITY	0.52	1.92	0.002	0.022
63	GO_ACTIVATION_OF_IMMUNE_RESPONSE	0.39	1.91	0.000	0.022
64	GO_BODY_MORPHOGENESIS	0.54	1.91	0.000	0.022
65	GO_CELLULAR_RESPONSE_TO_VASCULAR_ENDOTHELIAL_GROWTH_FACTOR_STIMULUS	0.60	1.91	0.002	0.023
66	GO_LEUKOCYTE_CHEMOTAXIS	0.48	1.91	0.002	0.023
67	GO_REGULATION_OF_LYMPHOCYTE_DIFFERENTIATION	0.44	1.91	0.000	0.023
68	GO_REGULATION_OF_INTERLEUKIN_2_BIOSYNTHETIC_PROCESS	0.66	1.91	0.000	0.023
69	GO_DNA_GEOMETRIC_CHANGE	0.46	1.91	0.000	0.023
70	GO_ACTIVATION_OF_PROTEIN_KINASE_ACTIVITY	0.39	1.90	0.000	0.023
71	GO_REGULATION_OF_PROTEIN_SERINE_THREONINE_KINASE_ACTIVITY	0.38	1.90	0.000	0.023
72	GO_SIDE_OF_MEMBRANE	0.38	1.90	0.000	0.024
73	GO_PEPTIDYL_THREONINE_MODIFICATION	0.54	1.90	0.000	0.024
74	GO_NEGATIVE_REGULATION_OF_CHROMOSOME_ORGANIZATION	0.46	1.90	0.000	0.024
75	GO_REGULATION_OF_ADHERENS_JUNCTION_ORGANIZATION	0.50	1.90	0.000	0.024
76	GO_REGULATION_OF_LEUKOCYTE_PROLIFERATION	0.41	1.89	0.000	0.025
77	GO_DNA_RECOMBINATION	0.41	1.89	0.000	0.025

78	GO_REGULATION_OF_INTERLEUKIN_2_PRODUCTION	0.53	1.89	0.000	0.025
79	GO_NUCLEOSIDE_TRIPHOSPHATASE_REGULATOR_ACTIVITY	0.38	1.89	0.000	0.025
80	GO_POSITIVE_REGULATION_OF_INTERLEUKIN_2_PRODUCTION	0.58	1.89	0.000	0.025
81	GO_POSITIVE_REGULATION_OF_B_CELL_ACTIVATION	0.48	1.88	0.002	0.026
82	GO_POSITIVE_REGULATION_OF_LIPID_KINASE_ACTIVITY	0.57	1.88	0.000	0.027
83	GO_DNA_REPLICATION	0.40	1.87	0.000	0.028
84	GO_MICROTUBULE_ORGANIZING_CENTER_PART	0.42	1.87	0.000	0.029
85	GO_POSITIVE_REGULATION_OF_FILOPODIUM_ASSEMBLY	0.61	1.87	0.005	0.029
86	GO_GUANYL_NUCLEOTIDE_EXCHANGE_FACTOR_ACTIVITY	0.39	1.87	0.000	0.029
87	GO_NEGATIVE_REGULATION_OF_CELL_ADHESION	0.40	1.87	0.000	0.029
88	GO_REGULATION_OF_PHOSPHOLIPID_METABOLIC_PROCESS	0.48	1.86	0.000	0.031
89	GO_COVALENT_CHROMATIN_MODIFICATION	0.38	1.86	0.000	0.031
90	GO_HISTONE_H4_ACETYLATION	0.54	1.86	0.002	0.031
91	GO_CELL_ACTIVATION	0.36	1.86	0.000	0.031
92	GO_REGULATION_OF_CHROMOSOME_ORGANIZATION	0.39	1.85	0.000	0.031
93	GO_REGULATION_OF_B_CELL_ACTIVATION	0.44	1.85	0.000	0.033
94	GO_RESPONSE_TO_FLUID_SHEAR_STRESS	0.57	1.85	0.000	0.033
95	GO_CYCLIN_DEPENDENT_PROTEIN_KINASE_HOLOENZYME_COMPLEX	0.59	1.85	0.002	0.033
96	GO_PEPTIDYL_SERINE_MODIFICATION	0.42	1.84	0.000	0.034
97	GO_REGULATION_OF_CHROMATIN_ORGANIZATION	0.43	1.84	0.000	0.034
98	GO_NEGATIVE_REGULATION_OF_CELLULAR_RESPONSE_TO_GROWTH_FACTOR_STIMULUS	0.42	1.84	0.000	0.034
99	GO_HISTONE_ACETYLTRANSFERASE_BINDING	0.59	1.84	0.000	0.034
100	GO_B_CELL_ACTIVATION_INVOLVED_IN_IMMUNE_RESPONSE	0.54	1.84	0.002	0.034
101	GO_MICROTUBULE_NUCLEATION	0.64	1.84	0.007	0.034

102	GO_ATP_DEPENDENT_DNA_HELICASE_ACTIVITY	0.55	1.84	0.003	0.034
103	GO_POSITIVE_REGULATION_OF_LEUKOCYTE_PROLIFERATION	0.42	1.84	0.000	0.034
104	GO_LEUKOCYTE_PROLIFERATION	0.48	1.84	0.000	0.034
105	GO_SOMATIC_DIVERSIFICATION_OF_IMMUNOGLOBULINS	0.58	1.83	0.005	0.035
106	GO_DOUBLE_STRAND_BREAK_REPAIR	0.41	1.83	0.000	0.034
107	GO_REGULATION_OF_PHOSPHATIDYLINOSITOL_3_KINASE_SIGNALING	0.41	1.83	0.000	0.034
108	GO_REGULATION_OF_B_CELL_PROLIFERATION	0.49	1.83	0.002	0.034
109	GO_RAB_GUANYL_NUCLEOTIDE_EXCHANGE_FACTOR_ACTIVITY	0.58	1.83	0.003	0.035
110	GO_RNA_HELICASE_ACTIVITY	0.48	1.83	0.002	0.035
111	GO_RAS_GUANYL_NUCLEOTIDE_EXCHANGE_FACTOR_ACTIVITY	0.39	1.83	0.000	0.035
112	GO_NEGATIVE_REGULATION_OF_EMBRYONIC_DEVELOPMENT	0.58	1.83	0.000	0.035
113	GO_CELL_ACTIVATION_INVOLVED_IN_IMMUNE_RESPONSE	0.44	1.83	0.000	0.035
114	GO_TAXIS	0.36	1.83	0.000	0.035
115	GO_MITOTIC_RECOMBINATION	0.53	1.82	0.000	0.035
116	GO_POSITIVE_REGULATION_OF_MAP_KINASE_ACTIVITY	0.39	1.82	0.000	0.035
117	GO_PLATELET_DERIVED_GROWTH_FACTOR_RECEPTOR_SIGNALING_PATHWAY	0.52	1.82	0.003	0.036
118	GO_REGULATION_OF_OSSIFICATION	0.39	1.82	0.000	0.037
119	GO_HISTONE_DEACETYLASE_BINDING	0.45	1.82	0.000	0.037
120	GO_NEGATIVE_REGULATION_OF_OSSIFICATION	0.48	1.82	0.000	0.037
121	GO_HISTONE_METHYLATION	0.46	1.82	0.002	0.037
122	GO_POSITIVE_REGULATION_OF_IMMUNE_RESPONSE	0.35	1.81	0.000	0.037
123	GO_NEGATIVE_REGULATION_OF_CYCLIN_DEPENDENT_PROTEIN_KINASE_ACTIVITY	0.61	1.81	0.003	0.037
124	GO_RESPONSE_TO_HEAT	0.45	1.81	0.003	0.037
125	GO_NONMOTILE_PRIMARY_CILIUM_ASSEMBLY	0.61	1.81	0.002	0.039
126	GO_ANATOMICAL_STRUCTURE_MATURATION	0.56	1.81	0.003	0.039

