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PhD thesis

Modern issues in poultry meat quality: heat stress mitigation by prebiotic delivered *in ovo* and emergent myopathies in breast muscle

Coordinator of the PhD Course: Prof. Giuseppe Maiorano
Supervisor: Prof. Giuseppe Maiorano

PhD Student: Daniela Prioriello
158075

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To my Family

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Content

Abstract

In recent years, to meet consumer demand, the poultry sector experienced a strong increase in production. To this end, genetic pressure has been intense to obtain highly productive commercial hybrids. However, modern poultry genotypes are more susceptible to heat stress (HS) and to the appearance of breast muscle anomalies, as Oregon Disease (OD), White Striping (WS), Wooden Breast (WB) and Spaghetti Meat (SM). One strategy to alleviate HS in poultry is the administration of pre-probiotics or synbiotics through the technology *in ovo* in order to improve intestinal health, one of the main factors influencing the vulnerability of chickens to heat. In order to be effective, pre-pro and synbiotics must be administered to animals under completely controlled conditions and as soon as possible to offer rapid protection and early and beneficial "formation" of intestinal microflora. Conventionally, these substances are added to feed and /or water in the first hours/days after hatching. Administration, at this stage, may be late since the chick's intestine is not sterile. Therefore, the *in ovo* technology has been developed, which consists in the early administration of pre-pro or synbiotics in the egg's air chamber on day 12 of embryonic incubation. This technique allows the embryo to receive an adequate amount of pre-pro or synbiotic able to promote proper development of the chick's intestinal microbiota. The thesis involved two experimental trials. The aim of the first trial was to estimate the incidence of the emerging anomalies of poultry meat (OD, WS, WB and SM) and to investigate the effect of some *ante-mortem* factors (genetic type, sex, diet, average live weight, age at slaughter, transport time, duration of the pre-slaughtering stop). In the second trial, it has been assessed the meat quality traits of broiler chicken *in ovo* stimulated with a commercial prebiotic (galactooligosaccharide, GOS) and exposed to HS.

The first investigation showed that the 43% of the analyzed breasts had at least one of the moderate stage defects and 23% of the breasts had at least one severe defect; WB was the highest myopathy (60%), followed by WS (31%), both of which were found mostly in heavy chickens. The 21% of breasts had the SM

defect; however, there was no relationship between breast weight and the presence of SM. The *ante-mortem* factors seem to have no role in the onset of myopathies. Conversely, the growth rate is a possible cause of WB and SM. Therefore, it is hypothesized that the critical factor causing the onset of myopathies could be genetic selection.

In the second study, at the day 12th of incubation 3,000 eggs, with viable embryos, were randomly divided equally into 3 experimental groups: group injected with prebiotic (GOS); group injected with physiological solution (S); control group (C), not injected. After hatching, 900 male chicks (300 chicks/treatment) were reared in floor pens in either thermoneutral (TN; 6 pens/group, 25 birds/pen) or heat stress conditions (HS; 30°C from 32 to 42d; 6 pens/group, 25 birds/pen). At 42 days of age, 15 animals were slaughtered per each subgroup. The pectoral muscle (PM) was taken from the all carcasses for physical-chemical analyses. This study showed that *in ovo* treatment did not have a significant effect ($P > 0.05$) on breast weight, pH, WHC, cooking losses, shear force, proximate composition, cholesterol and collagen content of PM; while, it affected ($P <0.05$) the color descriptors (L * and b*). Total PUFA and n-6 content was slightly lower in the GOS group than in S; while, the GOS did not affect the total content of SFA, MUFA, PUFA and nutritional indexes (n-6/n-3, P/S, AI, TI). HS has reduced ($P <0.01$) the weight of the PM, increased the pH ($P <0.01$) and the total lipid content, as well as influenced the color (L *, b *). HS had a marginal effect on the composition of lipids, as well as on the individual fatty acids and on the nutritional indexes. Significant interactions between factors were found for L*, a*, lipid, ash, SFA, MUFA and nutritional indexes. In conclusion, the injection *in ovo* of the prebiotic GOS seems to mitigate the negative effects of heat stress on some qualitative parameters of the meat.

Riassunto

La carne avicola è caratterizzata da un buon profilo nutrizionale. Grazie all'alto contenuto di proteine, vitamine e minerali, ad elevato valore biologico, e un basso contenuto di grassi. Da diversi anni, si riscontra una elevata richiesta di carne di pollo da parte dei consumatori, che ha indotto una selezione genetica molto spinta al fine di ottenere ibridi commerciali altamente produttivi con ritmi di crescita impensabili sino a qualche decennio fa, grazie soprattutto ad un migliore stato di salute degli animali, ad una alimentazione più efficiente e soprattutto all'uso di antibiotici promotori di crescita, che dal 2006 sono proibiti nei Paesi dell'Unione europea a causa del fenomeno dell'antibiotico resistenza. Tuttavia, la selezione continua, per ottenere ibridi commerciali con un più rapido accrescimento, un miglior indice di conversione alimentare, un petto più sviluppato e carcasse meno grasse, ha reso i moderni ibridi di pollo più sensibili allo stress da calore (HS) e alla insorgenza di anomalie del petto, come Oregon Disease (OD), White Striping (WS), Wooden Breast (WB) e Spaghetti Meat (SM). Una strategia per diminuire lo HS nel settore avicolo consiste nella somministrazione di prebiotici, probiotici o loro combinazione (simbiotici) al fine di migliorare la salute intestinale, uno dei principali fattori che influenzano la vulnerabilità dei polli allo HS. Pre-pro e simbiotici, per essere efficaci, devono essere somministrati agli animali in condizioni completamente controllate e prima possibile per offrire una protezione rapida e una "formazione" precoce e benefica della microflora intestinale. Convenzionalmente, queste sostanze vengono aggiunte al mangime e/o all'acqua alle prime ore/giorni dopo la schiusa. Tuttavia, la somministrazione in questa fase potrebbe essere tardiva poiché l'intestino del pulcino non è sterile. Pertanto, è stata sviluppata la tecnologia *in ovo*, che consta nella somministrazione anticipata di queste sostanze e dei probiotici, nella camera d'aria dell'uovo al 12° giorno d'incubazione embrionale. Questa tecnica permette all'embrione di ricevere una quantità adeguata di pre/pro/simbiotico in grado di favorire uno corretto sviluppo del microbiota intestinale del pulcino.

Il lavoro di tesi ha riguardato due prove sperimentali. Lo scopo della prima prova è stato quello di stimare l'incidenza delle miopatie (OD, WS, WB e SM) in relazione ad alcuni fattori *ante mortem* (tipo genetico, sesso, dieta, peso vivo medio, età, tempo di trasporto, sosta al macello). Nella seconda prova è stata valutata la qualità della carne di polli stimolati *in ovo* con un prebiotico commerciale (trans galatto-oligosaccaride, GOS) ed esposti a HS.

Il primo studio ha mostrato che il 43% dei petti aveva almeno un difetto allo stadio moderato e il 23% almeno un difetto allo stadio grave. Il WB è risultata la miopatia più elevata (60%), seguita dal WS (31%), entrambe riscontrate maggiormente nei polli pesanti e con petti più pesanti. Il 21% dei petti ha presentato il difetto SM. Comunque, non è emersa alcuna relazione tra il peso del petto e la presenza di SM. I fattori *ante-mortem* sembrerebbero non avere alcun ruolo nell'insorgenza delle miopatie. Al contrario, la velocità di crescita è una possibile causa di WB e SM. Pertanto, si ipotizza che il fattore critico causa dell'insorgenza delle miopatie potrebbe essere la selezione genetica.

Per il secondo studio sono state utilizzate 3.000 uova (Ross 308), con embrioni vitali. Al 12° giorno di incubazione, le uova sono state divise casualmente ed equamente in 3 gruppi sperimentali: gruppo iniettato con prebiotico (GOS); gruppo iniettato con soluzione fisiologica (S); gruppo controllo (C), non iniettato. Dopo la schiusa, 900 pulcini maschi (300 pulcini/trattamento: 6 gabbie/gruppo, 25 animali/gabbia) sono stati allevati in gabbie collettive in condizioni di termo-neutralità (TN) e di stress termico (HS) (30° C, dal giorno 32 fino al giorno 42). Al 42° giorno di età, sono stati macellati 15 animali per gruppo. Dalle carcasse è stato prelevato il muscolo pettorale (PM) per le analisi fisico-chimiche. Questo studio ha mostrato che il trattamento *in ovo* non ha avuto un effetto significativo sul: peso del petto, pH, WHC, perdite da cottura, sforzo di taglio, composizione centesimale, contenuto di colesterolo e collagene del PM; mentre, ha influenzato ($P< 0.05$) i descrittori del colore (L* e b*). Il contenuto totale di PUFA e n-6 è risultato lievemente inferiore nel gruppo GOS rispetto ad S; mentre, il GOS non ha influenzato il contenuto totale di SFA, MUFA e PUFA e gli indici nutrizionali (n-

6/n-3, P/S, AI, TI). HS ha: ridotto (P<0.01) il peso del PM, aumentato il pH (P<0.01) ed il contenuto di lipidi totali, influenzato il colore (L*, b*). HS ha avuto un effetto marginale sulla composizione del grasso, come anche sui singoli acidi grassi e sugli indici nutrizionali. Sono state trovate interazioni significative tra i fattori oggetto di studio per: L *, a*, lipidi, ceneri, SFA, MUFA e indici nutrizionali. In conclusione, l'iniezione *in ovo* del prebiotico GOS sembra mitigare gli effetti negativi dello stress da calore su alcuni parametri qualitativi della carne.

Chapter 1

1. Trends in poultry production

The global population is increasing continuously and is estimated to comprise about 9.6 billion individuals by 2050. Correspondingly, poultry production has intensified during the last year and it is predicted to produce about 130 million tons of chicken meat in 2020 (OECD/FAO 2018) to match the demands of a growing world population.

Meat consumption, measured in thousand tons of carcass weight (except for poultry expressed as ready to cook weight) and in kilograms of retail weight per capita, is related to living standards, diet, livestock production and consumer prices, as well as macroeconomic uncertainty and shocks to Gross Domestic Production (GDP). Compared to other commodities, meat is characterized by high production costs and high output prices. Meat demand is associated with higher incomes and a shift - due to urbanization - to food consumption changes that favor increased proteins from animal sources in diets.

Italy is largely self-sufficient as regards the supply of poultry meat. In Europe, it is in sixth place for quantities of poultry meat produced. At the end of the 1950s, less than 100.000 tons of poultry meat were produced in Italy; in a year, on average, each Italian ate only 24 kg of meat, of which just over 2 kg was poultry; the value of production was then just over 800 billion lire. In 2014 each Italian ate, on average about 90 kg of meat of which 19.45 Kg of poultry, in 2016, the production of poultry meat exceeded one million and 260 thousand tons.

Today, with a per capita consumption of 13.3 kg, poultry leads the world ranking of the most popular meat, followed by pork cuts with 12.2 kg, cattle (6.6 kg) and sheep (1.7 kg). And the prospect is to touch the 14.5 kg per capita in 2022, reaching an increase of 19%, which is definitely more favorable than the estimates of growth in demand in other sectors. This is what emerges in summary from the survey presented by UNAITALIA (National Union of agro-food chains for meat and eggs, 2018).

In Italy, the poultry supply chain with its 5.7 billion turnovers in 2012 and a production of 1,261 thousand tons (+ 2% compared to 2011), is showing strong anti-cyclical skills, which allow it to produce wealth and work despite the difficult economic context in recent years. Today there are almost 1,600 industrial companies employing about 25 thousand direct employees, whose gross wages have grown by 58% over the last 10 years, much more than the overall national food industry (+ 45%). More than 6,200 farms across the Country, divided into fattening farms, laying hens, breeding grounds and weaners. Completing the panorama are 400 factories for the production of feed, 174 small and large slaughterhouses, and more than 500 establishments for cutting and processing meat products and preparations. A sector, therefore, that travels in contrast to the crisis thanks, in particular, to the good trend of demand. The greatest satisfaction given to chicken meat by the Italian consumer lies also in the high content of service that in recent years has increasingly guided the productive choices of the processing industry. 26% of the chicken meat consumed in Italy refers in fact to preparations and processed (hamburgers, skewers, rolls, nuggets), while 61% to the product in parts and only 13% to the whole chicken. Ten years ago, the processed product was just 14% of the offer and in the 80s it was not even present on the market.

1.1 Poultry meat: EU production forecast for 2024

On the basis of the medium-term estimates released by the EU Agriculture Commission, for the next 10 years it will be expected a growth in production and consumption of poultry meat around 7% (UNAITALIA, 2018). In recent years, the production of poultry meat has served to fill the gap left by the reduced production of beef and pork. The production and consumption of poultry meat has been steadily increasing for many years. This increase was mainly concentrated in a number of Member States, which are then main producers: Germany, Spain, the Netherlands and Poland, these together account for 44% of total EU production in 2013. The production of poultry meat will continue to

grow steadily until 2024. The largest increase in production (around 1% per year) should concern only the so-called new Member States, mainly due to sustained production increases in Hungary, Poland and Romania. In the other major producer countries such as Germany and the Netherlands, output growth of 0.5% per year is expected. In detail, production in the EU is expected to reach 10 million tons (+ 1.5% compared to 2013), thanks to the favorable prices that continue to encourage consumption of chicken meat in the current economic scenario.