127	GO_PHAGOCYTOSIS	0.41	1.81	0.000	0.039
128	GO_HEAD_MORPHOGENESIS	0.52	1.81	0.002	0.039
129	GO_POSITIVE_REGULATION_OF_B_CELL_PROLIFERATION	0.52	1.81	0.002	0.039
130	GO_POSITIVE_REGULATION_OF_TRANSFERASE_ACTIVITY	0.34	1.81	0.000	0.038
131	GO_POSITIVE_REGULATION_OF_LOCOMOTION	0.36	1.80	0.000	0.039
132	GO_REGULATION_OF_ENDOTHELIAL_CELL_MIGRATION	0.42	1.80	0.001	0.039
133	GO_DEMETHYLASE_ACTIVITY	0.57	1.80	0.003	0.040
134	GO_POSITIVE_REGULATION_OF_MAPK_CASCADE	0.35	1.80	0.000	0.040
135	GO_RENAL_SYSTEM_VASCULATURE_DEVELOPMENT	0.62	1.80	0.003	0.040
136	GO_STRESS_ACTIVATED_PROTEIN_KINASE_SIGNALING_CASCADE	0.44	1.80	0.002	0.040
137	GO_BLOOD_VESSEL_MORPHOGENESIS	0.36	1.80	0.000	0.040
138	GO_MESENCHYME_DEVELOPMENT	0.39	1.79	0.000	0.040
139	GO_INTRINSIC_COMPONENT_OF_GOLGI_MEMBRANE	0.47	1.79	0.000	0.040
140	GO_REGULATION_OF_CYTOKINE_BIOSYNTHETIC_PROCESS	0.44	1.79	0.002	0.040
141	GO_CELL_PART_MORPHOGENESIS	0.34	1.79	0.000	0.040
142	GO_NEGATIVE_REGULATION_OF_CELL_SUBSTRATE_ADHESION	0.48	1.79	0.000	0.041
143	GO_REGULATION_OF_VASCULATURE_DEVELOPMENT	0.38	1.79	0.000	0.041
144	GO_CENTRIOLE	0.43	1.79	0.000	0.041
145	GO_REGULATION_OF_PHAGOCYTOSIS	0.49	1.79	0.002	0.041
146	GO_REGULATION_OF_IMMUNE_RESPONSE	0.34	1.79	0.000	0.041
147	GO_REGULATION_OF_CHEMOTAXIS	0.39	1.79	0.000	0.041
148	GO_SMOOTH_MUSCLE_CONTRACTION	0.47	1.79	0.000	0.041
149	GO_CILIARY_TRANSITION_ZONE	0.60	1.79	0.003	0.041
150	GO_KIDNEY_VASCULATURE_DEVELOPMENT	0.62	1.78	0.012	0.041
151	GO_REGULATION_OF_GTPASE_ACTIVITY	0.34	1.78	0.000	0.042
152	GO_CELL_CYCLE_PHASE_TRANSITION	0.37	1.78	0.000	0.042
153	GO_NEGATIVE_REGULATION_OF_SMOOTHENED_SIGNALING_PATHWAY	0.59	1.78	0.003	0.043

154	GO_CARDIAC_EPITHELIAL_TO_MESENCHYMAL_TRANSITION	0.58	1.78	0.000	0.042
155	GO_PHOSPHATIDYLINOSITOL_3_KINASE_COMPLEX	0.58	1.78	0.005	0.043
156	GO_REGULATION_OF_CELL_SIZE	0.39	1.78	0.000	0.043
157	GO_INOSITOL_PHOSPHATE_METABOLIC_PROCESS	0.47	1.78	0.000	0.043
158	GO_NEGATIVE_REGULATION_OF_CARTILAGE_DEVELOPMENT	0.56	1.78	0.005	0.043
159	GO_EXTRACELLULAR_STRUCTURE_ORGANIZATION	0.36	1.77	0.000	0.046
160	GO_MICROTUBULE_POLYMERIZATION	0.55	1.77	0.005	0.046
161	GO_NEGATIVE_CHEMOTAXIS	0.51	1.77	0.010	0.046
162	GO_REGULATION_OF_AXONOGENESIS	0.39	1.77	0.000	0.046
163	GO_DNA_BIOSYNTHETIC_PROCESS	0.42	1.77	0.000	0.046
164	GO_NEGATIVE_REGULATION_OF_ORGANELLE_ASSEMBLY	0.61	1.77	0.009	0.045
165	GO_CELLULAR_PROCESS_INVOLVED_IN_REPRODUCTION_IN_MULTICELLULAR_ORGANISM	0.38	1.77	0.000	0.045
166	GO_NEGATIVE_REGULATION_OF_LEUKOCYTE_MEDIATED_IMMUNITY	0.57	1.77	0.005	0.046
167	GO_WOUND_HEALING	0.35	1.76	0.000	0.046
168	GO_REGULATION_OF_NON_CANONICAL_WNT_SIGNALING_PATHWAY	0.60	1.76	0.007	0.046
169	GO_METHYLTRANSFERASE_COMPLEX	0.45	1.76	0.002	0.046
170	GO_HISTONE_METHYLTRANSFERASE_ACTIVITY	0.50	1.76	0.000	0.047
171	GO_ANGIOGENESIS	0.36	1.76	0.000	0.047
172	GO_NEGATIVE_REGULATION_OF_CELL_ACTIVATION	0.41	1.76	0.000	0.047
173	GO_METENCEPHALON_DEVELOPMENT	0.42	1.76	0.000	0.047
174	GO_REGULATION_OF_EXTRINSIC_APOPTOTIC_SIGNALING_PATHWAY_IN_ABSENCE_OF_LIGAND	0.51	1.76	0.005	0.047
175	GO_NEGATIVE_REGULATION_OF_BLOOD_CIRCULATION	0.53	1.76	0.006	0.048
176	GO_SWI_SNF_SUPERFAMILY_TYPE_COMPLEX	0.46	1.76	0.000	0.048
177	GO_NEGATIVE_REGULATION_OF_HISTONE_MODIFICATION	0.53	1.76	0.005	0.048
178	GO_PHOSPHATIDYLINOSITOL_3_KINASE_ACTIVITY	0.44	1.75	0.002	0.048

179	GO_REGULATION_OF_OSTEOBLAST_PROLIFERATION	0.58	1.75	0.002	0.049

5. FINAL CONCLUSIONS

Modern fast-growing broilers have been selected for decades for increased growth rate, feed efficiency and breast meat yield. These selection processes have significantly changed their body development, nutritional needs, and metabolic features, and hence an updated evaluation of these aspects in current broiler genotypes is necessary to ensure high productive efficiency.

Defining a proper ratio among dietary amino acids is a crucial aspect to improve efficiency in modern fast-growing broilers, as well as to promote the economic and environmental sustainability of the poultry industry. In this dissertation it has been shown that the dietary supplementation of crystalline L-arginine to increase the Arg:Lys ratio to levels higher than those currently recommended had a positive effect on feed efficiency without showing any negative outcome on meat quality attributes, foot pad condition and incidence of breast meat abnormalities. In addition, the application of a nuclear magnetic resonance (NMR) approach allowed to obtain important information about the changes exerted by the higher dietary Arg:Lys ratio on plasma and muscle metabolome. This analysis allowed to formulate hypothesis about the molecular mechanisms laying behind the improvements observed in FE in response to the dietary treatment. However, further studies are needed to clearly define and confirm the role of arginine on energy and protein metabolism considering also other tissues, such as liver and adipose tissue.

In the second trial, the growth performance and the whole gene expression profile in the small intestine, a key organ involved in several aspects which can deeply affect FE, were investigated in two fast-growing broiler chicken hybrids. The results showed that the two genotypes exhibited different growth pattern and feeding behavior. Similarly, the microarray analysis revealed a completely different gene expression profile in the intestinal mucosa of the two hybrids that were raised in the same environmental conditions and fed the same diet. Indeed, while the gene expression profile of the first genotype seems consistent with an overall healthy condition of the intestinal mucosa likely supported by an increased mitochondria functionality and antioxidant

capacity, the second one showed a potential inflammatory condition in the gut coupled with a marked activation of the immune system. Although the factors triggering gut inflammation and different feeding behavior are still not clear, the results pave the way to some nutritional and physiological considerations regarding the complex interaction between genotype and nutrition, and also to the importance of considering these aspects for the formulation of genotype-tailored diets to improve productive efficiency and thereby the sustainability of the poultry industry. Furthermore, the ongoing study of some physiological patterns, such as the expression of NPY and its effects on overall energy homeostasis processes, may allow a better comprehension of the feeding behavior mechanisms strictly related to feed efficiency in broiler chickens.

In conclusion, the results obtained combining both traditional and innovative techniques (transcriptomics and metabolomics) in a holistic approach can shed some light on important nutritional and molecular aspects involved in productive efficiency of broiler chickens, confirming the usefulness of these analytical platforms in investigating the molecular responses to different dietary treatments or genetic backgrounds in poultry. A better and detailed knowledge of these aspects in modern fast-growing broiler chicken hybrids may allow an optimization of productive strategies to efficiently sustain the increasing demand of poultry meat while improving animal welfare, product quality, and environmental sustainability.

References

Abdukalykova ST, Zhao X, Ruiz-Feria CA. Arginine and vitamin E modulate the subpopulations of T lymphocytes in broiler chickens. Poult Sci. 2008; 87:50-55.

Adamski J, Suhre K. Metabolomics platforms for genome wide association studies—linking the genome to the metabolome. Curr Opin Biotechnol. 2013; 24(1):39–47.

Aggrey SE, Karnuah AB, Sebastian B, Anthony NB. Genetic properties of feed efficiency parameters in meat-type chickens. Genet Sel Evol. 2010; 42(1):25. https://doi.org/10.1186/1297-9686-42-25.

Anderson JO, Dobson DC. Amino acid requirements of the chick. 2. Effect of total essential amino acid level in the diet on the arginine and lysine requirements. Poult Sci. 1959; 38:1140-1150.

Anderson JO, Combs GF. Effect of single amino acid excesses on glucose metabolism and chick growth as influenced by the dietary amino acid balance. J Nutr. 1952; 46:161-170.

Annison G, Choct M. Anti-nutritive activities of cereal non-starch polysaccharides in broiler diets and strategies minimizing their effects. World Poult Sci J. 1991; 47:232-242. doi:10.1079/WPS19910019

Association of Official Analytical Chemists. Official methods of analysis. 15th ed. AOAC, Washington, DC, USA; 1990.

Austic RE, Nesheim MC. Role of kidney arginase in variations of the arginine requirement of chicks. J Nutr. 1970; 100: 855-868.

Aviagen. Ross 308 nutrition specifications. 2014. http://eu.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-308-Broiler-Nutrition-Specs-plant-2014-EN.pdf http://eu.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-308-Broiler-Nutrition-Specs-plant-2014-EN.pdf Accessed 17 October 2018.

Baeza E, Jégou M, Gondret F, Lalande-Martin J, Tea I, Le Bihan-Duval E, et al. Pertinent plasma indicators of the ability of chickens to synthesize and store lipids. J Anim Sci. 2015;93:107-116.

Bai J, Greene E, Li W, Kidd MT, Dridi S. Branched-chain amino acids modulate the expression of hepatic fatty acid metabolism-related genes in female broiler chickens. Mol Nutr Food Res [Internet]. 2015;59(6):1171–81.https://doi.org/10.1002/mnfr.201400918.

Baker DH. Ideal amino acid profiles for swine and poultry and their applications in feed formulation. Biokyowa Technical Review 1997; 9: 1-24.

Baker DH, Han Y. Ideal amino acid profile for chicks during the first three weeks post-hatching. Poult Sci. 1994;73: 1441-1447.

Ball RO, Urschel KL, Pencharz PB. Nutritional consequences of interspecies differences in arginine and lysine metabolism. J Nutr. 2007;137: 1626S-1641S.

Balnave D, Brake J. Re-evaluation of the classical dietary arginine: lysine interaction for modern poultry diets: a review. World Poult Sci J. 2002;58: 275-289.

Barbara G, Scaioli E, Barbaro MR, Biagi E, Laghi L, Cremon C, et al. Gut microbiota, metabolome and immune signatures in patients with uncomplicated diverticular disease. Gut 2016; doi:10.1136/gutjnl-2016-312377.

Bedford MR. Mechanism of action and potential environmental benefits from the use of feed enzymes. Anim Feed Sci Technol. 1995; 53:145-155.

Berri C, Besnard J, Relandeau C. Increasing dietary lysine increases final pH and decreases drip loss of broiler breast meat. Poult Sci. 2008;87(3):480–4. https://doi.org/10.3382/ps.2007-00226.

Berry DP, Crowley JJ. Residual intake and body weight gain: a new measure of efficiency in growing cattle. J Anim Sci. 2012;90: 109-115.

Beski SS, Swick RA, Iji PA. Specialized protein products in broiler chicken nutrition: A review. Anim Nutr. 2015; 1:47-53.

Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. Physiol Rev. 2014; 94:329-354. https://doi.org/10.1152/physrev.00040.2012

Bilgili, S. F., Alley, M. A., Hess, J. B., & Nagaraj, M. (2006). Influence of age and sex on footpad quality and yield in broiler chickens reared on low and high density diets. Journal of Applied Poultry Research, 15, 433-441. https://doi.org/10.1093/japr/15.3.433

Bilgili, S. F., Moran Jr, E. T., & Acar, N. (1992). Strain-cross response of heavy male broilers to dietary lysine in the finisher feed: Live performance and further-processing yields. Poultry Science, 71(5), 850-858.

Bishop D. Dietary supplements and team-sport performance. Sports Med. 2010;40:995-1017.

Blankenship K, Gilley A, Piekarski A, Orlowski S, Greene E, Bottje W, et al. Differential expression of feeding-related hypothalamic neuropeptides in the first generation of quails divergently selected for low or high feed efficiency. Neuropeptides. 2016;58:31–40. https://doi.org/10.1016/j.npep.2015.12.007.

Bodle BC, Alvarado C, Shirley RB, Mercier Y, Lee JT. Evaluation of different dietary alterations in their ability to mitigate the incidence and severity of woody breast and white striping in commercial male broilers. Poult. Sci.2018; doi:10.3382/ps/pey166.

Boorman KN, Fisher H. The Arginine-Lysine Interaction In The Chick. Br Poult Sci. 1966;7: 39-44.

Boorman KN, Falconer IR, Lewis D. The effect of lysine infusion on the renal reabsorption of arginine in the cockerel. Proc of the Nutr Soc. 1968; 27:61 -62.

Borgström B, Erlanson C. Pancreatic juice colipase: physiological importance. Biochim. Biophys. Acta 1971; 242:509–513.

Bottje W, Tang ZX, Iqbal M, Cawthon D, Okimoto R, Wing T, et al. Association of mitochondrial function with feed efficiency within a single genetic line of male broilers. Poult Sci. 2002; 81(4):546–55. https://doi.org/10.1093/ps/81.4.546.

Bottje W, Pumford NR, Ojano-Dirain C, Iqbal M, Lassiter K. Feed efficiency and mitochondrial function. Poult Sci. 2006; 85(1):8–14. https://doi.org/10.1093/ps/85.1.8.

Bottje W, Brand MD, Ojano-Dirain C, Lassiter K, Toyomizu M, Wing T. Mitochondrial proton leak kinetics and relationship with feed efficiency within a single genetic line of male broilers. Poult Sci. 2009; 88(8):1683–93. https://doi.org/10.3382/ps.2009-00100.

Bottje WG, Carstens GE. Association of mitochondrial function and feed efficiency in poultry and livestock species. J Anim Sci. 2009; 87(E. Suppl):E48–63. https://doi.org/10.2527/jas.2008-1379

Bottje WG, Kong BW, Song JJ, Lee JY, Hargis BM, Lassiter K, et al. Gene expression in breast muscle associated with feed efficiency in a single male broiler line using a chicken 44K microarray. II. Differentially expressed focus genes. Poult Sci. 2012; 91(10):2576–87. https://doi.org/10.3382/ps.2012-02204.

Bottje W, Kong BW. Cell biology symposium: feed efficiency: mitochondrial function to global gene expression. J Anim Sci. 2013; 91(4):1582–93. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23148240.

Bottje WG, Kong BW, Lee JY, Washington T, Baum J, Dridi S, et al. Potential roles of mTOR and protein degradation pathways in the phenotypic expression of feed efficiency in broilers. Biochem Physiol. 2014; https://doi.org/10.4172/2168-9652.10001252.

Bottje W, Kong BW, Reverter A, Waardenberg AJ, Lassiter K, Hudson NJ. Progesterone signalling in broiler skeletal muscle is associated with divergent feed efficiency. BMC Syst Biol. 2017a;11(1) https://doi.org/10.1186/s12918-017-0396-2.

Bottje WG, Lassiter K, Piekarski-Welsher A, Dridi S, Reverter A, Hudson NJ, et al. Proteogenomics reveals enriched ribosome assembly and protein translation in pectoralis major of high feed efficiency pedigree broiler males. Front Physiol. 2017b;8 https://doi.org/10.3389/fphys.2017.00306.

Brake J, Balnave D. Essentiality of arginine in broilers during hot weather. Proceedings of 12th Annual Biokyowa Amino Acid Council Meeting. Oct. 3-5. 1995; St Louis, Mo, USA.

Brautigan DL, Li R, Kubicka E, Turner SD, Garcia JS, Weintraut ML, et al. Lysolecithin as feed additive enhances collagen expression and villus length in the jejunum of broiler chickens. Poult Sci. 2017; 96(8):2889–98. https://doi.org/10.3382/ps/pex078.

Brennan KM, Graugnard DE, Xiao R, Spry ML, Pierce JL, Lumpkins B, et al. Comparison of gene expression profiles of the jejunum of broilers supplemented with a yeast cell wall-derived mannan oligosaccharide versus bacitractin methylene disalicylate. Br Poult Sci. 2013; 54(2):238–46. https://doi.org/10.1080/00071668.2013.775404.

Bungo T, Shiraishi J, Kawakami S. Hypothalamic melanocortin system on feeding regulation in birds: a review. J Poult Sci. 2011; 48(1):1–13. https://doi.org/10.2141/jpsa.010117.

Cahill CM, Rogers JT, Walker WA. The role of phosphoinositide 3-kinase signaling in intestinal inflammation. J Signal Transduct. 2012. doi:10.1155/2012/358476.

Cain RJ, Ridley AJ. Phosphoinositide 3-kinases in cell migration. Biol Cell. 2009; 101:13-29. https://doi.org/10.1042/BC20080079

Christensen K, Owens C, Kidd MT. The effect of added l-arginine on the severity of woody breast and white striping in male broilers. Poultry Science Association Annual Meeting Abstracts. Poult Sci. 2015; 94 E-Suppl 1:147.

Classen HL. Diet energy and feed intake in chickens. Anim Feed Sci Technol. 2016; 233:13-21. https://doi.org/10.1016/j.anifeedsci.2016.03.004

Cobb-Vantress. Cobb 500 broiler performance and nutrition supplement. 2018. https://cobbstorage.blob.core.windows.net/guides/5a171aa0-6994-11e8-9f14-bdc382f8d47e Accessed 17 October 2018.

Choct M. Enzymes for the feed industry: past, present and future. World Poult Sci J. 2006; 62(1):5-16.

Choct M, Annison G. Anti-nutritive effect of wheat pentosans in broiler chickens: Roles of viscosity and gut microflora. Br Poult Sci. 1992; 33:821-834.

Cogburn LA, Porter TE, Duclos MJ, Simon J, Burgess SC, Zhu JJ, et al. Functional genomics of the chicken - a model organism. Poult Sci. 2007; 86: 2059-2094. https://doi.org/10.1093/ps/86.10.2059

Cogburn LA, Wang X, Carre W, Rejto L, Porter TE, Aggrey SE, Simon J. Systems-wide chicken DNA microarrays, gene expression profiling, and discovery of functional genes. Poult Sci. 2003; 82: 939-951. https://doi.org/10.1093/ps/82.6.939

Commission Internationale de l'Eclairage. Recommendations on uniform color spaces, color differences, equations. Psychometric color terms. 1978;15:Suppl 2. CEI, Paris, France.