The two main reasons that are driving the success of poultry meat in both developed and developing countries are mainly lower cost and the perceived healthy nutritional profile with respect to pork and beef meat. Regarding the nutritional aspects, poultry meat and in particular breast meat fits the modern consumers worldwide demand for a low-fat meat with a protein supply that is safe, wholesome, nutritious, abundant and affordable and also with high unsaturation degree of fatty acids and low sodium and cholesterol levels (Cavani et al., 2009). Poultry meat may also be considered as a functional food as it provides bioactive substances with favorable effects on human health, e.g. long-chain n-3 polyunsaturated fatty acids (PUFA), conjugated linoleic acid (CLA), bioactive peptides, vitamins and antioxidants (Cavani et al., 2009; Gibbs et al., 2010; Ryan et al., 2011). Besides it is economical and quick and easy to prepare and serve. Finally, the absence of religious or cultural barriers compared to other meat makes poultry the most favorite choice.

However, the concept of quality remains from the national origin of the cuts, information on the methods of breeding, the color and the tenderness of the meat. Although the consumer attributes great importance to the nutritional and organoleptic values of the meat, the price is still a variable that significantly affects the purchase choices. To this is added the attitude of many consumers who consider it appropriate to limit the quantity of meat to eat due to the spread of doubts about the relative healthiness and hygienic-sanitary

characteristics, often due to the lack of correct information on the breeding of animals, on their nutrition and, more generally, on the country of breeding.

Chapter 2

2. **Microbiota in Broiler Chickens**

2.1 Intestinal Microbiota in the Poultry

The primary function of the gut is the conversion and the digestion of food into its basics components and utilization by the birds. In chickens, the digestive tract begins at the mouth/beak, includes several important organs, and ends at the cloaca. The gastrointestinal tract (GIT) of the chickens is anatomically formed from the crops, proventriculus, gizzard, duodenum, jejunum, ileum, caeca, large intestine, and cloaca (Figure 1). Each gastrointestinal section hosts different microbial communities and has different metabolic functions. Chicken obtain feed by using their beak. Food picked up by the beak enters the mouth. Chickens do not have teeth, however, the mouth contains glands that secrete saliva, which wets the feed to make it easier to swallow, besides the saliva contains enzymes, such as amylase, that start the digestion process. Throw the tongue the feed is pushed to the back of the mouth to be swallowed into the esophagus. The esophagus is a flexible tube that connects the beak with the rest of the digestive tract. It carries food from the mouth to the crop and from the crop to the proventriculus. It contains mucus glands that help to lubricate the passage of the food to the crop where it is stored temporarily. The crop is an out-pocketing of the esophagus and is located just outside the body cavity in the neck region. Swallowed feed and water are stored in the crop until they are passed to the rest of the digestive tract. When the crop is empty or nearly empty, it sends hunger signals to the brain so that the chicken will eat more. The food passes to the proventriculus. The proventriculus is the glandular stomach where digestion primarily begins. Hydrochloric acid and digestive enzymes, such as pepsin, are added to the feed here and begin to break it down more significantly than the enzymes secreted by the salivary glands. At this point, however, the food has not

yet been ground and passes into the ventriculus. The ventriculus, or gizzard, consists of two sets of strong muscles that act as the bird's teeth and has a thick lining that protects those muscles. Consumed feed and the digestive juices from the salivary glands and proventriculus pass into the gizzard for grinding, mixing, and mashing. From the gizzard the food passes to the small intestine. The small intestine is made up of the duodenum, jejunum and the ileum. The small intestine is an organ distinguished histologically by the presence of villi, which complete the digestion of proteins through the secretion of intestinal juice and digestive enzymes such as amino peptidase, amylase, maltase and invertase. Another function is to absorb the nutrients in the digested foodstuffs so they can enter the bloodstream; finally, the small intestine provides peristaltic action that passes undigested materials to the ceca. The ceca consists of a pair of tubes where undigested food is fermented and it is emptied every 24h. The water and the undigested food are absorbed in the large intestine, a section of the digestive tract that leads from the junction with the ceca, through the colon and ends in the external opening of the cloaca. In the cloaca, the digestive wastes mix with wastes from the urinary system (urates).

The integrity of the GIT and the gut microbial community play vital roles in nutrition absorption, development of immunity, and disease resistance (Shang et al., 2018). Alterations of this relationship have adverse effects on feed efficiency, productivity, and health of chickens.

The microbial communities inhabiting the gastrointestinal tract (GIT) of chickens are essential for the gut homeostasis, the host metabolism and affect the animals' physiology and health. They play an important role in nutrient digestion, pathogen inhibition and interact with the gut-associated immune system.

The microbiota within the GIT, influences the health and the physiology of vertebrates host, because it has important roles in protection from pathogens, detoxification and modulation of immune system development (Mead et al., 1989; Brisbin et al., 2008).

Microbiota in the animal intestine has evolved together with the host. As a consequence, the GIT could be considered a meta-community, comprising many local microbial communities in different ecological niches. Each individual gut compartment has its unique physiochemical characteristics and each location is inhabited by a specialized microbial composition (Dethlefsen et al., 2007). The microorganisms inhabiting the intestinal tract of humans and animals outnumber the cells of a host (Savage et al., 1977). The vast majority of gut bacteria reside in the distal intestine, where densities approach 10^{11} to 10^{12} cells/g, the highest recorded for any microbial habitat (Whitman et al., 1998). The gut microbiota therefore extends the host's genome such that the host can be described as host-microbe super-organism (Kleerebezem et al., 2009).

The development of the intestinal chicken microbiota begins at the time of hatching. In the first moments of life the chicks come into contact with bacteria that are present on the surface of the egg shells and that come from the mother's intestine and from the surrounding environment (Rinttilä et al., 2013). Therefore, in the initial phase of the post-hatched period the bacterial population is fundamental for the creation of the intestinal microbial community. The presence of this initial microbial population can last for the whole life of a chicken influencing the development of the intestinal microbiota and of the immune system (Apajalahti et al., 2004). The chicken GIT harbors a very complex microbiota, with over 600 different bacterial species from more than 100 bacterial genera (Torok et al., 2011). In general, the most abundant phylum in the chicken intestinal microbiota is Firmicutes followed by two minor phyla, Proteobacteria and Bacteroidetes (Figure 2). In addition, members of phyla Actinobacteria, Tenericutes (Waite and Taylor, 2014), Cyanobacteria and Fusobacteria (Qu et al., 2008) can be found in very low abundance. Bacterial communities vary considerably by locations along the GIT of chickens. Crop, gizzard and duodenum share similar microbiota, dominated by the genus Lactobacillus, as high as 99% in some birds (Gong et al., 2007; Sekelja et al., 2012). The highest diversity of Lactobacillus was observed in the crop (Gong et

al., 2007). The jejunum is also dominated by Lactobacillus species, mainly *L. salivarius* and *L. aviarius* (Gong et al., 2007; Feng et al., 2010). The microbial composition of the ileum is more diverse and less stable compared with the duodenum and the jejunum. The ileum is dominated by Lactobacillus, Candidatus Arthromitus, Enterococcus, Escherichia coli/Shigella and Clostridium XI (Asrore et al., 2015; Pourabedin et al., 2015). The cecum is by far the most densely colonized microbial habitat in chickens and its bacterial diversity is much higher than those in the upper GIT. The most detailed information regarding chicken gut microbiota is available for the cecum. The cecum is a key region for bacterial fermentation of non-digestible carbohydrate and a main site for colonization by pathogens. Chickens have two paired ceca, both harboring similar bacterial communities (Stanley et al., 2015). In a study by Gong et al. (2007), the cecum was mainly occupied by the Clostridia genus followed by genera Lactobacillus and Ruminococcus. The majority of Clostridia detected in the cecum fall primarily into three main families, Clostridiaceae, Lachnospiraceae and Ruminococcaceae (Danzeisen et al., 2011). Enterococcaceae, Enterobacteriaceae and Bacteroidaceae are other reported abundant families in the cecal microbiota (Yin et al., 2010). The cecum is also rich in unknown and unclassified bacterial residents (Stanley et al., 2013). At the species level, *Bacteroides fragilis*, *L. crispatus*, *L. johnsonii*, *L. salivarius* and *L. reuteri* comprise more than 40% of cecal microbiota (Stanley et al., 2015).

To study GI microbiota, fecal samples are often used because of easy sampling. The composition of fecal microbiota highly fluctuates depending on varying contributions of microbiota from different GI segments (Sekelja et al., 2012). Lactobacillaceae, Peptostreptococcaceae, Streptococcaceae, Clostridiaceae and Enterobacteriaceae were identified as common families of the fecal microbiota (Videnska et al., 2014a). Stanley et al. (2015) indicated that about 88% of all operational taxonomic units, comprising 99.25% of sequences, were shared between cecal and fecal samples in broiler chickens. The GI microbiota of chickens could be separated into four potential robust clusters, referred to as

enterotypes (Kaakoush et al., 2014), similar to the presence of three enterotypes in human gut microbiome (Arumugam et al., 2011). Enterotypes are in fact distinct bacterial communities, each dominated by different bacteria genera. Enterotypes in humans are correlated with long-term dietary patterns but independent of host phenotypes such as gender, age or body mass index (Wu et al., 2011). Despite existence of such enterotypes, there is a strong individual variation among chickens of a same breed, on a same diet and even under highly controlled experimental conditions (Nordentoft et al., 2011; Sekelja et al., 2012; Stanley et al., 2013b). This variation could be due to the fact that in the modern industrial poultry production, chickens are being hatched in highly hygiene incubators and reared without exposure to maternally derived bacteria. The random colonization by surrounding environmental bacteria is assumed to be a key reason for a high variation in the intestinal microbiota (Stanley et al., 2013b). The chicken gut microbiota has been found to be affected by diet (Torok et al., 2008), gender (Lumpkins et al., 2008), background genotype (Zhao et al., 2013), housing condition (Nordentoft et al., 2011), floor litter (Torok et al., 2009; Cressman et al., 2010), feed restriction (Callaway et al., 2009) and stocking density (Guardia et al., 2011). Furthermore, as a bird ages, the microbiota complexity increases (Yin et al., 2010; Crhanova et al., 2011; Danzeisen et al., 2011; Sekelja et al., 2012). Certain bacteria may disappear over time or emerge in the intestinal microbiota of older chickens while others remain stable throughout the life (Pourabedin et al., 2015). Firmicutes species are dominant in young chickens while the representatives of Bacteroidetes are most common in adult birds (older than 7 months) (Callaway et al., 2009; Videnska et al., 2014). In layers, four different profiles of cecal microbiota have been identified from the day of hatching until 60 weeks of age (Videnska et al., 2014). However, temporal characterization of gut microbiota in poultry varies among studies and needs more frequent sampling and robust sequencing and analyses (Table 1).

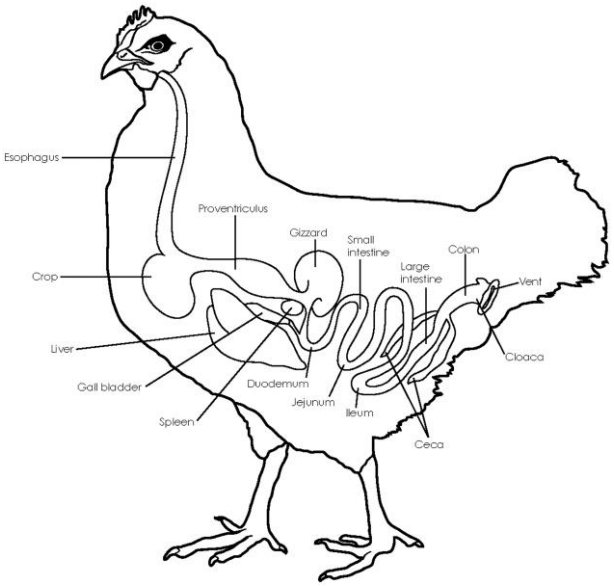

Figure 1. Gastrointestinal tract in chickens (Source: Clavijo et al., 2018)

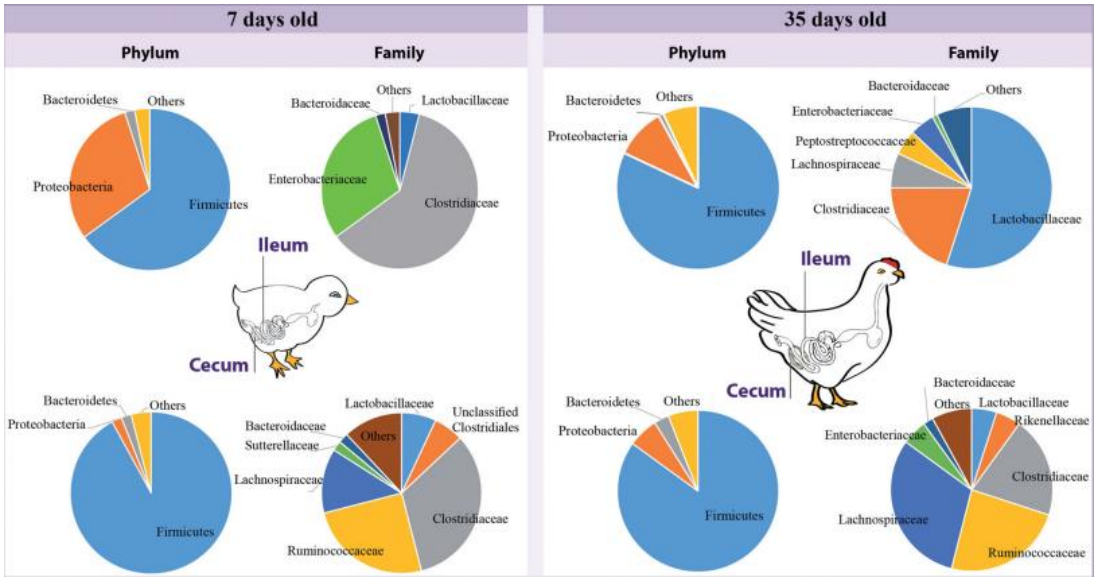

Figure 2. The chicken gut microbiome . The graphs provide an overview of the relative abundance of dominant bacterial phyla and families of the broiler chicken ileal (top level) and cecal (bottom level) microbiota in two different ages, 7 and 35 days. (Source: Pourabedin et al., 2015)

Table 1. Spatial distribution of most common and abundant bacterial taxa (phylum, order (o), family (f), (genus) in the gastro-intestinal tract of chickens irrespective of age, diet and technique differences.

GIT location (per g of content)	Bacterial phyla	Bacteria genera	Techniques used
Crop (108-109/ g)	Firmicutes Actinobacteria Proteobacteria	*Lactobacillus* *Bifidobacterium* *Enterobacter*	16 S rDNA sequencing and cloning
Gizzard (10^7-10^8/ g)	Firmicutes	*Lactobacillus*, *Enterococcus*	
Small Intestine (most of the studies are conducted in Ileum; 10^8-10^9/ g)	Firmicutes/Low G+C, Gram positive bacteria	Enterococcaceae (f.), *Enterococcus*, Clostridiaceae (f.), *Clostridium*, Lactobacillacae (f.) *Lactobacillus*, *Candidatus Arthomitus*, *Weisella*, *Ruminococcus*, *Eubacterium*, *Bacillus*, Stapylococcaceae (f.), *Staphylococcus*, *Streptococcus*, *Turicibacter*, *Methylobacterium*	Finger printing: T-RFLP, 16S rRNA qPCR, Cloning and sequencing and Next Generation Sequencing
	Cytophaga/ Flexibacter/ Bacteroides/ High G+C, Gram positive bacteria Protobacteria	Bacteroidaceae (f.), *Bacteroidetes*, *Flavibacterium*, *Fusobacterium*, *Bifidobacterium* *Ochrobaterium*, *Alcaligenes*, *Escherichia*, *Campylobacter*, *Hafnia*, *Shigella*	
	Actinobacteria/ Cyanobacteria	*Corynebacterium*	
Caeca (10^{10}-10^{11}/ g)	Methanogenic Archaea (0.81%)	*Methanobrevibacter*, *Methanobacterium*, *Methanothermobacter*, *Methanosphaera*, *Methanopyrus*, *Methanothermus*, *Methanococc*	Finger printing: T-RFLP, 16S rRNA qPCR, Cloning and sequencing and Next Generation Sequencing
	Firmicutes/ Low G+C, Gram positive bacteria (44–56%)	*Anaerotruncus*, Ruminococcaceae (f) *Ruminoccoccus*, *Faecalibacterium*,	Finger printing: T-RFLP, 16S rRNA qPCR, Cloning and

		Lachnospirceae, *Bacillus*, *Streptococcus*, Clostridiales (o), *Clostridium*, *Megamonas*, *Lactobacillus*, *Enterococcus*, *Weisella*, *Eubacterium*, *Staphylococcus*, *Streptococcus*,	sequencing and Next Generation Sequencing
	Bacteroides/ Cytophaga/ Flexibacter/ High G+C, Gram positive bacteria (23–46%) Actinobacteria Proteobacteria (1–16%)	Rikenellaceae (f), *Bacteroidetes*, *Alistipes*, *Fusobacterium*, *Bifidobacterium*, *Flavibacterium*, *Odoribacter*, *Corynebacterium* *Ochrobaterium*, *Alcaligenes*, *Escherichia*, *Campylobacter*	
Large Intestine	Firmicutes Proteobacteria	*Lactobacillus* *Escherichia*	16 S rDNA sequencing and cloning

2.2 The role of gastrointestinal microbiota.

Between the poultry, host and its intestinal microbiota occur different interactions, influencing many physiological functions within the host. Kogout (2013) in a study suggested that these bacteria play crucial roles in digestion and in the development of gastrointestinal tract. In addition, these bacteria are involved in the development of the immune system and in the production of the mucus layer, epithelial monolayer, of the intestinal immune cells (e.g., cytotoxic and helper T cells, immunoglobulin producing cells and phagocytic cells), and the lamina propria (Oakley et al., 2014). The gut microbiota creates a protective barrier by attaching to the epithelial walls of the enterocyte (Yegani et al., 2008), a gel-like mucus layer formed from mucin glycoprotein secreted by the calceiform cells constitutes this barrier. The layer of mucin consists of an outer loose layer in which microorganisms can colonize and an inner compact layer,

which repels most bacteria, thereby prevents gut microorganisms from penetrating into the intestinal epithelium and serves as the first line of defense against infections. Another important component of the innate immune system that functions in the avian gut is the antimicrobial peptides β- defensins, present on the epithelial surface. They are small cationic peptides produced by avian macrophages, heterophils, and epithelial cells, and they can kill various intestinal pathogens by disrupting cell membrane permeability, which leads to cell lysis. Brisbin et al., (2008) indicated that *Salmonella* infections increased the expression of β- defensins genes in chicken, whereas administrations of probiotics prior to *Salmonella* inoculation resulted in a decline in the gene expression of β- defensins.

Furthermore, microbiota is involved in the production of energy and nutrients from the undigested feed that become available for the host: vitamins, amino acids and short chain fatty acids (SCFA). The most common SCFAs are butyrate, acetate, lactate and propionate. SCFA have antimicrobial properties that are able to slow down or eliminate the growth of foodborne microbes (Shang et al., 2018). SCFA can further increase the gastrointestinal absorption surface because they can stimulate gut epithelial cell proliferation, they are able to affect the immune response of the intestine because regulate the production of mucin and they regulate blood flow (Clavijo et al., 2018). Moreover, their production improves growth performance and carcass quality characteristics in chickens (Kogut et al., 2017). Gut microbiota also contributes to metabolism of host nitrogenous compounds. The nitrogen from the diet gets incorporated into bacterial cellular protein and therefore, bacteria themselves can be a source of proteins/amino-acids (Metges et al., 2000). Intestinal health is a highly critical component for an efficient poultry production: when the animals are not threatened by disease, they have more capability to convert feed into carcass as efficiently possible. Rinttilä et al. (2013) demonstrated that there is a correlation between cecal microbiota composition and efficiency of the host to extract energy from the diet and to deposit that energy into improved feed conversion

rate (FCR). However, the question of how the intestinal bacterial community composition relate to relevant metabolic changes and to broiler chicken performance is not completely understood (Rinttilä et al., 2013).

2.3 Chicken diet and its influence on the Microbiota

The nutrition of chicken is based on plants diet that are supplemented with a variety of amino acids, minerals, vitamins, enzymes to improve growth performance. These nutrients can also modulate the growth of the microbiota. Therefore, it is possible to modify the gut microbial population, concomitant with the growth of favorable bacteria in the gut of chicken. Over the past decade the use of antibiotics at sub-therapeutic level has been the most popular and the most effective strategy to enhance feed efficiency and to keep animal healthy. However, this practice has been criticized, given the increasing prevalence of resistance to antibiotics in chicken (Kabir, 2009) and its potential spread to humans' pathogens. In recent years, nutrition-based research has been greatly intensified in farm animals: some food have been reported to modulate the gut microbiota and the immune system which may be beneficial for the chicken, referred as nutraceuticals (Huyghebaert et al., 2011). In general, nutraceuticals can be defined as food or food components that have a role in modifying and maintaining normal physiological functions that support the healthy host (Das et al., 2012). Nutraceuticals are isolated nutrients (vitamins, minerals, amino acids, fatty acids), herbal products (polyphenols, herbs, spices), and food supplements (prebiotics, organic acids, antioxidants, enzymes). The use of nutraceuticals is also considered for the prevention and the treatment of enteric infections in chickens. Thanks to improved intestinal morphology and nutrient absorption, nutritionists include these compounds in the diet to promote bird growth performance. Another integration to diet consists in the use of probiotics. Probiotics are living microorganism that improve gut health and animal performance if added to the diets in adequate amounts. These microorganisms

compete with pathogenic bacteria for adhesion sites at the intestinal epithelium (Salminen et al., 1996). Moreover, mechanism of action from probiotics consists of the enhancement of activity of digestive enzymes like proteases, lipases and amylases (Fuller et al., 2001), the improvement of mucosa ultrastructure, thus also increasing nutrient absorption (Gheisar et al., 2016).

A frequent mistake when talking about gut health and diet is to relate them only to the prevention and control of the intestinal disease. However, this is, in reality, the consequence and not the cause of the problem (Edgar et al., 2019). The true problem is an excess of nutrients in the gut which causes the proliferation of these microbes in the GIT (Chan et al., 2013) with the consequence disruption of gut microbiota equilibrium (Round et al., 2009; Weiss et al., 2017) causing the metabolic or pathogenic inflammation (Kogut et al., 2018). In particular, the cause of the excess of nutrients in the gut can be searched in high nutrient levels in the diet or suboptimal digestion (Brown et al., 2012). Bacteria, fungi, protozoa and even virus proliferate when there are more undigested nutrients in the gut available for their use (Apajalahti et al., 1999). This proliferation results in deregulation of the microbiota that can lead microorganisms to become pathogens (Round et al., 2009). As reviewed by Clavijo (2018), the principal characteristics of feed that may affect the microbiota are the form of cereal (whole or milled grains, or pellets); the kind of cereal; the quantity of water-soluble non-starch polysaccharides and the sources of fat, starch and proteins. Modifying the concentration of diet constituents is favored the growth of group of bacteria: diets rich in water soluble non starch polysaccharides favor the proliferations of *C. perfringens*, this happens because the high level of non-starch polysaccharides improve digesta viscosity and decrease the passage of feed, in this way there is a reduction of nutrient digestibility that favors the colonization of *Clostridium perfringens* and the occurrence of necrotic enteritis disease (Borda-Molina et al., 2018). On the other hand, corn based-diet or the addiction of feed additives in the diet stimulate the growth of microorganism that improve gut health (Rodriguez et al., 2012). For

instance, the enzymes xylanase and β-glucanase improve the growth of lactobacilli. These are commensal bacteria that are known for their ability to activate the intestinal immune system and to increase the resistance to disease, throw the release of low-molecular weight peptides which induce immune activation (Muir et al., 2000). Besides, lactobacilli adhere to the gut epithelium and compete with pathogens for its colonization, such as *E. coli*, reduce the digesta viscosity and protect chicken from enteric disease (Pan et al., 2014), furthermore they are able to produce SCFAs with bacteriostatic properties and favored the renewal and barrier function of the gastrointestinal epithelium (Kogut et al., 2013). Even the dietary protein content and their source can have effect on the gut microbiota. In fact, Sun (2013), demonstrated that fermented cottonseed meal as a protein source rather than soybean diet, increased the population of lactobacilli and decreased the coliforms in the cecum of broiler chickens. In addition, diets with high content of animal protein and enriched with animal fat (lard and tallow) favor the growth of *C. perfringens* compared to soil oil diet (Drew et al., 2004). To avoid the growth of pathogens in the gut, poultry farms pay more attention to the choice of raw materials to minimize the conditions that could increase the presence of mycotoxins and of rancid fats in feed exceeding the recommended limits (Grenier and Applegate, 2013; Murugesan et al., 2014). Some mycotoxins facilitate the persistence of intestinal pathogens and the likelihood of intestinal inflammation. Rancid fats and oils should be rejected in relation to the pathogenesis of enteric diseases (Hoerr, 1998; Butcher and Miles, 2000; Collet, 2005). The bacteria population is also influenced by hygiene conditions of house farming, (dirty environment, pathogen load of the ingredients, humidity of the shed, litter type, etc.), by feed additives (antibiotics, coccidiostats, buffers that influence gut pH) and stress (change of feed, sudden disturbances, heat or water stress) (Cocht, 2009).

Chapter 3

3. The use of Antibiotic Growth Promoters in Poultry

3.1 Antibiotics in Poultry production

The discovery of antibiotics dates back to the 1920s, since this period they have been administered in the progress and prosperity of the poultry industry. Antibiotics have been used in animal feed at sub-therapeutic doses to improve growth and feed conversion efficiency and to prevent infections (Castanon, 2007). The first experiments reported the effect of antibiotics on improving performance of chickens were published by Moore et al. (1946), they observed that birds fed streptomycin exhibited increased growth responses. In 1959s, many other experiments in chickens corroborated these results (Groschke and Evans, 1950; McGinnis, 1950; Whitehill et al., 1950). In-feed antibiotic use became a well-established practice in the animal industry and increased with the intensification of livestock production (Gadde et al., 2017).

Antibiotics, either of natural or synthetic origin are used to both prevent proliferation and destroy bacteria. They are not effective against fungal and viral pathogens. Antibiotics are classified according to their chemical family, mode of action and the specie of bacteria on which they act (Mehdi et al., 2018). They function by altering certain properties of bacterial cellular metabolism resulting in impaired growth or death. Some antibiotics interfere with the building and maintenance of the cell wall, while others interrupt proper protein translation at the ribosomal level. Because of their elevated rate of growth and proliferation, bacteria are vulnerable to antibiotics that target active cellular metabolism. Limiting the growth and proliferation of certain bacteria and inhibiting the production of various toxins restricts the influence that the microbe has upon the host organism. This enables the host to grow and perform better than if grown under normal challenge conditions. For the ultimate eradication of microorganisms, immune responses of the host are required. Antibiotics are

used by the poultry industry and poultry veterinarians to enhance growth and feed efficiency and reduce disease. Antibiotic usage has facilitated the efficient production of poultry, allowing the consumer to purchase, at a reasonable cost, high quality meat and eggs. Antibiotic usage has also enhanced the health and well-being of poultry by reducing the incidence of disease. Although these uses benefit all involved, unfortunately, consumer perceptions are that edible poultry tissues are contaminated with harmful concentrations of drug residues. The right choice of an antimicrobial drug requires the observation of the basic principles for any antimicrobial therapy. For these reasons the WHO (World Health Organization) proposed global principles for judicious use of antibiotics to minimize bacterial resistance in food animals. From a safety standpoint, extensive toxicology and pharmacology studies are required to demonstrate consumers will not be exposed to harmful concentrations of antibiotic residues in edible poultry tissues. According to these guidelines, it is required to veterinarians that any use of antibiotics is based on the following:

- An exact diagnosis through microbiological tests. Antimicrobial therapy requires a clinical examination of the animal, including a laboratory examination, with the identification of the pathogens and their antimicrobial sensitivity (the bacteria must be sensitive to the drug);
- The selection of the most appropriate antibiotic must with respect to: use of narrow-spectrum antibiotics rather than a less specific broad spectrum antibiotic and restricted use of newer classes of antibiotics used in human medicine as only as a last resort;
- The choice of an appropriate dose regimen with respect to the pharmacokinetics and pharmacodynamics of the antibiotic;
- Dose interval that take into account bacteriostatic or bactericidal or post-antibiotic effects of the antibiotic;
- Avoidance of under-dosing;
- Treatment that lasts as long as required and as short as possible.