Corzo A, Kidd MT, Dozier WA, Shack LA, Burgess SC. Protein expression of pectoralis major muscle in chickens in response to dietary methionine status. Br J Nutr. 2006;95(4):703–8. https://doi.org/10.1079/BJN20051716.

Corzo A, Kidd MT. Arginine needs of the chick and growing broiler. Int J Poult Sci. 2003;2:379-382.

Corzo A, Moran Jr. ET, Hoehler D. Arginine need of heavy broiler males: Applying the ideal protein concept. Poult Sci. 2003;82:402-407.

Cox CM, Dalloul RA. Immunomodulatory role of probiotics in poultry and potential in ovo application. Benefic Microbes. 2015;6:45–52. https://doi.org/10.3920/BM2014.0062.

D'mello JPF, Lewis D. Amino acid interactions in chick nutrition. 1. The interrelationship between lysine and arginine. Br Poult Sci. 1970;11: 299-311.

D'Alessandro A, Zolla L. Meat science: from proteomics to integrated omics towards system biology. J Proteome. 2013;78:558–77. https://doi.org/10.1016/j.jprot.2012.10.023.

Davenport HW. Physiology of the digestive tract. Year Book. 1980. Medical Publishers Inc., London, UK.

Dean WF, Scott HM. The development of an amino acid reference diet for the early growth of chicks. Poult Sci. 1965; 44:803-808.

Dean WF, Scott HM. Ability of arginine to reverse the growth depression induced by supplementing a crystalline amino acid diet with excess lysine. Poult Sci. 1968; 47:341-342.

Denbow M. Gastrointestinal Anatomy and Physiology. In: Sturkie's Avian Physiology. 2015; Pp-. 337-366. Ed: Scanes C. Academic Press, San Diego, CA, USA.

Deng K, Wong CW, Nolan JV. Long-term effects of early life L-arginine supplementation on growth performance, lymphoid organs and immune responses in Leghorn-type chickens. Br Poult Sci. 2005; 46:318-324.

Denton J, Coon C, Pettigrew J, Parsons C. Historical and scientific perspectives of same species feeding of animal by-products. J Appl Poult Res.2005; 14:352-361.

Deusch S, Tilocca B, Camarinha-Silva A, Seifert J. News in livestock research—use of omics-technologies to study the microbiota in the gastrointestinal tract of farm animals. Comput Struct Biotechnol J. 2015;13:55–63. https://doi.org/10.1016/j.csbj.2014.12.005.

Dibner JJ, Richards JD. Antibiotic growth promoters in agriculture: history and mode of action. Poult Sci. 2005; 84(4):634-643.

Dieterle F, Ross A, Schlotterbeck G, Senn H. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics. Anal Chem. 2006; 78:4281-4290.

Dozier III WA, Kidd MT, Corzo A. Dietary amino acid responses of broiler chickens. J Appl Poult Res. 2008; 17:157-167.

Dridi S, Anthony N, Kong BW, Bottje W. Feed efficiency: A key pro-duction trait and a global challenge. Adv Food Technol Nutr Sci Open J. 2015 1(3), e11-e13.

Dridi S, Swennen Q, Decuypere E, Buyse J. Mode of leptin action in chicken hypothalamus. Brain Res. 2005;1047(2):214–23. https://doi.org/10.1016/j.brainres.2005.04.034.

Duke GE. Alimentary canal: secretion and digestion, special digestive functions, and absorption. In Avian physiology (pp. 289-302). 1986. Springer, New York, NY.

Duke GE. Anatomy and physiology of the digestive system in fowl. In: Proc. 21st Annual Carolina Poultry Nutrition Conference, 1994, Charlotte, NC, pp.46–49.

Ebrahimi M, Zare Shahneh A, Shivazad M, Ansari Pirsaraei Z, Tebianian M, Ruiz-Feria CA, et al. The effect of feeding excess arginine on lipogenic gene expression and growth performance in broilers. Br Poult Sci. 2014; 55:81-88.

Ekstrand C, Algers B, Svedberg J. Rearing conditions and foot-pad dermatitis in Swedish broiler chickens. Prev Vet Med. 1997; 31:167-174. https://doi.org/10.1016/S0167-5877(96)01145-2

Eltzschig HK, Faigle M, Knapp S, Karhausen J, Ibla J, Rosenberger P, et al. Endothelial catabolism of extracellular adenosine during hypoxia: the role of surface adenosine deaminase and CD26. Blood 2006; 108:1602-1610.

Enser M. The chemistry, biochemistry and nutritional importance of animal fats. 1984. J. Wiseman (Ed.), Fats in Animal Nutrition, Butterworths, London, UK. Pp. 23-51.

Escobar J, Frank JW, Suryawan A, Nguyen HV, Kimball SR, Jefferson LS, et al. Physiological rise in plasma leucine stimulates muscle protein synthesis in neonatal pigs by enhancing translation initiation factor activation. Am J Physiol Endocrinol Metab. 2005; 288:E914-E921.

European Commission. Council Regulation 999/2001/EC of of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. OJ L 147, 31.5.2001, p. 1–40.

European Commission. Council Directive 2007/43/EC of 28 June 2007 laying down minimum rules for the protection of chickens kept for meat production. Off J. 2007; L182:19–28.

European Commission. Council Regulation (EC) No 1099/2009 of 24 Sept. 2009 on the protection of animals at the time of killing. Off J. 2009; L303:1–30.

European Commission. Council Directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes. Off J. 2010; L276:33–79.

European Food Safety Authority. Safety and efficacy of L-arginine produced by Corynebacterium glutamicum KCCM 80099 for all animal species. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). EFSA Journal 2017; 15(6):4858. doi: 10.2903/j.efsa.2017.4858

European Food Safety Authority. Safety and efficacy of L-arginine produced by fermentation with Escherichia coli NITE BP-02186 for all animal species. EFSA Panel on Additives and

Products or Substances used in Animal Feed (FEEDAP). EFSA Journal 2018; 16(5):5276. doi: 10.2903/j.efsa.2018.5276.

European Food Safety Authority. Scientific Opinion on the safety and efficacy of L-arginine produced by Corynebacterium glutamicum KCTC 10423BP for all animal species. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). EFSA Journal 2016; 14(1):4345 doi:10.2903/j.efsa.2016.4345.

FAO. FAO statistics. 2013. http://www.fao.org/faostat/en/

FAO. How to feed the world in 2050. High-level expert forum. 2009. Rome, 12-13 October 2009.

Ferket PR, Gernat AG. Factors that affect feed intake of meat birds: A review. Int J Poult Sci. 2006; 5:905-911.

Fernandes JIM, Murakami AE. Arginine metabolism in uricotelic species. Acta Scient Anim Sci. 2010; 32(4):357-366.

Fernandez SR, Aoyagi S, Han Y, Parsons CM, Baker DH. Limiting order of amino acids in corn and soybean meal for growth of the chick. Poult Sci. 1994; 73(12):1887-1896.

Fisher H, Scott HM. The essential amino acid requirements of chicks as related to their proportional occurrence in the fat-free carcass. Archives Biochem. 1954; 51:517-519.

Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem. 1957; 226:497-509.

Fontanesi L. Metabolomics and livestock genomics: insights into a phenotyping frontier and its applications in animal breeding. Animal Frontiers. 2016; 6(1):73–9.

Foschi C, Laghi L, D'Antuono A, Gaspari V, Zhu C, Dellarosa N, et al. Urine metabolome in women with Chlamydia trachomatis infection. PloS one 2018; 13(3), e0194827.

Fouad AM, El-Senousey HK, Yang XJ, Yao JH. Dietary L-arginine supplementation reduces abdominal fat content by modulating lipid metabolism in broiler chickens. Animal 2013;7:1239-1245.

Fouad AM, El-Senousey HK, Yang XJ, Yao JH. Role of dietary L-arginine in poultry production. Int J Poult Sci. 2012;11:718-729.

Gao S, Zhang J, He C, Meng F, Bu G, Zhu G, et al. Molecular characterization of neuropeptide Y (NPY) receptors (Y1, Y4 and Y6) and investigation of the tissue expression of their ligands (NPY, PYY and PP) in chickens. Gen Comp Endocrinol. 2017;240:46–60. https://doi.org/10.1016/j.ygcen.2016.09.005.

García-Cañas V, Simó C, Herrero M, Ibáñez E, Cifuentes A. Present and future challenges in food analysis: Foodomics. Anal Chem. 2012;84(23):10150–9. https://doi.org/10.1021/ac301680q.

Garriga C, Barfull A, Planas JM. Kinetic characterization of apical D-fructose transport in chicken jejunum. J Membrane Biol. 2004; 197(1):71-76.

Gilbert ER, Wong EA, Webb Jr KE. Board-invited review: peptide absorption and utilization: implications for animal nutrition and health. J Anim Sci. 2008; 86(9): 2135-2155.

Glista WA, Mitchell HH, Scott HM. The amino acid requirements of the chick. Poult Sci. 1951; 30(6): 915-915.

Gong J, Si W, Forster RJ, Huang R, Yu H, Yin Y, et al. 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. FEMS Microbiol Ecol. 2007; 59(1):147-157.

Hack B, Witting PK, Rayner BS, Stocker R, Headrick JP. Oxidant stress and damage in post-ischemic mouse hearts: effects of adenosine. Mol Cell Biochem. 2006; 287:165-175.

Hajati H, Rezaei M. The application of prebiotics in poultry production. Int J Poult Sci. 2010;9(3):298–304.

Han K, Lee JH. The role of synthetic amino acids in monogastric animal production. Asian-Australas J Anim Sci. 2000; 13(4):543-560.

Havenstein GB, Ferket PR, Scheideler SE, Rives DV. Carcass composition and yield of 1991 vs 1957 broilers when fed typical 1957 and 1991 broiler diets. Poult Sci. 1994; 73:1795-1804.

Havenstein GB, Ferket PR, Qureshi MA. Carcass composition and yield of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets., Poult Sci. 2003a; 82:1509-1518.

Havenstein GB, Ferket PR, Qureshi MA. Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult Sci. 2003b; 82:1500-1508.

Hazarika M. Utilization of animal by-products as animal feed. Livest Advis. 1994; 19:14

Hermier D. Lipoprotein metabolism and fattening in poultry. J. Nutr. 1997; 127:805S–808S.

Hickling D, Guenter W, Jackson ME. The effects of dietary methionine and lysine on broiler chicken performance and breast meat yield. Can J Anim Sci. 1990; 70(2):673-678.

Honda K, Saneyasu T, Kamisoyama H. Gut hormones and regulation of food intake in birds. J Poult Sci. 2017; 54:103–10. https://doi.org/10.2141/jpsa.0160100.

Hristakieva P, Mincheva N, Oblakova M, Lalev M, Ivanova I. Effect of genotype on production traits in broiler chickens. Slovak J Anim Sci. 2014; 47:19-24.

Huber K, Zeller E, Rodehutscord M. Modulation of small intestinal phosphate transporter by dietary supplements of mineral phosphorus and phytase in broilers. Poult Sci. 2015; 94(5):1009–17. https://doi.org/10.3382/ps/pev065.

Hubert M, Rousseeuw PJ, Vanden Branden K. ROBPCA: a new approach to robust principal component analysis. Technometrics 2005; 47:64-79.

Hurwitz S, Bar A, Katz M, Sklan D, Budowski P. Absorption and secretion of fatty acids and bile acids in the intestine of the laying fowl. J. Nutr. 1973; 103:543–547.

Huston RL, Scott HM. Effect of varying the composition of a crystalline amino acid mixture on weight gain and pattern of free amino acids in chick tissue. In Federation proceedings 1968; 27(5): 1204-1209.

Iqbal M, Pumford NR, Tang ZX, Lassiter K, Ojano-Dirain C, Wing T, et al. Compromised liver mitochondrial function and complex activity in low feed efficient broilers are associated with higher oxidative stress and differential protein expression. Poult Sci. 2005; 84(6):933–41. https://doi.org/10.1093/ps/84.6.933.

Iqbal M, Pumford NR, Tang ZX, Lassiter K, Wing T, Cooper M, et al. Low feed efficient broilers within a single genetic line exhibit higher oxidative stress and protein expression in breast muscle with lower mitochondrial complex activity. Poult Sci. 2004; 83(3):474–84. https://doi.org/10.1093/ps/83.3.474.

Jahanian R, Khalifeh-Gholi M. Marginal deficiencies of dietary arginine and methionine could suppress growth performance and immunological responses in broiler chickens. J Anim Physiol Anim Nutr. 2018; 102:e11-e20.

Jamroz D. Comparative characteristic of gastrointestinal tract development and digestibility of nutrients in young chickens, ducks and geese. World Poultry Science Association, 15th European Symposium on Poultry Nutrition, Balatonfüred, Hungary, 25-29 September, 2005;74-83.

Jeacocke RE. Continuous measurement of the pH of beef muscle in intact beef carcasses. J Food Tech. 1977;12:375–386.

Jerrett SA and Goodge WR. Evidence for amylase in avian salivary glands. J. Morphol., 1973; 139: 27-45.

Jiao P, Guo Y, Yang X, Long F. Effects of dietary arginine and methionine levels on broiler carcass traits and meat quality. J Anim Vet Adv. 201; 9:1546-1551.

Jones JD. Lysine-arginine antagonism in the chick. J Nutr. 1964; 84:313-321.

Jones JD, Petersburg SJ, Burnett PC. The mechanism of the lysine-arginine antagonism in the chick: effect of lysine on digestion, kidney arginase and liver transamidinase. J Nutr. 1967; 93:103-1 16.

Joshi A, Paratkar SG, Thorat BN. Modification of lecithin by physical, chemical and enzymatic methods. Eur J Lipid Sci Technol. 2006; 108:363–373.

Junot C, Fenaille F, Colsch B, Bécher F. High resolution mass spectrometry based techniques at the crossroads of metabolic pathways. Mass Spectrom Rev. 2014;33(6):471–500.

Kabir SML. The role of probiotics in the poultry industry. Int J Mol Sci. 2009; 10(8):3531–46. https://doi.org/10.3390/ijms10083531.

Kaiser P, Balic A. The avian immune system. Sturkie's Avian Physiology, 6th ed. C.G. Scanes, ed. Elsevier Inc. Academic Press, San Diego, CA, USA. 2015; 403-418.

Kestin SC, Gordon S, Su G, Sørensen P. Relationships in broiler chickens between lameness, liveweight, growth rate and age. Vet Record 2001; 148:195-197.

Khajali F, Wideman RF. Dietary arginine: metabolic, environmental, immunological and physiological interrelationships. Worlds Poult Sci J. 2010; 66:751-766.

Khonyoung D, Yamauchi K, Suzuki K. Influence of dietary fat sources and lysolecithin on growth performance, visceral organ size, and histological intestinal alteration in broiler chickens. Livest Sci. 2015;176:111–20. https://doi.org/10.1016/j.livsci.2015.03.011.

Kidd MT, Kerr BJ, Anthony NB. Dietary interactions between lysine and threonine in broilers. Poult Sci.1997; 76:608-614.

Kidd MT, Kerr BJ, Halpin KM, McWard GW, Quarles CL. Lysine levels in starter and grower-finisher diets affect broiler performance and carcass traits. J Appl Poult Res. 1998;7(4):351–8. https://doi.org/10.1093/japr/7.4.351.

Kidd MT, Peebles ED, Whitmarsh SK, Yeatman JB, Wideman Jr. RF. Growth and immunity of broiler chicks as affected by dietary arginine. Poult Sci. 2001; 80:1535-1542.

Klain GJ, Scott HM, Johnson BC. The amino acid requirement of the growing chick fed a crystalline amino acid diet. Poult Sci. 1960; 39(1):39-44.

Kneen MA, Annegarn HJ. Algorithm for fitting xRF, SEM and PIxE x-ray spectra backgrounds. Nucl Instrum Methods Phys Res B. 1996; 109:209-213.

Koch RM, Swiger LA, Chambers D, Gregory KE. Efficiency of feed use in beef cattle. J Anim Sci. 1963;22: 486-494.

Kogut MH, Genovese KJ, Swaggerty CL, He H, Broom L. Inflammatory phenotypes in the intestine of poultry: not all inflammation is created equal. Poult Sci. 2018; 97:2339–2346. https://doi.org/10.3382/ps/pey087

Kong BW, Lassiter K, Piekarski-Welsher A, Dridi S, Reverter-Gomez A, Hudson NJ, et al. Proteomics of breast muscle tissue associated with the phenotypic expression of feed efficiency within a pedigree male broiler line: I. Highlight on mitochondria. PLoS One. 2016; 11(5):e0155679. https://doi.org/10.1371/journal.pone.0155679. PMID: 27244447.

Kong BW, Song JJ, Lee JY, Hargis BM, Wing T, Lassiter K, et al. Gene expression in breast muscle associated with feed efficiency in a single male broiler line using a chicken 44K oligo microarray. I. Top differentially expressed genes. Poult Sci. 2011; 90(11):2535–47. https://doi.org/10.3382/ps.2011-01435.

Konwar D, Barman K. Potential of utilization of animal by-products in animal feed. North East Vet 2005; 5: 28-31.

Krogdahl A. Digestion and absorption of lipid in poultry. J Nutr. 1985; 115:675–685.

Kuenzel WJ, Beck MM, Teruyama R. Neural sites and pathways regulating food intake in birds: a comparative analysis to mammalian systems. J Exp Zool A: Ecol Genet Physiol. 1999; 283(4-5):348–64. https://doi.org/10.1002/(SICI)1097-010X(19990301/01)283:4/5<348::AID-JEZ5>3.0.CO;2-5.

Kuenzel WJ, Douglass LW, Davison BA. Robust feeding following central administration of neuropeptide Y or peptide YY in chicks, Gallus domesticus. Peptides 1987; 8(5): 823-828.

Kuttappan VA, Lee Y, Erf GF, Meullenet JF, Owens CM. Consumer acceptance of visual appearance of broiler breast meat with varying degrees of white striping. Poult Sci. 2012; 91:1240–1247.

Kwak H, Austic RE, Dietert RR. Influence of dietary arginine concentration on lymphoid organ growth in chickens. Poult Sci. 1999; 78:1536-1541.

Laika M, Jahanian R. Increase in dietary arginine level could ameliorate detrimental impacts of coccidial infection in broiler chickens. Livest Sci. 2017; 195:38-44.