To ensure that meat does not contain residues in quantities in excess of the maximum residue limits there is an interval of time (Withdrawal Period) between the last administration of an antibiotic to animals and the production of food from those animals. The length of the withdrawal period for each antibiotic is set to ensure that the animal tissues and products are clear of prohibited levels of residues.

Antibiotics limit the growth of detrimental microbes, such as *Clostridium perfringens* (Truscott and Al-Sheikhly, 1977). They also limit the growth and colonization of numerous non-pathogenic species of bacteria in the gut, including lactobacilli, bifidobacteria, bacteroides, and enterococci (Tannock, 1997). Antibiotics reduce the production of antagonistic microbial metabolites, such as ammonia (Zimber and Visek, 1972), which adversely affect the physiology of the host animal. Sub therapeutic levels of antibiotics in the diet also reduce weight and length of the intestines (Visek, 1978; Postma et al., 1999). A thinner intestinal epithelium in antibiotic-fed animals may enhance nutrient absorption (Visek, 1978) and reduce the metabolic demands of the gastrointestinal system. The minimization of gastrointestinal bacteria may also ease the competition for vital nutrients between the bird and the microbes (Ferket, 1991). Finally, antibiotics may reduce the adverse effects of immunological stress on growth performance by lowering the enteric microbial load.

3.2 Antibiotics as Growth Promoters

Some antibiotics are used therapeutically to improve the health and well-being of animals (Castanon, 2007), most are given for prophylactic purposes and to improve growth rate and feed conversion efficiency (as antimicrobial growth performance promoters, AGPs) (Huyghebaert et al., 2010). The antibiotics used in poultry as feed additives included penicillin, neomycin, erythromycin, chlortetracycline, streptomycin, chloramphenicol and fluoroquinolones. However, due to the emergence of microbes resistant to antibiotics which are

used to treat human and animal infections, the European Commission (EC) decided to phase out, and ultimately ban (January 2006), the marketing and use of antibiotics as growth promoters in feed (EC Regulation No. 1831/2003). In other countries, such as the USA, consumer pressure is pushing the poultry industry to rear animals without AGPs (Dibner and Richards, 2005; Castanon, 2007). AGP removal has led to animal performance problems, feed conversion increases, and a rise in the incidence of certain animal diseases, such as (subclinical) necrotic enteritis (Wierup, 2001; Dibner and Richards, 2005). One disease syndrome that is clearly emerging in the EU broiler industry simultaneously with the ban of growth promoting antibiotics is often referred to as 'dysbacteriosis'. This is a poorly described condition of the gut and may be synonymous with conditions such as 'wet litter', 'small intestinal bacterial overgrowth', 'malabsorption', and 'feed passage syndrome' (Huyghebaert et al., 2010).

At sub-therapeutic doses, antibiotics promote growth and protect the health of birds by modifying the immune status of broiler chickens (Mehdi et al., 2017). This is mainly due to the control of gastrointestinal infections and microbiota modification in the intestine (Singh et al., 2014). The mechanism of the growth promotion by antibiotics are not completely understood and are thought to be by inhibition of subclinical infections, reduction of metabolites which can affect growth such as end products of bile degradation, reduction of nutrient availability to pathogens, thinning of intestinal epithelium and exhibition of anti-inflammatory action on macrophages and granulocytes (Brussow, 2015). Another school of thought (Niewold, 2007) states that the term 'Growth Promoter' has been used for years to describe the use of sub-therapeutic levels of antibiotics to improve growth performance, but it is an inappropriate term to describe this use of antibiotics because they do not promote growth as do anabolic hormones, such as growth hormone or estrogen-like compounds. This may be why the public confuses this term with the use of anabolic hormones. The poultry industry does not use anabolic hormones. Hormones are not approved for use in

poultry, are illegal to use, and are closely monitored by the federal government to ensure they are not used in poultry (Donoghue, 2003). Instead of calling them 'Growth Promoters', they should be called 'Growth Permitters' because they allow the animal to express its genetic potential for growth without compromise (Niewold, 2007). He hypothesized that antibiotics lower the inflammatory response and thus the production of pro-inflammatory cytokines, which reduce the appetite and promote muscle catabolism. The anti-inflammatory role of AGP reduces wasted energy and promote muscle metabolism (Niewold, 2007).

Though a clear consensus on how AGP acts still does not exist in the scientific community, it is now clear that shits in microbiota composition do occur when antibiotics are included in animals diets (Gadde et al., 2017). These shifts may ultimately result in an optimal and balanced microbiota that is less capable of evoking an inflammatory response, increases energy harvest from nutrients, and helps animal perform to their genetic potential (Huyghebaert et al., 2011; Lin, 2011).

3.3 Antimicrobial resistance and new alternatives to AGPs

Since their advent during the 1930s, antibiotics have not only had a dramatic impact on human medicine, but also on food production. Scientific evidence suggests that the use of antimicrobials in livestock production can promote bacterial resistance in treated animals (O'Brien, 2002). Antibiotic resistance is defined as the ability of microorganism to proliferate in presence of an antibiotic that generally inhibits or kills microorganisms of the same species (RUMA, 2016). Resistance is by mutation or acquisition of genes carried by mobile genetic elements such as transposons, integrons, plasmids or phages (Kemp and Zeitouni, 2012). Resistant microorganism can spread between food-producing animals and humans (Roth et al., 2019). Bacterial resistance to animal antibiotics is a public health issue. The abusive use of antibiotics and the associated selection pressure have led to decreased therapeutic efficacy and created

populations of antibiotic- resistant microorganisms (Mehdi et al., 2018). Antibiotic resistance may spread over time despite the suspension of antibiotic use. The administration and restriction of antibiotics in the EU are regulated by Directive 96/23/EC (European Commission, 1996). This directive focuses on measures to monitor residues in animal products. Antibiotic growth promoters were banned in the EU in 2006, in the US in 2017 and are currently allowed in Brazil and China (European Commission, 2005, Access Science Editors, 2017). Instead, antibiotic usage for disease prevention is permitted in all large poultry producing countries (Roth et al., 2019). Certain limits of antibiotics residues are imposed in food and animal product (meat and eggs). The tide of public opinion is forcing animal agriculture to develop alternatives, or at least substantially reduce the amount of antibiotics used to maintain production efficiency and produce safe meat and egg products. Alternatives to antibiotics promote gut health by several possible mechanisms including altering gut pH, maintaining protective gut mucins, selection for beneficial intestinal organisms or against pathogens, enhancing fermentation acids, enhancing nutrient uptake, and increasing the humoral immune response. Strategic use of these alternative compounds will help optimize growth provided they are used in a manner that complements their modes of action.

Considering the proposed mechanism of action of AGPs (microbiome and immune-modulating activities), a practical alternative should possess both of these properties in addition to having a positive impact on feed conversion and/or growth (Huyghebaert et al., 2011; Seal et al., 2013).

Reviewing the literature shows that removal of in feed antibiotics has been resulted in significantly negative effects on the performance of poultry. Yakhkeshi et al. (2011) studied the effects of different natural growth promoters in broiler chickens to compare the results with the groups feeding the diets with/without antibiotics. Their results indicated that feeding the birds with the diets containing antibiotic alternatives alleviated the negative effects of removing antibiotics from the diet of commercial poultry. Several classes of

alternatives have been proposed and tested in poultry production, including probiotics, prebiotics, synbiotics, organic acids, enzymes, phytobiotics etc... (Figure 3).

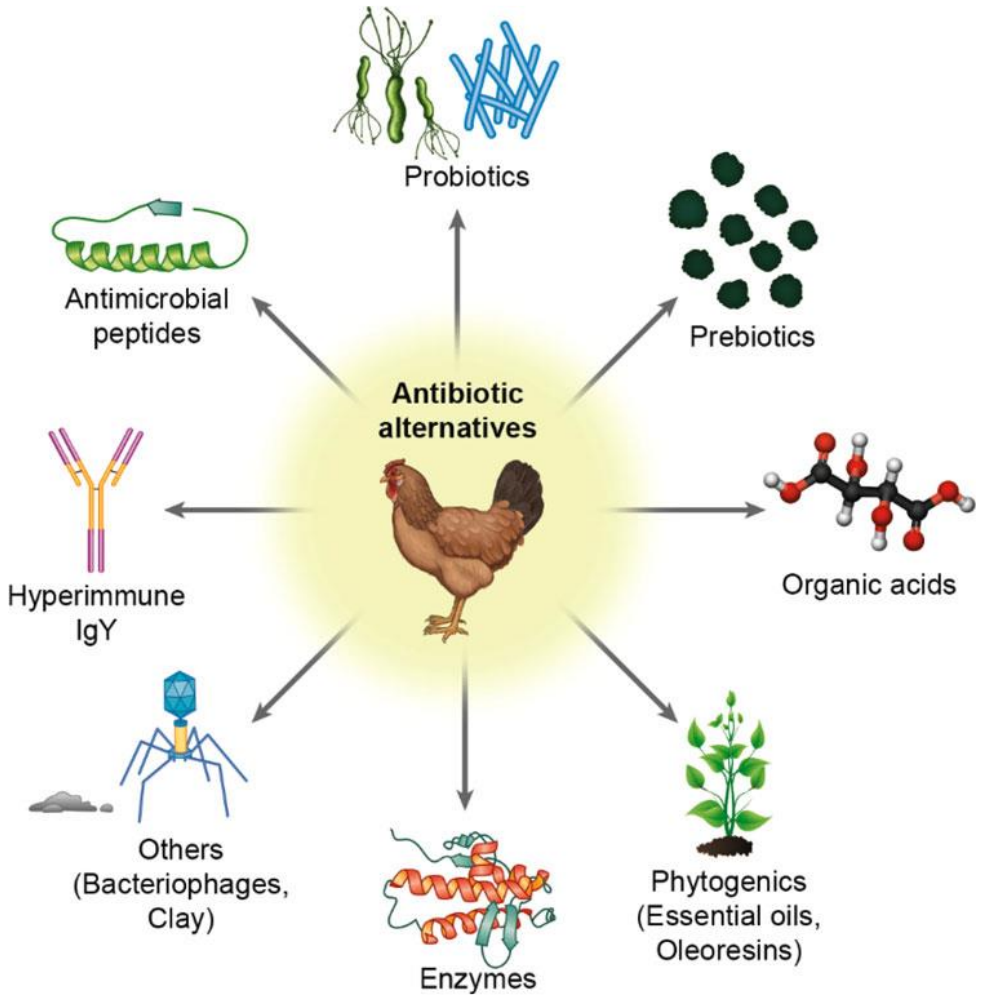

Figure 3. Various classes of antibiotic alternatives that are available for use in poultry production. (Source: Gadde et al., 2017).

3.4 Probiotics

Probiotics are gaining acceptance as potential alternatives to antibiotics to improve production efficiency (Lee et al., 2010). Initially, they were defined as *"live microbial feed supplements which beneficially affect the host animal by*

improving its intestinal microbial balance" (Fuller, 1989). A recent definition adopted by FAO /WHO (2001) states that *"Probiotics are mono or mixed cultures of live organisms which when administered in adequate amounts confer a health benefit in the host".* Probiotics may contain one or more strains of microorganisms and may be given alone or in combination with other additives in feed or water (Thomke and Elwinger, 1998). Novel application strategies such as spray on chicks are also practiced and potential methods such as *in ovo* application are being explored (Wolfenden et al., 2007; Cox and Dalloul, 2015). The characteristics of an ideal probiotic are: be of host origin; non-pathogenic; withstand processing and storage; resist gastric acid and bile; adhere to epithelium or mucus; persist in the intestinal tract; produce inhibitory compounds; modulate immune response and alter microbial activities (Simmering et al., 2001). The species of microorganisms currently being used in probiotic preparations are varied (*Bacillus, Bifidobacterium, Enterocoocus, Lactobacillus, Streptococcus and Lactococcus* spp.) and the majority of research is specifically aimed to reduce the number of pathogenic microorganisms in the gastrointestinal tract, improve growth, feed efficiency and intestinal health of birds. The definite mechanism through which probiotics may improve the defense and performance of chickens remains unclear, the improvement is achieved by reducing intestinal pH, intestinal bacteria composition and digestive activity. Two most important mechanisms through which probiotics exert beneficial effects include balancing the gut microflora and immune regulation (Gadde et al., 2017). Probiotics help establish a microenvironment in the gut that favors beneficial microorganisms and reduces the colonization of pathogenic bacteria (competitive exclusion) by creating a hostile environment for harmful bacterial species (through production of lactic acid, SCFA, and reduction in pH), competing for nutrients with undesired bacteria, producing and secreting antibacterial substances (e.g. bacteriocins by *Lactobacillus*, *Bacillus* spp.); and inhibiting the bacterial adherence and translocation (Nurmi and Rantala, 1973; Fuller, 1989; Netherwood et al., 1999; Schneitz, 2005; Ng et al., 2009; Brown,

2011). Probiotics are also known to improve intestinal function by maintaining epithelial cell homoeostasis, promoting cytoprotective responses and cell survival (through production of cytokines that enhance epithelial cell regeneration and inhibit apoptosis), improving barrier function (modulation of cytoskeletal and epithelial tight junctions), and increasing mucin synthesis (Chichlowski et al., 2007; Ng et al., 2009; Brown, 2011). They also play an important role in digestion and nutrient retention by increasing digestive enzyme activity and improving the breakdown of indigestible nutrients (Jin et al., 2000; Ng et al., 2009; Wang and Gu, 2010; Ciorba, 2012). Probiotics also exert their action by reducing toxic amine production and ammonia levels in the gut (Chiang and Hsieh, 1995). Another important mechanism of probiotics action includes modulating and regulating intestinal immune responses by reducing pro-inflammatory cytokines, increasing secretory IgA production, and promoting specific and non-specific immune responses against pathogens (activation of macrophages, increase cytokine production by intraepithelial lymphocytes) (Ng et al., 2009; Lee et al., 2010, 2011).