Lassiter K, Ojano-Dirain C, Iqbal M, Pumford NR, Tinsley N, Lay J, et al. Differential expression of mitochondrial and extramitochondrial proteins in lymphocytes of male broilers with low and high feed efficiency. Poult Sci. 2006; 85(12):2251–9. https://doi.org/10.1093/ps/85.12.2251. 19.

Lassiter K, Greene E, Piekarski A, Faulkner OB, Hargis BM, Bottje W, et al. Orexin system is expressed in avian muscle cells and regulates mitochondrial dynamics. Am J Physiol Regul Integr Comp Physiol. 2014; 308(3):R173-R187.

Leclercq B. Lysine: Specific effects of lysine on broiler production: comparison with threonine and valine. Poult Sci. 1998; 77(1):118-123.

Lee J, Aggrey SE. Transcriptomic differences of genes in the avian target of rapamycin (avTOR) pathway in a divergent line of meat-type chickens selected for feed efficiency. Genet Mol Res. 2016;15(2):gmr.15027120.

Lee J, Karnuah AB, Rekaya R, Anthony NB, Aggrey SE. Transcriptomic analysis to elucidate the molecular mechanisms that underlie feed efficiency in meat-type chickens. Mol Gen Genomics. 2015;290(5):1673–82. https://doi.org/10.1007/s00438-015-1025-7.

Lee SA, Bedford MR. Inositol - an effective growth promotor? World Poult Sci J. 2016;72:743–60. https://doi.org/10.1017/S0043933916000660.

Lee JE, Austic RE, Naqi SA, Golemboski KA, Dietert RR. Dietary arginine intake alters avian leukocyte population distribution during infectious bronchitis challenge. Poult Sci. 2002; 81:793-798.

Leeson S, Summers JD. Scott's nutrition of the chicken. 4th Edition. 2001. University books, Guelph, Ontario, Canada.

Liebler DC. Introduction to proteomics: tools for the new biology. New York: Springer Science & Business Media; 2001.

Liland KH, Almøy T, Mevik BH. Optimal choice of baseline correction for multivariate calibration of spectra. Appl Spectrosc. 2010; 64:1007-1016.

Lundbaek JA, Andersen OS. Lysophospholipids modulate channel function by altering the mechanical properties of lipid bilayers. J Gen Physiol. 1994; 104:645–673.

Luo J, Zheng A, Meng K, Chang W, Bai Y, Li K, et al. Proteome changes in the intestinal mucosa of broiler (Gallus gallus) activated by probiotic enterococcus faecium. J Proteome. 2013; 91:226–41. https://doi.org/10.1016/j.jprot.2013.07.017.

Marcato SM, Sakomura NK, Munari DP, Fernandes JBK, Kawauchi IM, Bonato MA. Growth and body nutrient deposition of two broiler commercial genetic lines. Rev Bra Ciênc Avíc. 2008; 10:117-123. http://dx.doi.org/10.1590/S1516-635X2008000200007

Marchaim U, Kulka RG. The non-parallel increase of amylase, chymotrypsinogen and procarboxypeptidase in the developing chick pancreas. Biochim Biophys Acta Enzymology 1967; 146:553-559.

McConell GK, Shinewell J, Stephens TJ, Stathis CG, Canny BJ, Snow RJ. Creatine supplementation reduces muscle inosine monophosphate during endurance exercise in humans. Med Sci Sports Exerc. 2005; 37:2054-2061.

Meluzzi A, Sirri F. Welfare of broiler chickens. Ital J Anim Sci. 2009; 8:161-173. https://doi.org/10.4081/ijas.2009.s1.161

Mignon-Grasteau S, Narcy A, Rideau N, Chantry-Darmon C, Boscher MY, Sellier N, et al. Impact of selection for digestive efficiency on microbiota composition in the chicken. PLoS One. 2015;10(8) https://doi.org/10.1371/journal.pone.0135488.

Mine Y, Chiba K, Tada M. Effect of phospholipids on conformational change and heat stability of ovalbumin. Circular dichroism and nuclear magnetic resonance studies. J Agric Food Chem. 1993; 41:157–161.

Mitchell HH. Comparative nutrition of man and domestic animals. 1964. Academic Press, New York, USA.

Mitchell HH, Block RJ. Some relationships between the amino acid contents of proteins and their nutritive values for the rat. J Biol Chem. 1946; 163(3):599-620.

Moran Jr ET, Bilgili SF. Processing losses, carcass quality, and meat yields of broiler chickens receiving diets marginally deficient to adequate in lysine prior to marketing. Poult Sci. 1990; 69(4):702-710.

Nahm KH. Efficient feed nutrient utilization to reduce pollutants in poultry and swine manure. Crit Rev Environ Sci Technol. 2002; 32:1-16.

National Research Council. Nutrient requirements of poultry. Subcommittee on Poultry Nutrition, Committee on Animal Nutrition, Board on Agriculture 1994;176.

Newmyer BA, Nandar W, Webster RI, Gilbert E, Siegel PB, Cline MA. Neuropeptide Y is associated with changes in appetite-associated hypothalamic nuclei but not food intake in a hypophagic avian model. Behavioural brain research. 2013; 236:327-331.

Newsholme P, Lima MMR, Procopio J, Pithon-Curi TC, Bazotte RB, Curi R. Glutamine and glutamate as vital metabolites. Braz J Med Biol Res. 2003;36:153-163.

Noy Y, D. Sklan. Digestion and absorption in the young chick. Poult. Sci. 1995; 74:366–373.

O'Dell BL, Savage JE. Arginine-lysine antagonism in the chick and its relationship to dietary cations. J Nutr. 1966; 90: 364-370.

Ojano-Dirain C, Toyomizu M, Wing T, Cooper M, Bottje WG. Gene expression in breast muscle and duodenum from low and high feed efficient broilers. Poult Sci. 2007; 86(2):372–81. https://doi.org/10.1093/ps/86.2.372.

Ojano-Dirain CP, Iqbal M, Cawthon D, Swonger S, Wing T, Cooper M, et al. Determination of mitochondrial function and site-specific defects in electron transport in duodenal mitochondria in broilers with low and high feed efficiency. Poult Sci. 2004; 83(8):1394–403. https://doi.org/10.1093/ps/83.8.1394.

Olukosi OA, Cowieson AJ, Adeola O. Age-related influence of a cocktail of xylanase, amylase, and protease or phytase individually or in combination in broilers. Poult. Sci. 2007; 86:77-86.

Patterson JA, Burkholder KM. Application of prebiotics and probiotics in poultry production. Poult Sci. 2003; 84:627–31. https://doi.org/10.1093/ps/82.4.627.

Petracci M, Mudalal S, Soglia F, Cavani C. Meat quality in fast-growing broiler chickens. World Poult Sci J. 2015; 71:363-374.

Phang JM, Liu W, Zabirnyk O. Proline metabolism and microenvironmental stress. Annu Rev Nutr. 2010; 30:441-463.

Phang JM, Pandhare J, Liu Y. The metabolism of proline as microenvironmental stress substrate. J Nutr. 2008;138:2008S–2015S.

Picard B, Berri C, Lefaucheur L, Molette C, Sayd T, Terlouw C. Skeletal muscle proteomics in livestock production. Brief Funct Genomic Proteomic. 2010;9:259–78.

Piekarski A, Greene E, Anthony NB, Bottje W, Dridi S. Crosstalk between autophagy and obesity: potential use of avian model. Adv Food Technol Nutri Sci. 2015; 1:32-37. http://dx.doi.org/10.17140/AFTNSOJ-1-106

Piekarski-Welsher A, Bottje W, Dridi S. Adipokines-AMPK cross-talk in avian species: A translational model for human obesity. Obes Res Open J. 2016; 3:40-42. http://dx.doi.org/10.17140/OROJ-3-126

Pond WG, Church DC, Pond KR, Schoknecht PA. Basic animal nutrition and feeding. 5th ed. 2005. John Wiley & Sons, Inc., New York, NY.

Ravindran V. Poultry feed availability and nutrition in developing countries. Poultry development review 2013; 60-63.

Ravindran V, Tancharoenrat P, Zaefarian F, Ravindran G. Fats in poultry nutrition: Digestive physiology and factors influencing their utilisation. Anim Feed Sci Technol. 2016; 213:1-21.

European Commission. Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. Off J. 2003; 268:29–43.

Richards MP, Proszkowiec-Weglarz M. Mechanisms regulating feed intake, energy expenditure, and body weight in poultry. Poult Sci. 2007; 86(7):1478–90. https://doi.org/10.1093/ps/86.7.1478.

Richards MP. Genetic regulation of feed intake and energy balance in poultry. Poult Sci. 2003;8 2(6):907–16. https://doi.org/10.1093/ps/82.6.907.

Ritz CW, Fairchild BD, Lacy MP. Implications of ammonia production and emissions from commercial poultry facilities: A review. J Appl Poult Res. 2004; 13:684-692.

Rodeheaver DP, Wyatt RD. Distribution of alpha-amylase activity in selected broiler tissues. Poult. Sci. 1986; 65:325-329.

Romero LF, Zuidhof MJ, Renema RA, Naeima AN, Robinson F. Effects of maternal energetic efficiency on egg traits, chick traits, broiler growth, yield, and meat quality. Poult Sci. 2009; 88: 236-245.

Rueda E, Michelangeli C, Gonzalez-Mujica F. L-canavanine inhibits L-arginine uptake by broiler chicken intestinal brush border membrane vesicles. Br Poult Sci. 2003; 44:620-625.

Ruiz-Feria CA, Kidd MT, Wideman Jr RF. Plasma levels of arginine, ornithine, and urea, and growth performance of broilers fed supplemental l-arginine during cool temperature exposure. Poult Sci 2001; 80:358-369.

Sabio G, Davis RJ. TNF and MAP kinase signalling pathways. Semin immunol 2014; 26:237-245. https://doi.org/10.1016/j.smim.2014.02.009

Saneyasu T, Honda K, Kamisoyama H, Ikura A, Nakayama Y, Hasegawa S. Neuropeptide Y effect on food intake in broiler and layer chicks. Comp Biochem Physiol A Mol Integr Physiol. 2011; 159(4):422-426.

SAS. SAS/STAT Guide for Personal Computers, Version 6.03 edition. 1988; SAS Institute Inc., Cary, NC, USA.

Sasse CE, Baker DH. Modification of the Illinois reference standard amino acid mixture. Poult Sci. 1973; 52:1970-1972.

Savory CJ, Mitchell MA. Absorption of hexose and pentose sugars in vivo in perfused intestinal segments in the fowl. Comp Biochem Physiol A Mol Integr Physiol. 1991; 100(4):969-974.

Scanes C, Brant G., Ensminger M. Poultry science (4th ed.). 2004. Pearson Prentice Hall, New Jersey, USA 100-118.

Scanes CG, Pierzchala-Koziec K. Biology of the gastro-intestinal tract in poultry. Avian Biol Res. 2014;7(4):193–222. https://doi.org/10.3184/175815514X14162292284822.

Scanes CG. Carbohydrate metabolism. In: C.G. Scanes, editor. Sturkie's Avian Physiology, 6th ed. Elsevier Inc. Academic Press, San Diego, CA, USA. 2015; p. 421-437.

Schiavone A. Nutrizione e alimentazione degli avicoli. In: Avicoltura e Coniglicoltura. Pp. 179-260. Ed. Cerolini S, Marzoni Fecia di Cossato M, Romboli I, Schiavone A, Zaniboni L. 2008. Le Point Veterinaire Italy, Milano, Italy.

Schmeisser J, Séon AA, Aureli R, Friedel A, Guggenbuhl P, Duval S, et al. Exploratory transcriptomic analysis in muscle tissue of broilers fed a phytase-supplemented diet. J Anim Physiol Anim Nutr. 2017;101(3):563–75. https://doi.org/10.1111/jpn.12482.

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C T method. Nat Prot. 2008; 3(6):1101.

Schwarzer K, Adams CA. The influence of specific phospholipids as absorption enhancer in animal nutrition. Lipid/Fett. 1996; 98:304–308.

Selle PH, Ravindran V. Microbial phytase in poultry nutrition. Anim Feed Sci Technol. 2007; 135:1–41. https://doi.org/10.1016/j.anifeedsci.2006.06.010.

Shepherd EM, Fairchild BD. Footpad dermatitis in poultry. Poult Sci. 2010; 89:2043-2051. https://doi.org/10.3382/ps.2010-00770

Sherwood DH. Modern broiler feeds and strains: What two decades of improvement have done. Feedstuffs 1977; 49:70

Shier WT, Baldwin JH, Nilsen-Hamilton M, Hamilton RT, Thanassi NM. Regulation of guanylate and adenylate cyclase activities by lysolecithin. Proc Natl Acad Sci USA. 1976; 73:1586–1590.

Siegel PB. Evolution of the modern broiler and feed efficiency. Annu. Rev. Anim. Biosci., 2014; 2: 375-385.

Siegert W, Wild KJ, Schollenberger M, Helmbrecht A, Rodehutscord M. Effect of glycine supplementation in low protein diets with amino acids from soy protein isolate or free amino acids on broiler growth and nitrogen utilisation. Br Poult Sci. 2016; 57:424-434.

Sihvo HK, Immonen K, Puolanne E. Myodegeneration with fibrosis and regeneration in the pectoralis major muscle of broilers. Vet Pathol. 2014;51:619-623.

Sintubin P, Greene E, Collin A, Bordas A, Zerjal T, Tesseraud S, et al. Expression profile of hypothalamic neuropeptides in chicken lines selected for high or low residual feed intake. Neuropeptides. 2014; 48(4):213–20. https://doi.org/10.1016/j.npep.2014.04.007.

Sirri F, Maiorano G, Tavaniello S, Chen J, Petracci M, Meluzzi A. Effect of different levels of dietary zinc, manganese, and copper from organic or inorganic sources on performance, bacterial chondronecrosis, intramuscular collagen characteristics, and occurrence of meat quality defects of broiler chickens. Poult Sci. 2016; 95:1813-1824.

Sirri F, Petracci M, Zampiga M, Meluzzi A. Effect of EU electrical stunning conditions on breast meat quality of broiler chickens. Poult Sci. 2017; 96:3000-3004.

Snetsinger DC, Scott HM. Efficacy of glycine and arginine in alleviating the stress induced by dietary excesses of single amino acids. Poult Sci. 1961; 40:1675-1 68 I.

Soares R, Franco C, Pires E, Ventosa M, Palhinhas R, Koci K, et al. Mass spectrometry and animal science: protein identification strategies and particularities of farm animal species. J Proteome. 2012; 75:4190–206.

Spratt RS, McBride BW, Bayley HS, Leeson S. Energy metabolism of broiler breeder hens. 2. Contribution of tissues to total heat production in fed and fasted hens. Poult Sci. 1990; 69: 1348-1356. https://doi.org/10.3382/ps.0691348

Spring P, Wenk C, Connolly A, Kiers A. A review of 733 published trials on bio-Mos®, a mannan oligosaccharide, and Actigen®, a second generation mannose rich fraction, on farm and companion animals. J Appl Anim Nutr. 2015; 3:E8. https://doi.org/10.1017/jan.2015.6.

Stanley D, Denman SE, Hughes RJ, Geier MS, Crowley TM, Chen H, et al. Intestinal microbiota associated with differential feed conversion efficiency in chickens. Appl Microbiol Biotechnol. 2012; 96(5):1361–9. https://doi.org/10.1007/s00253-011-3847-5.

Stanley D, Hughes RJ, Geier MS, Moore RJ. Bacteria within the gastrointestinal tract microbiota correlated with improved growth and feed conversion: challenges presented for the identification of performance enhancing probiotic bacteria. Front Microbiol. 2016; 7:1897. https://doi.org/10.3389/fmicb.2016.00187.

Sterling KG, Pesti GM, Bakalli RI. Performance of different broiler genotypes fed diets with varying levels of dietary crude protein and lysine. Poult Sci. 2006;8 5(6):1045–54. https://doi.org/10.1093/ps/85.6.1045.

Suarez ME, Wilson HR, Mather FB, Wilcox CJ, McPherson BN. Effect of strain and age of the broiler breeder female on incubation time and chick weight. Poult Sci. 1997; 76:1029-1036. https://doi.org/10.1093/ps/76.7.1029

Sugiharto S. Role of nutraceuticals in gut health and growth performance of poultry. J Saudi Soc Agri Sci.2016; 15:99-111. https://doi.org/10.1016/j.jssas.2014.06.001

Sun X, Zhang H, Sheikhahmadi A, Wang Y, Jiao H, Lin H, et al. Effects of heat stress on the gene expression of nutrient transporters in the jejunum of broiler chickens (Gallus gallus domesticus). Int J Biometeorol. 2015; 59(2):127–35. https://doi.org/10.1007/s00484-015-0996-8.

Tagesson C, Franzen L, Dahl G, Westrom B. Lysophosphatidylcholine increases rat ileal permeability to macromolecules. Gut 1985; 26:369–377.

Tallentire CW, Leinonen I, Kyriazakis I. Artificial selection for improved energy efficiency is reaching its limits in broiler chickens. Sci Rep. 2018; 8(1):1168.

Tamir H, Ratner S. Enzymes of arginine metabolism in chicks. Arch Biochem Biophys. 1963;102:249-258.

Tan J, Applegate TJ, Liu S, Guo Y, Eicher SD. Supplemental dietary l-arginine attenuates intestinal mucosal disruption during a coccidial vaccine challenge in broiler chickens. Br J Nutr. 2014a;112(7):1098–109. https://doi.org/10.1017/S0007114514001846.

Tan J, Liu S, Guo Y, Applegate TJ, Eicher SD. Dietary l-arginine supplementation attenuates lipopolysaccharide-induced inflammatory response in broiler chickens. Br J Nutr. 2014b; 111:1394–404. https://doi.org/10.1017/S0007114513003863.

Tan JZ, Guo YM, Applegate TJ, Du EC, Zhao X. Dietary L-arginine modulates immunosuppression in broilers inoculated with an intermediate strain of infectious bursa disease virus. J Sci Food Agric. 2015; 95(1):126–35. https://doi.org/10.1002/jsfa.6692.

Tan X, Pan JQ, Li JC, Liu YJ, Sun WD, Wang XL. L-arginine inhibiting pulmonary vascular remodelling is associated with promotion of apoptosis in pulmonary arterioles smooth muscle cells in broilers. Res Vet Sci. 2005; 79: 203-209.