Probiotics have positive effects on poultry meat quality (Maiorano et al., 2012; Popova 2017; Tavaniello et al., 2018). They improve pH, color, fatty acid profile, chemical composition, water retention capacity and oxidation stability (Popova, 2017). The probiotics affects the protein and fat contents of meat and thus the meat quality. The lipid oxidation is one of the main causes of deterioration in feed quality (Abdurrahman et al., 2016). Saleh et al., (2012) showed that the inclusion of *Aspergillus awamori* and *Saccharomyces cerevisae* in chicken feed reduced blood saturated fatty acids and increased the polyunsaturated. Another study showed feed containing *B. licheniformis* improves meat color, juiciness and flavor of broiler chickens (Liu et al., 2012). These factors are very important in terms of consumers' appreciation especially the color.

3.5 Prebiotics

One of the alternatives for antibiotic growth promoters that are receiving much attention is dietary fibers with prebiotic functions. Prebiotics are defined as non-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one limited number of bacteria in the ileum and caecum (Gibbson and Roberfroid 1995; Patterson and Burkholder, 2003). This definition was recently refined to shift the focus from selective targets to microbial ecological functions within the gut. The new definition of a prebiotic is *"a non-digestible compound that, through its metabolization by microorganisms in the gut, modulates composition and /or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host"* (Bindles et al., 2015). Large amounts of bacteria present in the monogastric small intestine are potentially capable of utilizing these indigestible carbohydrate sources for energy (Hajati et al., 2010). Some researchers have been conducted to manipulate beneficial bacteria in the gastrointestinal tract and the use of prebiotic is a promising approach for enhancing the role of endogenous beneficial organisms in the gut. The potential effects of prebiotics in diets for farm animals and pets has been documented by Mul and Perry (1994, farm and pet animals), Houdijk (1998, swine), Iji and Tivey (1998; 1999, poultry), Flickinger and Fachey (2002, pets, poultry and rabbits). The use of prebiotics or fermentable sugars instead of antibiotics is going to be popular in the birds in order to improve the useful microbial population of the gastrointestinal tract (Kermanshahi and Rostami, 2006). Prebiotics have been shown to alter GIT microflora, alter the immune system (Babu et al., 2012; Yitbarek et al., 2012), prevent colon cancer (Cummings and Macfarlane, 2002), produce antimicrobial factors (Munoz et al., 2012) and reduce pathogen invasion including pathogens such as *Salmonella enteritidis* and *E. coli* (Callaway et al., 2008). The mechanism by which prebiotics perform these functions remains less clear, but it is likely that the ability of prebiotics to increase the number of host beneficial bacteria in

the gut can help the competitive exclusion of the pathogen from the gastrointestinal tract of birds (Alloui et al., 2013). A limited set of carbohydrate compounds have traditionally considered possessing all the characteristics that define the classic prebiotic and its associated properties when consumed by animals and humans.

An ideal prebiotic should be neither hydrolyzed or absorbed in the upper part of the gastrointestinal tract; it would be a selective substrate for one or a limited number of bacteria commensal to caecum/colon, which are stimulated to grow or metabolically activated; it should be able to alter the colonic flora in favor of a healthier composition; it should induce systemic effects that are beneficial to host's health; it must have known structure, which can be documented; it should be palatable as food ingredient and large-scale processing must be easy. The classification has expanded to include a variety of oligosaccharides varying chain length all of which share the common characteristic of not being digestible by the host. The most common prebiotics used in poultry include inulin, fructooligosacchaides (FOS), mannanooligosaccharides (MOS), galactooligosaccharides (GOS), soya-oligosaccharides (SOS), xylo-oligosaccharides (XOS), pyrodextrins, isomaltooligosaccharides (IMO) and lactulose (Huyghebaert et al., 2011; Kim et al., 2011; Alloui et al., 2013). FOS and its long chain, inulin, are among the most studied prebiotics in humans and animals. FOS are natural linear polymers, up to 10 monomeric, of β-(2-1) - linked fructosyl units, terminated by one glucose residue. FOS are not hydrolyzed by mammalian or avian digestive enzymes and thereby reach the colon undigested, allowing fermentation by gut microbiota (Roberfroid et al., 2010). Mannanoligosaccharides (MOS) are mannose-based oligomers linked together by β-1, 4 glycosidic bonds. They are naturally found in certain plants, beans and mannoprotein portion of the cell wall of the yeast *Saccharomyces cerevisiae.* Because birds do not have enzymes to break down the mannan backbone, these oligosaccharides is believed to reach the lower GI tract undigested (Pourabedin et al., 2015). Xylooligosaccharides (XOS) are chains of β-1, 4- linked D-

xylopyranoside units, produced by partial hydrolytic degradation of lignocellulosic materials, commonly arabinoxylans, which are found in abundance in the cereal grains (Carvalho et al., 2013). Chickens lack enzymes required to degrade the glycoside link between xylose monomers; therefore, XOS reach the lower intestine tract and cecum, where they are metabolized by xylantolytic microorganisms. GOS are naturally found in human milk and it has been received a notable attention as a supplement for infant formulas. GOS are manufactured from lactose found in cow's milk using enzymatic reactions. These use *β*-Galactosidase that is a hydrolase that attacks the O-glucosyl group of lactose. The general mechanism of enzymatic lactose hydrolysis has a trans-galactosylic nature, involving a multitude of sequential reactions with disaccharides (other than lactose) and higher saccharides, collectively named galacto-oligosaccharides (GOS), as intermediate products (Wallenfels and Malhotra, 1960). The ability of these oligosaccharides, when added to infant milk formulas, to replicate the bifidogenic effect of human milk, not only in bacterial numbers, but also with respect to the metabolic activity of the colonic microbiota (Knol et al., 2005), has significantly increased interest in their production and application in various food and pharmaceutical processes. GOS were recently defined as "a mixture of those substances produced from lactose, comprising between 2 and 8 saccharide units, with one of these units being a terminal glucose and the remaining saccharide units being galactose and disaccharides comprising 2 units of galactose" (Tzortzis and Vulevic, 2009). Several *in vitro* and *in vivo* experiments have demonstrated the indigestibility and stability to hydrolysis by digestive enzymes of GOS. In a consensus report, it was concluded that more than 90% of GOS passes into the colon (van Loo et al., 1999).

The effects of these prebiotics on gut microbiota are several: FOS and MOS reduce intestinal colonization by *Salmonella*, decrease populations of *C. perfringens* and *E. coli*, provide nutrients for the growth of beneficial bacteria in the gut, increase the population of *Bifidobacterium* in the small intestine and

colon, increase the population and diversity of lactobacilli in the ileum. In particular, inulin increases the population of *Bifidobacterium* counts and decreased *E. coli* counts in cecal contents. GOS increase *Bifidobacterium* spp. and decrease *Campylobacter* spp. in fecal samples. The best-characterized properties identified with GIT microbial antagonism of foodborne pathogens is the production of short chain fatty acids (SCFA, primarily acetate, propionate and butyrate) and lactate during fermentation (Ricke, 2018). Van Immerseel et al. (2006) suggested that the presence of butyrate might down regulate *Salmonella* invasion genes while propionate can inhibit epithelial cells invasion. Acetate can elicit other impacts on foodborne pathogens. Fukuda et al. (2011) reported that increased acetate generated by bifidobacteria in mice inhibited translocation of Shiga toxins produced by *E. coli* O157:H7 from the GIT lumen to the blood stream.

Prebiotics have also been reported to enhance the immune response of chickens, resulting in rapid clearance of pathogens from the gut (Kim et al., 2015). Immunomodulation by prebiotics is thought to be due to the activation of innate immunity by the interaction of the sugars with the certain receptors present on the surface of dendritic cells and macrophages, which can stimulate production of cytokines, proliferation of lymphocytes and activity of NK (natural killers) cells (Hashim, 2012; Saad et al.,2013). Extensive studies have been carried out to MOS, mannans present in MOS bind to mannose-specific type I fimbriae on Gram-negative pathogens (such as *E. coli* and *Clostridium*), thus preventing them from adhering to, and colonizing the gut (Sinovec and Markovic, 2005). Xiao et al. (2012) reported the association of MOS with increased expression of genes responsible for oxidative phosphorylation, mitochondrial electron transport chain and antioxidant enzymes (Xiao et al., 2012). Prebiotics have also been shown to affect globet cell number and mucin production in host gut and to decrease the expression of IL-1β, a pro inflammatory cytokine (Babu et al., 2012; Tsurumaki et al., 2015).

Dietary prebiotic supplementation is attributable to the improved bird performance and energy utilization (Yang et al., 2008; Choct, 2009; Nabizadeh, 2012). Other authors reported that supplementation of prebiotics to the diet improved the weight gain of broiler when compared with the control. Addition of prebiotics *in ovo* have been explored and these may be a promising means to achieve greater consistency in prebiotic efficacy and immune function modulation (Roto et al., 2016; Slawinska et al., 2016). Villaluenga et al. (2004) compared the dietary prebiotic inclusion and the *in ovo* injection technique, they showed that *in ovo* injection increased the population of beneficial microflora on the day of hatch, and leaded to a high and stable level of bifidobacteria throughout the broiler chickens growing period (Villaluenga et al., 2004).

3.6 Synbiotics

There is a great potential for synbiotics to be used as antibiotics alternatives for improving performance and reducing pathogenic load in the intestines of poultry. Synbiotics are additives that combine the use of probiotics and prebiotics such that they can act synergistically (Alloui et al., 2013). This combination could improve the survival and persistence of health-promoting organism in the gut of birds because its specific substrate is available for fermentation (Yang et al., 2009; Adil and Magray, 2012). Several studies have shown the potential benefits of synbiotics on the intestinal microbial ecosystem: synbiotics increased the lactobacilli populations and reduced *E.coli* and total *coliform* populations in the intestine (Dibaji et al., 2014), reduced *C. jejuni* concentration in poultry feces (Baffoni et al., 2012), reduced the intestinal colonization by *C. perfringens* (El-Ghany 2010), reduced intestinal *S. enteritidis* colonization. In terms of effects on immune system, synbiotics increase antibody production (Hassanpour et al., 2013), stimulate the expression of IL-6 and IFN-γ during *in vitro* culturing of chickens lymphocytes (Slawinska et al., 2012) and

improve the antibody response to infectious bronchitis virus vaccines (El-Sissi and Mohamed, 2011). Awad et al. (2008) showed that supplementation of diets with a synbiotic significantly improve body weight, average daily gain, feed efficiency and carcass yield percentage compared with probiotic-fed broilers.

3.7 Organic acids

Dietary organic acids have been considered as potential alternatives to AGPs, owing to their antimicrobial nature (Gadde et al., 2017). For decades they have been used as feed additives because they are considered as Generally Recognized as Safe (GRAS) for meat products (Suresh et al., 2018). The term "organic acid" refers to a broad class of compounds used in fundamental metabolic processes of the body. Chemically, organic acids share the common features of water solubility, acidity, and ninhydrin-negativity (no primary or secondary amines). The term is generally considered to include all carboxylic acids, with or without keto, hydroxyl, or other non-amino functional groups, but does not include most amino acids. Short chain fatty acids are also contained in this group. They are either simple monocarboxylic acids such as formic, acetic, propionic and butyric acids or carboxylic acids with hydroxyl group such as lactic, malic, tartaric and citric acids or short chain carboxylic acids containing double bonds like fumaric and sorbic acids. As a group of chemicals, organic acids are considered to be any organic carboxylic acid of the general structure R-COOH. Organic acids with specific antimicrobial activity are short-chain acids (C1–C7) and they are widely distributed in nature as normal constituents of plants or animal tissues. They can be administered in the feed or drinking water and can be used individually as organic acids or their salts (sodium, potassium, or calcium) or as blends of multiple acids or their salts (Huyghebaert et al., 2011). They are also products of microbial fermentation of carbohydrates especially in the caeca of birds (Huyghebaert et al., 2011). The SCFA produced during carbohydrate fermentation are used by the host as a source of energy.

Organic acids use has been shown to have significant benefits in swine and poultry production over the years (Gadde et al., 2017). Their use improves weight gain and feed efficiency (Patten and Waldroup, 1988; Skinner et al., 1991; Banday et al., 2015). Recent studied demonstrated that combinations of organic acids are more effective than supplements that contain only one type of acid (Samanta et al., 2008, 2010).