Tan X, Sun WD, Hu XY, Li JC, Pan JQ, Wang JY, Wang XL.Changes in pulmonary arteriole protein kinase Cα expression associated with supplemental L-arginine in broilers during cool temperature exposure. Br Poult Sci. 2006; 47:230-236.

Tancharoenrat P, Ravindran V, Zaefarian F, Ravindran G. Digestion of fat and fatty acids along the gastrointestinal tract of broiler chickens. Poult Sci. 2014; 93:412–419.

Taylor RL, Austic RE, Dietert RR. Dietary arginine influences Rous sarcoma growth in a major histocompatibility B complex progressor genotype. Proceedings of Society of Experimental Biology and Medicine 1992; 199: 38-41.

Tesseraud S, Bouvarel I, Collin A, Audouin E, Crochet S, Seiliez I, et al. Daily variations in dietary lysine content alter the expression of genes related to proteolysis in chicken pectoralis major muscle. J Nutr. 2009; 139(1):38–43.https://doi.org/10.3945/jn.108.095752.

Tesseraud S, Everaert N, Boussaid-Om Ezzine S, Collin A, Métayer-Coustard S, Berri C. Manipulating tissue metabolism by amino acids. World Poult Sci J. 2011; 67:243–51. https://doi.org/10.1017/S0043933911000274.

Tesseraud S, Peresson R, Lopes J, Chagneau AM. Dietary lysine deficiency greatly affects muscle and liver protein turnover in growing chickens. Br J Nutr. 1996; 75(6):853–65. http://www.ncbi.nlm.nih.gov/pubmed/8774230

Tesseraud S, Temim S, Le Bihan-Duval E, Chagneau AM. Increased responsiveness to dietary lysine deficiency of pectoralis major muscle protein turnover in broilers selected on breast development. J Anim Sci. 2001; 79(4):927–33. https://doi.org/10.2527/2001.794927x.

Tinsley N, Iqbal M, Pumford NR, Lassiter K, Ojano-Dirain C, Wing T, et al. Investigation of mitochondrial protein expression and oxidation in heart muscle in low and high feed efficient male broilers in a single genetic line. Poult Sci. 2010; 89(2):349–52. https://doi.org/10.3382/ps.2009-00138.

Tomas-Cobos T, Minambres R, Rodrigo A, Navarro A, Tomas D. Arginine and the immune system. Proceedings of Nutrition Society 2008; 67(OCE)E3.

Tona K, Onagbesan OM, Kamers B, Everaert N, Bruggeman V, Decuypere E. Comparison of Cobb and Ross strains in embryo physiology and chick juvenile growth. Poult Sci. 2010; 89:1677-1683. https://doi.org/10.3382/ps.2009-00386

Twyman RM. Principles of proteomics. New York: Garland Science (Taylor and Francis Group), USA; 2013.

Uni Z, Tako E, Gal-Garber O, Sklan D. Morphological, molecular, and functional changes in the chicken small intestine of the late-term embryo. Poult. Sci., 2003; 82:1747-1754.

Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, et al. Global trends in antimicrobial use in food animals. Proceedings of the National Academy of Sciences of the United States of America 2015; 112(18):5649–5654. http://doi.org/10.1073/pnas.1503141112

Ventrella D, Laghi L, Barone F, Elmi A, Romagnoli N, Bacci ML. Age-related 1 H NMR characterization of cerebrospinal fluid in newborn and young healthy piglets. PLoS one 2016;11:e0157623.

Vignale K, Greene ES, Caldas JV, England JA, Boonsinchai N, Sodsee P, et al. 25-hydroxycholecalciferol enhances male broiler breast meat yield through the mTOR pathway. J Nutr. 2015; 145(5):855–63. https://doi.org/10.3945/jn.114.207936.

Villamor E, Ruijtenbeek K, Pulgar V, De Mey JGR, Blanco CE. Vascular reactivity in intrapulmonary arteries of chicken embryos during transition to ex ovo life. Am J Physiol. 2002; 282: R917-R927.

Wallner S, Hermetter A, Mayer B, Wascher TC. The alpha-amino group of l-arginine mediates its antioxidant effect. Eur J Clin Invest. 2001; 31:98-102.

Wang K, Gan L, Lee I, Hood L. Isolation and characterisation of the chicken trypsinogen gene family. Biochem J. 1995; 307:471-479.

Weaver SA, Ward SG. Phosphoinositide 3-kinases in the gut: a link between inflammation and cancer?. Trends Mol Med. 2001; 7:455-462. https://doi.org/10.1016/S1471-4914(01)02107-4

Wen C, Chen X, Chen GY, Wu P, Chen YP, Zhou YM, et al. Methionine improves breast muscle growth and alters myogenic gene expression in broilers. J Anim Sci. 2014a; 92(3):1068–73. https://doi.org/10.2527/jas.2013-6485.

Wen C, Wu P, Chen Y, Wang T, Zhou Y. Methionine improves theperformance and breast muscle growth of broilers with lower hatching weight by altering the expression of genes associated with the insulin-like growth factor-I signalling pathway. Br J Nutr. 2014b; 111(2):201–6. https://doi.org/10.1017/S0007114513002419.

Wideman Jr RF, Kirby YK, Forman M, Tacket CD, Marson NE, Mcnew RW. Cardiopulmonary function during acute unilateral occlusion of pulmonary artery in broilers fed diets containing normal or high levels of arginine-HCl. Poult Sci. 1996; 75:1587-1602.

Wideman Jr RF, Kirby YK, Ismail M, Bottje WG, Moore RW, Vardeman RC. Supplemental L-arginine attenuates pulmonary hypertension syndrome (ascites) in broilers. Poult Sci. 1995; 74:323-330.

Willems OW, Miller SP, Wood BJ. Aspects of selection for feed efficiency in meat producing poultry. World Poult Sci J. 2013; 69:77-88.

Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, et al. HMDB: the human metabolome database. Nucleic Acids Res. 2007; 35(suppl 1), D521-D526.

Wu G, Bazer FW, Burghardt RC, Johnson GA, Kim SW, Knabe DA, et al. Proline and hydroxyproline metabolism: implications for animal and human nutrition. Amino Acids 2011; 40:1053-1063.

Wu LY, Fang YJ, Guo XY. Dietary L-arginine supplementation beneficially regulates body fat deposition of meat-type ducks. Br Poult Sci. 2011; 52:221-226.

Wu G. Dietary requirements of synthesizable amino acids by animals: a paradigm shift in protein nutrition. Journal of Animal Science and Biotechnology, 2014a; 5(1), 34.

Wu G, Bazer FW, Dai Z, Li D, Wang J, Wu Z. Amino acid nutrition in animals: protein synthesis and beyond. Annu Rev Anim Biosci. 2014b; 2(1):387-417.

Xiao R, Power RF, Mallonee D, Routt K, Spangler L, Pescatore AJ, et al. Effects of yeast cell wall-derived mannan-oligosaccharides on jejunal gene expression in young broiler chickens. Poult Sci. 2012; 91(7):1660–9. https://doi.org/10.3382/ps.2011-02035.

Xu YQ, Guo YW, Shi BL, Yan SM, Guo XY. Dietary arginine supplementation enhances the growth performance and immune status of broiler chickens. Livest Sci. 2018;209:8-13.

Zampiga M, Flees J, Meluzzi A, Dridi S, Sirri F. Application of omics technologies for a deeper insight into quali-quantitative production traits in broiler chickens: A review. J Anim Sci Biotechnol, 2018;9(1), 61.

Zampiga M, Meluzzi A, Sirri F. Effect of dietary supplementation of lysophospholipids on productive performance, nutrient digestibility and carcass quality traits of broiler chickens. Ital J Anim Sci. 2016; 15(3):521-528.

Zhai W, Araujo LF, Burgess SC, Cooksey AM, Pendarvis K, Mercier Y, et al. Protein expression in pectoral skeletal muscle of chickens as influenced by dietary methionine. Poult Sci. 2012; 91(10):2548–55. https://doi.org/10.3382/ps.2012-02213.

Zheng A, Luo J, Meng K, Li J, Bryden WL, Chang W, et al. Probiotic (enterococcus faecium) induced responses of the hepatic proteome improves metabolic efficiency of broiler chickens (Gallus gallus). BMC Genomics. 2016;17(1):89. https://doi.org/10.1186/s12864-016-2371-5.

Zheng A, Luo J, Meng K, Li J, Zhang S, Li K, et al. Proteome changes underpin improved meat quality and yield of chickens (Gallus gallus) fed the probiotic enterococcus faecium. BMC Genomics. 2014;15(1):1167. https://doi.org/10.1186/1471-2164-15-1167.

Zhou N, Lee WR, Abasht B. Messenger RNA sequencing and pathway analysis provide novel insights into the biological basis of chickens' feed efficiency. BMC Genomics. 2015a;16(1) https://doi.org/10.1186/s12864-015-1364-0.

Zhou Z, Lamont SJ, Lee WR, Abasht B. RNA-seq analysis of abdominal fat reveals differences between modern commercial broiler chickens with high and low feed efficiencies. PLoS One. 2015b;10(8) https://doi.org/10.1371/journal.pone.0135810.

Zubay G. Biochemistry. 1983. Addison-Wesley Publishing Company. Reading, MA, USA.

Zuidhof MJ, Schneider BL, Carney VL, Korver DR, Robinson FE. Growth, efficiency, and yield of commercial broilers from 1957, 1978, and 2005. Poult Sci. 2014; 93:2970-2982.