The mechanism of action of organic acids is not clearly understood, it can be attributed to their antibacterial activity. Organic acid can diffuse through the semi-permeable membrane of bacteria into the cell cytoplasm in the undissociated form (Van Immerseel et al., 2006). Once in the cell, where the pH is maintained near 7, the acids will dissociate and suppress bacterial cell enzymes (e.g. decarboxylase and catalases) and nutrient transport system (Huyghebaert et al., 2011). The dissociation constant (pKa), that is the pH at which the acid is half dissociated, is one of the most important characteristics of organic acids. In general, organic acids with higher pKa values are more effective antimicrobials compounds (Huyghebaert et al., 2011). The bacterial cell wall contains lipid material, hydrophobicity is an essential feature of organic acids to exert its antimicrobial activity, in this way they can interact with this lipid material and disrupts microbial activity (Kuroda et al., 2009; Huyghebaert et al., 2011). The beneficial effects of organic acids can be enhanced by using them as blends rather than a single acid because different types of organic acids diffuse through the bacterial cell wall and membrane and into the cell cytoplasm at different rates. The antibacterial activity plays a crucial role in controlling the population of pathogenic bacteria in the gut of birds (Partanen and Mroz, 1999). Thanks to the reduction of pathogenic intestinal bacteria, which produce toxin causing damage of intestinal villi and crypt structure, the gut structure of chickens is improved by increasing the height of villus (Adil et al., 2010, 2011). The extension of the height of villus increases the nutrient absorption and consequently the growth performance in broiler chicken improves (Adil et al., 2010). Mista et al., (2010) reported that these histopathological changes in the small intestine can

be averted through the use of short chain fatty acids; SCFA up-regulate genes involved in epithelial cell growth, division, differentiation, proliferation and apoptosis (Hashemi and Davoodi, 2011). SCFAs are commonly included in feed as they have been shown to improve performance, feed quality and modulate disease resistance of broiler (Abdel- Fattah et al., 2008; Sohail et al., 2015; Reda et al., 2016). Among the SCFA, butyrate has gained particular interest, as it the preferred energy source for the enterocytes and is known to regulate cellular differentiation and proliferation within the intestinal mucosa, thereby increasing tissue weight (Le Blay et al., 2000). Yet, butyric acid has been reported to down-regulate the expression of the Salmonella pathogenicity island I genes responsible for virulence and invasion of epithelial cells (Van Immerseel et al., 2006). Butyrate has been shown to increase production of tight junction proteins in the cell, thus decreasing permeability of intestinal epithelium to invasion by pathogens (Van Deun et al., 2008; Andreopoulou et al., 2014). Butyrate has been studied extensively also for its anti-inflammatory properties. Sunkara et al., (2011) reported that butyrate could induce the synthesis of host defence peptides in chicken, as well as increase the activity of chicken monocytes against *S. enteritidis* with minimum production of inflammatory cytokines.

Organic acids have also been studied for their effect on gut mucosa and their immunomodulatory action. Dietary supplementation of organic acids has shown to increase the counts of CD4 cells and T-Cell Receptor II lymphocytes, which corresponds to a faster immune response (Khan and Iqbal, 2016). Additionally, organic acids are also being studied for their role in improvement of phytate phosphorus utilization in chickens (Rafacz-Livingston et al., 2005).

3.8 Enzymes

Dietary enzymes are biologically active proteins that facilitate chemical breakdown of nutrients to smaller compounds for further digestion and absorption (Thacker, 2013). The different classes of enzymes, produced through

fungi and bacteria fermentations, commonly employed include phytase, carbohydrases (xylanase, cellulase, α-galactosidase, β-mannanase, α-amylase, and pectinase), and proteases. The use of exogenous enzymes is of importance in poultry because chicken diets are composed primarily of corn and soybean meal, which contain varying levels of different anti-nutritive factors (e.g., non-starch polysaccharides (NSP) and protease inhibitors) that can impede normal digestion and absorption processes of nutrients in the digestive tract (Yegani and Korver, 2013). The possible mechanisms of action of in-feed enzymes include the increase in the digestibility of nutrients that are otherwise not degraded by host enzymes; the elimination of the nutrient-encapsulating effect of cell-wall polysaccharides and an increase in the availability of starches, amino acids, and minerals; the inactivation of anti-nutritional factors and the reduction of intestinal viscosity; the increase in the solubility of non-soluble NSP and promotion of cecal fermentation; and the supplementation of endogenous enzymes that may be in insufficient amounts, especially in young animals in which the digestive system is not fully developed (Choct, 2009; Kiarie et al., 2013). Exogenous enzymes have been reported to modulate the gut microbiota of birds (Bedford and Cowieson, 2012). The enzyme-induced microbiota changes are indirect and are mediated by two mechanisms: the first is the reduction of the undigested substrates and the second is the formation of short-chain oligosaccharides from cell-wall NSP with potential prebiotic effects (Bedford, 2000; Bedford and Cowieson, 2012; Kiarie et al., 2013). These mechanisms influence the nutrient supply and intestinal environment thus altering selection pressures on bacterial species (Bedford and Cowieson, 2012; Cheng et al., 2014). Adeola and Cowieson (2011) revealed that carbohydrase supplementation increased the proportion of lactic and organic acids, reduced ammonia production, and increased the concentration of fatty acids which is indicative of hydrolysis fragmentation of NSP and supporting growth of beneficial bacteria. The potential for use of in feed enzymes, as antibiotic alternatives, to improve performance in poultry is significant: Yang et al. (2008) reported that the crypt

depth of jejunum was reduced by xylanase and this was associated with the increased growth of chicken fed xylanase; Adeola and Cowieson (2011) showed that carbohydrase supplementation improved villi length and supported the growth of chicken. Moreover, various meta-analysis conducted corroborate these beneficial effects in broilers upon enzyme supplementation (Gadde et al., 2016). An example is given by Jackson and Hanford (2014) that conducted a meta-analysis investigating the effects of β-mannase supplementations in male broilers raised to market age. They reported that the weight gain and FCR were improved and concluded that the β-mannase supplementation is effective in broilers. However, the beneficial effects of enzyme supplementation are sometimes inconsistence owing to the difference in enzyme type, source, amount of enzyme used, presence of side enzyme effects, diet composition and genetic variations among animals (Ravindran and Son, 2011).

3.9 Phytobiotics

Phytobiotics are natural active compounds that are derived from plants and can be added to the diet of commercial animals to improve their productivity through enhancing feed properties, promoting animals' production performance, and improving the quality of products derived from these animals (Windisch et al., 2008). In recent years, they have been used as natural growth promoters (Ghasemi et al., 2014; Toghyani et al., 2011) in poultry industries and for their potential applications as AGP alternatives (Franz et al., 2010). A wide range of plants and their products fall under this category and, based on their origin (part of plant), they can be classified as herbs (flowering, non woody and non-persistent plants), spices (non-leaf parts of plants, including seeds, fruits, bark or root with intensive taste or smell), essential oils (volatile lipophilic substances obtained by cold extraction or by steam or alcohol distillation) and oleoresins (extracts derived by non-aqueous solvents) (Windisch et al., 2008; Van Der Klis and Vinyeta-Punti, 2014). They can be used in solid, dried and ground form or as

extracts (crude or concentrated). The main bioactive compounds of the phytobiotics are phenolic substances such as thymol, carvacrol, phenylpropane, limonene, geraniol and citronellal, their composition and concentration vary according to the plant, parts of the plant, geographical origin, harvesting season, environmental factors, storage conditions, and processing techniques (Windisch et al., 2008; Applegate et al., 2010). The mechanism by which the phytobiotics exert their benefits on the gut remain unclear, but possible mechanisms proposed are: modulation of the cellular membrane of microbes leading to membrane disruption of the pathogens; increased of the hydrophobicity of the microbial species which may influence the surface characteristics of microbial cells and thereby affect the virulence properties of the microbes, stimulation of the growth of favorable bacteria such as lactobacilli and bifidobacteria in the gut, acting as an immunostimulatory substance and protecting the intestinal tissue from microbial attack (Vidanarachchi et al., 2005; Windisch and Kroismayr, 2007).

The principal use of phytobiotics in aviculture has been the administration of essential oils, which have been used for a long time in the preparation of feed as artificial flavors and preservatives. Most essential oils have been classified as Generally Recognized as Safe (GRAS), by the US Food and Drug Administration (FDA). These oils are characterized as engaging in antimicrobial activities and having growth promoting properties. The antimicrobial activity (either bactericidal or bacteriostatic) of phytogenic compounds against foodborne organisms such as protozoa and fungi has been investigated by several researchers (Chao et al., 2000; Burt 2004; Si et al., 2006; Stein and Kil 2006; Michiels et al., 2009; Panghal et al., 2011; Giannenas et al., 2013). Dorman and Deans (2000) reported that some oils such as carvacrol and thymol obtained from oregano and eugenol from the clove plant inhibit several pathogens bacteria. Other studies showed that oils have been used as feed additives to reduce the presence of different pathogens in the intestine, including *Salmonella*

(Vicente et al., 2007), *Campylobacter* (Ali, 2014) and *C. perfringens* (Mitsh et al., 2004).

It has been mostly reported that addition of herbal products to diets has growth promoting effect on poultry (Wenk, 2003; Kim et al., 2010). Mohammadi Gheisar et al. (2015) reported that feeding broiler chickens with diet containing 0.075% of a phytogenic blend led to 3.9% and 3.4% improvement in BWG and FCR, respectively. Phytobiotics have a beneficial effect on the taste and palatability of feed, thus enhancing the production performance (Windisch et al., 2008).

The antioxidant activity of phytobiotics is another biological property of great interest. Their ability of eliminating free radicals may play an important role in preventing some diseases caused by free radicals, such as cancer and heart diseases (Kamatou and Viljoen, 2010; Miguel, 2010). The ability of donating hydrogen or an electron to free radicals and also delocalizing the unpaired electron within the aromatic structure are the main mechanisms of protecting other biological molecules against oxidation (Fernandez-Panchon et al., 2008; Giannenas et al., 2013). Researchers have investigated the potential effect of phytobiotics feed additives from the *Labiatae* plant family containing phenolic compounds on improving the oxidative stability of poultry meat (Young et al., 2003; Basmacioglu et al., 2004; Florou- Paneri et al., 2006). Cherian et al. (2013) reported that feeding broiler chickens with phytobiotics feed additives (Artemisia annua) resulted in a significant reduction in thiobarbituric acid reactive substances (TBARS) value in breast and thigh meat. They suggested that the reduction in TBARS value could be due to individual or combined antioxidant properties of polyphenolic compounds or vitamin E in Artemisia annua. Cuppett and Hall (1998) have suggested that the antioxidative activity of *Labiatae* family plants is due to their contents of phenolic terpenes (e.g. rosmarinic acid and rosmarol). Placha et al. (2014) have demonstrated that supplementing the diet of broiler chickens with thymol can reduce the oxidation of fatty acids indicated by the lower malondialdehyde level in duodenal mucosa. Franz et al. (2010) have suggested that phytobiotics can beneficially affect some antioxidant enzymes

such as glutathione peroxidase and superoxide dismutase, consequently affecting lipid metabolism in animals.

A positive influence of phytobiotics has been demonstrated on the immune system such as increased proliferation of immune cells, elevated expression of cytokines and increased antibody titers (Kim et al., 2010; Lee et al., 2010; Park et al., 2011; Pourhossein et al., 2015). The addition of phytobiotics to the diet was also shown to increase intestinal and pancreatic enzyme production and activity and increase bile flow (Lee et al., 2003; Jang et al., 2007; Malayoğlu et al., 2010; Hashemipour et al., 2013, 2014). Phytobiotics also help maintain and improve gut histology, increase villi height and thus expand absorptive surface of the intestine (Ghazanfari et al., 2015; Murugesan et al., 2015). Increase in digestive enzyme secretion and absorption results in improved apparent nutrient digestibility and thus improves performance (Jamroz et al., 2003; Hernández et al., 2004; Jørgensen et al., 2008; Wang et al., 2008; Amad et al., 2011; Amerah et al., 2011; Issa and Omar, 2012). They also might play a role in maintaining the intestinal barrier function as evidenced by the increase in the trans-epithelial electrical resistance of duodenal mucosa of broilers that included thymol in their diets (Placha et al., 2014).

Our knowledge on the mode of action of phytobiotics and applicative aspects is still rather limited, further research is needed to confirm whether they can improve production parameters and animal health (Diaz-Sanchez et al., 2015).

Chapter 4

4. *In ovo* technique

With increasing concerns about antibiotic resistance and the ban on AGP usage in Europe, there is growing interest in finding alternatives to antibiotics for poultry production. One possible alternative is to control the GIT microbiota, through the use of a wide range of potential feed additives, to make up the negative effects on growth performance and immune system response observed in poultry.

There are different ways to deliver feed additives into avian gastrointestinal tract. To achieve desired efficacy, feed additives must be administered to an animal as early in life as possible and under fully controlled conditions (Schneitz, 2005). Conventionally, in-feed or in-water supplementation has been used at first hours/days post hatching. This approach relies on amount of feed and/or water intake, the quality of water and other experimental factors (Waldroup et al., 2003; Ciesiolka et al., 2005; Schneitz, 2005; Huyghebaert et al., 2011). As a consequence, consumed dose of additives varies in the first hours/days after hatching. Therefore, *in ovo* approach for injection of feed additives directly to the incubating egg has been developed. This method born after the success with the *in ovo* injection of vaccine against Marek's disease (MD). In the 1980s was established the *in ovo* vaccination against MD to ward off the infection due to the exposure to the virus (Sharma et al., 1982). Prior to *in ovo* injection, the MD vaccine was distributed post-hatch; however, it was observed in vaccinated flocks high rate of mortality due to the MD virus. Sharma et al. (1982) hypothesized that one of the most possible reason of the observed loss was that post-hatch vaccinated birds were prematurely exposed to MD, so young chicks had insufficient time to mature immunity following vaccination. (Sharma et al., 1982). Sharma and Burmester (1982), recognized the ability of late stage embryos and fetuses to support immune responses to viral and bacterial

antigens (Brown et al., 1979; Richardoson et al., 1972), used the *in ovo* injection for the MD vaccine in embryonic chickens. They observed significantly greater protective indices when vaccinated at embryo stage compared to those vaccinated at hatch while having no effect on hatchability (Sharma et al., 1982). After, extensive experimentation has been conducted with the injections of various biologics, such as nutrient supplementation, hormones and immunostimulants. However, skepticism of the technique was also raised based on lack of optimization in deliverance (age, volume, location of injection, as well as other factors), stress caused to the embryo by disruption of the internal environment or osmotic balance, and insufficient evaluation for the optimal individual or mixed substances for injection or their appropriate concentrations for delivery. Since the initial introduction of *in ovo* technique, there have been numerous patents for automated deliverance with variations in site of injection, solution injected, age of injection, and method of automation (Lewis US patent 5056464; Hebrank US patent 4903635; Paul US patent 5136979; Miller US patent 4469047). Among the various patents for auto-mated injections, Uni and Ferket patented a method for the delivery of *in ovo* injections that has come to be widely accepted among investigating researchers in terms of the age, location of injection, volume of injection, and a validated array of biologics that may be injected (Uni and Ferket, patent US 6592878 B2 (2003)).

4.1 Approaches used in ovo technology: in ovo feeding and in ovo stimulation

For optimizing bird health and performance it would be better to establish a healthy and balanced GIT microbiota at the beginning of its formation rather than trying to alter an already established GIT microbiota. The most crucial time in the development of a young chick is the perinatal period (the last few days prior to hatch and the first few days after hatch); this time period is when intestinal development is occurring most rapidly (Iji et al., 2001; Ferket et al.,2012). The perinatal period is a transitional time in which the chicks undergo

metabolic and physiological shifts from the utilization of egg nutrients to exogenous feed (Ferket et al., 2012). For the operation of hatcheries and the delivery of newly hatched chicks to broiler farms, the chicks are exposed to delayed feeding for 48–72 h. The starvation period introduced by poultry farmers is brief, but it occurs at a crucial time in chicks development causing stress on the young chicks, which can retard GIT development (Ferket et al.,2012). During the starvation time the reserves intended for muscle protein are being mobilized to continue gluconeogenesis, while the newly hatched chick gains the capability of digesting exogenous glucose sources (Vieira et al.,1999). The chick allocates the limited reserves to the upkeep of thermal regulation and metabolism, which restricts growth and development (Ricklefs et al., 1987; Pinchasov et al., 1993). Avian species with rapid early development of the intestine and liver have been correlated with high growth rates overall (Lilja, 1983). Studies have revealed connections of early feeding not only to the overall performance but also specifically to intestinal, muscle, and immunological development and yolk-reserve utilization (Uni et al., 2004; Careghi et al., 2005). Early feeding allows the small intestine to continue its growth and development rapidly because, at the time of hatch, the GIT is not yet fully developed (Noy et al.,1998; Nitsan et al., 1991). Increasing villi height and crypt depth allows for increasing absorption and digestive capabilities (Sell et al.,1991; Sklan et al.,2001). The transition from the lipid-rich yolk contents to carbohydrate and protein rich exogenous nutrient source is only possible by the appropriate development of the GIT (Uni et al.,1999; Sklan et al., 2001).

In ovo technology has been developed to facilitate manipulation of chicken embryo before hatching. The literature describes two major time points of chicken embryo development that have been successfully used for substance delivery through *in ovo* technology. The first time point is around days 17/18 of egg incubation. This method is referred to as "*in ovo* feeding" and has been patented by Uni and Ferket. *In ovo* technology used at this time point aims to mitigate the negative effects of starvation period. In particular, this technology

was designed for a late-stage poultry embryo (day 17/18 of egg incubation) to facilitate its adaptation to a switch from embryonic nutrition based on fat and proteins deposited in the yolk (during the embryonic stage) to autonomous nutrition, which is based on carbohydrates and proteins (during the post-hatching stage) (Roto et al., 2016). The key parameters, which were measured to evaluate the efficacy of *in ovo* feeding (i.e., the transition from embryonic to independent nutrition), included the proliferation of enterocytes, the enzymatic activity of digestive organs and gut morphology (Roto et al., 2016). The second time point for the in ovo delivery of bioactive compounds is around day 12 of egg incubation and has been used only for the delivery of prebiotics and synbiotics. This approach is based on the studies made by Pedroso et al., (2016) who demonstrated that neonatal chicks possess intestinal microbiota even before hatching, but at this stage the microbiota is not effective enough for the competitive exclusion of pathogens (Pedroso et al., 2016). So, *in ovo* injection of prebiotic stimulate the microbiota. This method has been developed and patented by Gulewicz and Bednarczyk (2008). *In ovo* stimulation refers to the delivery of prebiotics or synbiotics in the early-stage embryo. It aims to stimulate selective growth of indigenous microflora, which colonize embryonic guts. The agent that triggers this stimulation is a prebiotic, which is metabolized by the indigenous bacteria, stimulating their growth. Prebiotics and synbiotics can be delivered *in ovo* on day 12 of egg incubation to program the microbiota colonization and modulate gene expression related to the microbiome. Such modulation poses life-long beneficial effects in performance and mitigating life stressors.

During the embryonic development, there are five regions through which an *in ovo* injection may be delivered: the air cell, the allantoic membrane, the amniotic fluid, the yolk, and the embryo body (Figure 4).

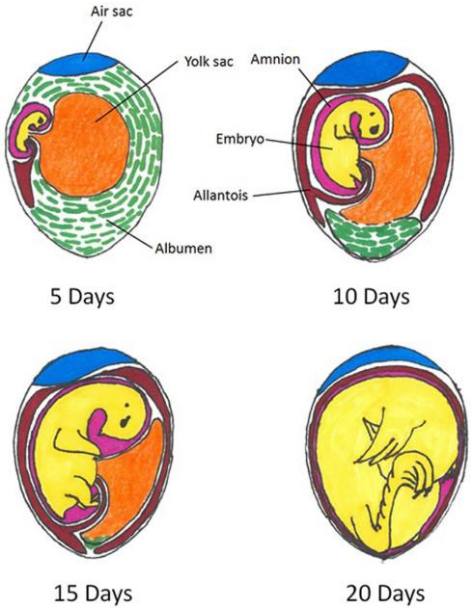

Figure 4. Spatial relations within an embryonated chicken egg at 5, 10, 15, and 20 days of incubation. Colors indicate differing compartments: embryo = yellow; air sac = blue; amnion = pink; allantois = red; albumen = green. (Source: Roto et al., 2016)

There are differences between *in ovo* stimulation and *in ovo* feeding in the delivery of bioactive compounds. Uni and Ferket (2003) proposed to inject the bioactive substances directly into the amniotic fluid. The embryo consumes the amniotic fluid and its contents are exposed to the intestines and the enteric cells that comprise them. Therefore, substances administered to this region will be consumed along with the amniotic fluid and presented to enteric tissues (Uni and Ferket, 2003). The injected volume amounts to 1-1.7ml solution of specific nutrients (carbohydrates, proteins and others). The possible consequences of the *in ovo* feeding injection were evaluated by Wakenell et al., (2002). These authors indicated that the needle should pass through the air cell and the allantoic fluid in order to inject and dispense the vaccine to either the amniotic fluid or the embryo body to achieve the greatest protection efficacy (Figure 5). The precision in the depth of the injection is crucial; the needle not being deep enough into the egg will result in the dispersion of the vaccine to the air cell or allantoic fluid, while injecting the needle too deep may cause trauma to the embryo (Figure 5).

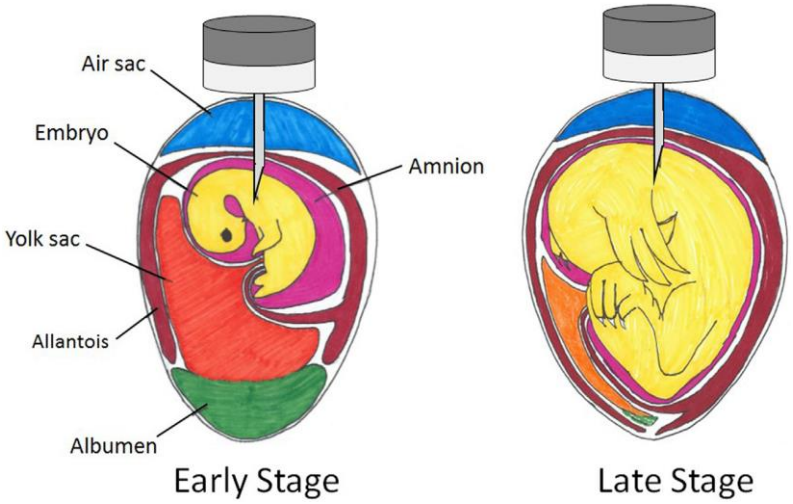

Figure 5. Spatial relations within an embryonated chicken egg at early and late stages of incubation with possible *in ovo* injection sites. (Source: Roto et al., 2016)

In ovo stimulation on day 12 of egg incubation provide the deposition of the prebiotic/synbiotic solution into the air cell and the consecutive transfer of small-weight oligosaccharides through the membrane into the blood vessels surrounding the embryo. The prebiotic is transferred through the blood system to the embryo. For this purpose, the prebiotic has to be soluble in water or it does not pass through the membrane. The volume injected is of 0.2 ml, because a larger volume would infiltrate the junction between the inner and outer egg membrane and immediately kill the embryo. Applying prebiotic on day 12 of egg incubation gives the opportunity to stimulate endogenous microbiota before hatching, because at this time, the chorioallantoic membrane is highly vascularized and allows the transfer of the prebiotic from the air cell into the circulatory system and further to the developing intestine. To demonstrate the transfer of the prebiotic and probiotic after *in ovo* stimulation on day 12 of egg incubation, Plowiec et al. (2018) performed an experiment in which hatching eggs were injected with a solution of blue dye, with a molecular weight similar to a GOS prebiotic, and a probiotic strain (*Lactococcus lactis* subsp. *Cremoris)*. Either prebiotic and probiotic were injected into the air cell on day 12 of egg incubation. In this way, the authors were able to monitor the migration of prebiotic (the blue dye) and probiotic through the outer and inner shell

membranes (Figure 6). To analyze the migration rate of the prebiotic and probiotic, the eggs were dissected and analyzed daily from day 13 to 18 of egg incubation. The author showed that prebiotic migrated through the shell membrane and entered the blood circulation on day 3 after injection, while the probiotic bacteria stay in the air cell until the beginning of hatching (Plowiec et al., 2018). For this reason, probiotic, even though it might be injected on day 12 of egg incubation (in the form of synbiotic), should be considered *in ovo* feeding due to its availability to the late-stage embryo. The major obstacle to using *in ovo* stimulation is the need for technological adjustment of production lines in hatcheries to add another time point for egg manipulation (day 12 of egg incubation). A prototype of such an automated injection system for early-stage embryos exists and has been tested in commercial settings (Bednarczyk et al., 2011). The pipeline includes three procedures: a hole is drilled in the eggshell, a solution is injected, and then the hole is sealed. This allows for *in ovo* injections of 30,000 eggs per hour while maintaining superior hatchability.

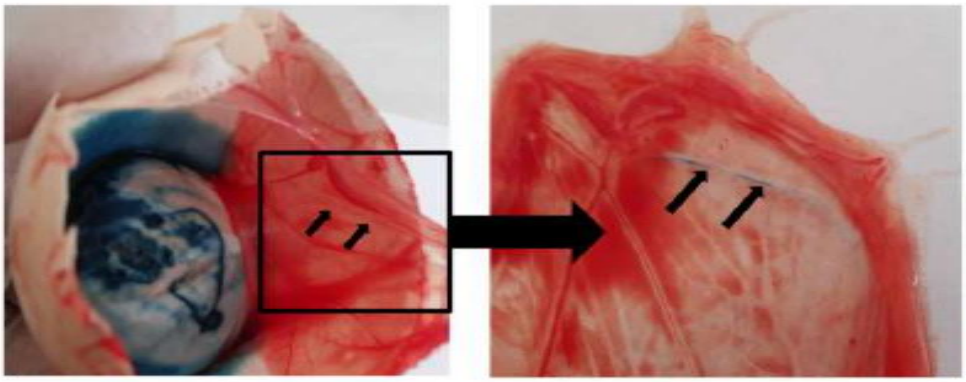

Figure 6. Model of *in ovo* delivery and penetration of the bioactive solution through the chorioallantoic membrane into the circulatory system of the chicken embryo. (Source: Siwek et al., 2018)

4.2 In ovo technology: performance and meat quality

In the last decades, *in ovo* technology has been applied to improve production traits such as growth rate, feed intake (Bednarczyk et al., 2011), meat quality (Maiorano et al., 2012) and immune system development. However, the mechanisms of these processes are still difficult to identify. Maiorano et al., (2012) studied the influence of prebiotic (RFO-raffionose family oligosaccharides) and three different synbiotics on growth performance and meat quality of broiler chickens. They observed a decrease in the density of the muscle fiber per mm^2 in the synbiotic group (RFO with *L. lactis* spp. *cremoris*) in comparison to the control group. Moreover, intramuscular collagen was reduced in the prebiotic and synbiotic groups. There were no effects of treatment on abdominal fat, ultimate pH, and cholesterol in the pectoral muscle of chickens (Maiorano et al., 2012).

Tavaniello et al. (2018) studied the effect of prebiotics administration on the meat-quality traits of broiler chicken by evaluating different ways of their delivery (*in ovo vs* in-water *vs in ovo* and in-water combined). Authors reported that irrespective of the delivery method, prebiotics showed a positive impact on breast muscle weight and yield, which was also associated with a greater thickness of muscle fibers, and a decreased of the redness (a*) of fillets. In addition, they observed a higher content of PUFA (polyunsaturated fatty acids) and n-3 fatty acid in the meat from the prebiotic groups, displaying more favorable indexes for human health. Moreover, Tavaniello et al. (2018) compared the effect of *in ovo* administration of two different synbiotic formulations (GOS with *L. salivarius* - SYN1 and RFO with *L. plantarum* - SYN2) on carcass- and meat-quality traits in broiler chickens. In *in ovo* SYN1-injected chickens they recorded the highest color lightness (L*) in 45 minutes after slaughter; in the SYN2 group was observed the highest content of MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids) and n-6 fatty acids in breast muscle.

By influencing the intestinal microbiota through the administration *in ovo* of prebiotics and synbiotics it is possible to modulate the immune response (Slawinska et al., 2014). In literature there are few information regarding the effects of *in ovo* delivered prebiotics and synbiotics in broiler (Slawinska et al., 2014). However, Madey et al. (2015) demonstrated that the *in ovo* delivery of prebiotics and synbiotics, at day 12 of incubation, when intensive growth, maturation and selection of lymphocytes take place, can influence the maturation and the development of immune system. Their hypothesis is that the early colonization of bacteria from the treatment with synbiotic can increase the potential of immune system to respond to foreign antigens (Madej *et al.* 2015).

Chapter 5

5. Poultry meat quality

Intensive research on poultry meat quality began after World War II, mainly in industrialized countries, at that time the main objectives were to satisfy the growing demand for animal protein in human nutrition and to increase the safety of meat (Grashorn, 2010). Poultry products are in demand in all parts of the world: poultry meat continue to be the most efficient and economical way to convert feed grains to animal protein. When there are no religious or cultural barriers, poultry meat usually leads in consumer preference (Van der Sluis, 2001). Poultry meat also enjoys popularity in developed markets, due to its price and perceived safety health advantages compared to other meat sources (FAS, 2001).

There are many definitions of “quality”. Groom (1990), considering the need to make products that meet the demands of the market place, defined quality as: *"The composite of those characteristics that differentiate individual units of a product and which have significance in determining the degree of acceptability of that unit to the user*". Hoffman (1990) described meat quality as the *“sum of all quality factors of meat in terms of the sensoric, nutritive, hygienic and toxicological and technological properties”.* According to Northcutt (1997), the main attributes related to poultry meat that may determine its quality are: the eating quality (appearance, texture and flavor), stability (shelf life, quality retention); wholesomeness (safety, purity), convenience (availability, ease of preparation) and nutritive value (nutrient content, nutrient availability, caloric value). The concept of “quality” includes also views or perceptions about the conditions of animal production in relation to animal welfare, the impact of animal production on the environment and food safety. Production and management practices, from farm to processing plant and the use of technologies to reduce risk factors throughout the production chain will allow the production of better quality poultry meat.

5.1 Sensory attributes: appearance, texture and flavor

5.1.1 Appearance

When consumers buy a poultry meat product if the characteristics do not meet their expectations the product is considered to be of inferior quality. Consumer perceptions are dynamic, and there are often differences between what consumers perceive and their behavior. The most critical feature for the selection of meat product is the appearance (including shape and colour). Consumers select o reject a product based only on its appearance. One of the major contributing components of appearance is the colour. For poultry meat products, colour is important for skin and for meat, in fact, in the mind of the average consumer about to purchase meat, colour becomes synonymous with fresh red meat quality (Renerre and Labas, 1987). In reality, the colour of fresh meat is not well correlated with the eating quality, however, the consumer still demands beef to be a bright cherry-red colour (Taylor, 1996), lamb a brick red colour and pork and chicken an even pink colour (Troy and Kerry, 2010). The colour of meat can be determined visually or using instruments (colorimeters). For the visual evaluation of the meat color, it is necessary to have trained panelists, who evaluate the appearance of meat by using the hedonic scale. The instrumental determination of meat color is more efficient and the methods of reflection or extraction are used to quantify the amount of pigment. The color of foods can be defined as the interaction of a light, an object, an observer and the surroundings of the food. The International Commission on Illumination has described how background can influence the appreciation of color. Instruments used for evaluation of meat color by reflection method are colorimeters, for example, CR Minolta 300 or 400 that work on the principle of meat color comparison in regard to standard color values. The International Commission on Illumination lists three values: CIE L*, a* and b*. CIE L* indicates lightness, where values range from 0 (black) to 100 (white). The value of CIE a* shows redness

while CIE b* indicates yellowness. Negative a* and b* values indicate the appearance of green and blue color of the meat.

Many factors affect poultry meat colour, such as bird strain, age, sex, processing procedures, chemical exposure, cooking temperature (Mugler and Cunningham, 1972). The most important factors that influence the meat colour are the concentration and chemical state of the meat pigments, primarily myoglobin and hemoglobin, and the physical characteristics of meat, such as its light scattering and absorbing properties (Kropf, 1993). The myoglobin concentration of muscle varies between and within species and is affected by factors such as age, exercise, diet of the animal, as well as genetic and environmental factors (Livingston and Brown, 1981). Myoglobin can exist in one of three forms: deoxymyoglobin, oxymyoglobin or metmyoglobin. Interconversion of the three pigment states is possible and the dominant pigment form depends on localized conditions (Kropf, 1993). Deoxymyoglobin, frequently referred to as myoglobin or reduced myoglobin, contains iron in the ferrous (Fe2+) state, it is purplish-red in colour and is responsible for the colour of meat immediately after cutting into a deep muscle, or of meat stored under a vacuum (Renerre, 1990). Oxymyoglobin, a cherry-red form of the pigment, forms very quickly after exposure of deoxymyoglobin to oxygen (Livingston and Brown, 1981). In red meats oxymyoglobin imparts the colour that consumers associate with freshness (Faustman and Cassens, 1990). The colour of red meats is relatively short-lived and both deoxymyoglobin and oxymyoglobin readily oxidise to metmyoglobin, in which the haem iron has been oxidised to the ferric (Fe3+) state. Metmyoglobin is incapable of binding oxygen and is thus physiologically inactive (Faustman and Cassens, 1990). Metmyoglobin gives meat a brown colour which consumers associate with a lack of freshness and unacceptability (Hood and Riordan, 1973). The rate of metmyoglobin accumulation is related to intrinsic factors such as muscle pH, muscle fiber type and the age, breed, sex and diet of animals, as well as extrinsic factors such as pre-slaughter treatment of animals and hot-boning, electrical stimulation and chilling of carcasses. Besides, during retail display

environmental factors such as temperature, oxygen concentration, type of lighting, microbial growth and packaging storage atmosphere all influence the shelf-life and potential retail sale of meat.

Skin colour is most critical for the marketing of fresh whole birds or parts. As reviewed by Fletcher (2002), consumers generally prefer broiler skin colours ranging from white, to pale yellow, to deeply pigmented based on traditional regional supplies. Thus consumers tend to favor skin colours which were traditionally available and which were based on local feeding practices as well as genetic stock (Fletcher, 2002). In modern markets, consumers still tend to favor their traditional market forms: in the North Italy deeply pigmented birds are the most desired, because a pale color of the skin makes them think to an animal in a bad state of health; while in the South Italy pale skin colour is preferred because the yellow color of the skin gives an impression of fatty meat. Pigmentation depends upon the genetic capability of the bird to deposit carotenoid pigments, dietary source of pigments, health of the bird, and processing (Fletcher, 1989). In last decades, the importance of the skin colour is decreased because the demand for skinless products, deboned meat and fully cooked products is increasing.

Other visual defects that can affect the appearance of the meat are the bruising and the hemorrhages. These defects are not associated with the pigments, chemical property of the skin or meat, but they are due to physical trauma (bruising) or to capillary or blood vessel rupture (hemorrhages). The bruising affect the colour of meat because the damaged tissue initially appears darken/blue black, then green and finally green and yellow. Hemorrhages affect negatively the appearance because the consumer notes a blood accumulation. If these visual defects are severs the product will be rejected by the consumer. Factors identify to affect bruising and hemorrhages include litter conditions, disease, mycotossins, season, stress, vitamins, holding condition, picking, electrical stunning and killing. Also the diet influence the presence of hemorrhages: Tung et al., (1971) showed that feed aflatoxins can result in capillary fragility and increased incidences of hemorrhages.

5.1.2 Texture

Texture is a complex set of characteristics that includes everything from thickness and gumminess to chewiness. One of the most important textural properties in meat is tenderness, which is defined as the ease with which a piece of meat can be cut and chewed. Juiciness (succulence), a property related to the fat and moisture contents of meat, and water holding capacity (WHC) are also important textural components in meat. Tenderness is the most important attribute that strongly influence consumer purchase for a poultry product. Many external factors contribute to the broad variation in meat tenderness. These factors can be related to the bird and environment, processing conditions, and cooking methods. Age, strain, and sex as well as environmental and nutritional stresses have all been shown to influence the variation in tenderness among meat samples (Owens *et al*., 2004). The intrinsic factors that contributing to poultry meat tenderness include the composition and contractile state of muscle fibers, the amount and the maturity of connective tissue (Fletcher, 2002) and the extent of proteolysis in rigor muscle (Joo et al., 2013). The contractile state of the muscle (myofibrillar component) is probably the most important influence in meat tenderness in market aged (6- 8 weeks) broilers (Owens, 2014). The contractile state of the muscle can be determined by measuring the length of the sarcomeres, with shorter sarcomeres length being strongly correlated with tough meat (Herring et al., 1967; Locker, 1960). The contractile state of the myofibrillar proteins depend on the rate and severity of *rigor mortis* development. Slaughtering of a bird stops blood circulation which in turn blocks supply of oxygen and energy resources (glycogen, ATP, CK) to the muscles. The resultant biochemical processes provoke significant changes in the structure of muscles resulting mainly in the denaturation of muscular proteins: muscles run out of energy, contract and become stiff. This stiffening, called *rigor mortis*, is followed by softening again making meat tender when cooked (Northcutt, 2009). Shrimpton (1960) found that under commercial conditions *rigor* can be expected

to develop within 10 minutes of slaughter unless the birds have been specially starved. Besides, he argued that adverse conditions during the life of the bird may negatively affect tenderness of the meat and may also be accentuated by bad practice in the packing station (Shrimpton, 1960). The major factors affecting *rigor* development and accelerated processing are *ante mortem* handling, electrical stunning, gas stunning and electrical stimulation. The stress due to the stunning conditions significantly affecting degradation of glycogen resources in muscle tissues (Raj et al., 1990; Raj and Johnson, 1997). Raj et al. (1990) found that after argon stunning broiler meat was more tender in comparison to electrical stunning, despite accelerated glycolysis. They assumed that in argon stunning, anaerobic glycolysis may start before the broiler has died, and that the anoxic convulsions occurring during stunning will accelerate the onset of *rigor mortis*. A possible explanation for this is that wing flapping in broilers is an act very similar to that of flight and the breast muscles are morphologically designed to cope with it, irrespective of its severity over a short duration. On the contrary, the tonic muscular spasm induced by electrical stunning appears to be relatively more detrimental to meat quality (Raj et al., 1990).

Maturity of the connective tissue involves the chemical cross bonding of the collagen in the muscle. Collagen is the major component of the intramuscular connective tissue. Since collagen cross-linking increases with age, meat is generally tougher from older animals (Fletcher, 2002). Today, broiler chickens are slaughtered in 6-7 weeks to meet the needs of consumers, so the age-related toughness problem (connective tissue cross-linking) has disappeared. According to Roy et al. (2006) age-related changes in collagen content are very small in chickens compared to mammalian species, but supplying high dietary energy enhances the deposition of insoluble collagen. In the study of Roy et al. (2006) it was reported that chickens fed high energy diets had a thick perimysium with large collagen fibers composed of compact accumulated fibrils. The authors suggested that the rapid rate of growth of the pectoral muscle, induced by the feeding of high nutritional value diets, was accompanied by myofibre

hypertrophy and by the development of large bundles or perimysal collagen fibers. Therefore, this can lead to an increase in the toughness of the meat.

5.1.3 Flavor

Flavor is another important factor in terms of meat quality. Bloody, metallic, and salty taste is generally a unique characteristic of fresh uncooked meat. However, chicken meat is more susceptible for quality deterioration mainly due to lipid oxidation and resulting off-flavours because chicken meat contains higher levels of unsaturated fatty acids compared to red meat (Hunton, 1995; Jayasena et al., 2013). This off-flavour development is supposed to be one of the main problems regarding the quality of the chicken meat. However, significant changes take place in the flavor of meat during cooking. The main reactions involved during cooking that are responsible for flavour development are Maillard reaction, thermal degradation of lipids and Maillard-lipid interactions (Brunton et al., 2002). Flavor gets developed during cooking through complex reactions between components found in raw meat combining with heat. More than 1,000 chemicals have so far been identified in the volatiles of different muscle foods (Shahidi et al., 1994). Majority of the volatile compounds identified in cooked poultry meat, have been recognized in chicken (Brunton et al., 2002). However many of these have little influence on flavor of meat and relatively few make a key contribution to the odor and flavor of cooked meat (Aliani and Farmer, 2005).

Findings of researches conducted over years have shown that several *pre*- and *post-mortem* factors affect the flavor of chicken meat. The main determinants that are considered are the strain of the chicken, diet of the bird, presence of free amino acids and nucleotides, irradiation, high pressure treatment, cooking, antioxidants, pH, sex, and ageing (Farmer, 1999; Jayasena et al., 2013). Effect of sex on chicken meat flavor was demonstrated by many researchers. Meat from male birds received higher scores for flavor as opposed to that from female birds (Ramaswamy and Richards, 1982; Farmer, 1999). However, it was also shown that the breast and leg meat of female birds were preferred to those of male

birds. Many other researchers reported no significant relationship between the two parameters (Farmer, 1999). The diet of the bird also plays a vital role towards the flavor of chicken meat (Fanatico et al., 2007; Perez-Alvarez et al., 2010). Diet can either positively or negatively influence the flavor of chicken meat. Corn-fed chicken and arachidonic acid enriched diet fed chicken have produced tastier meat (Lyon et al., 2004; Kiyohara et al., 2011; Takahashi et al., 2012) while diets supplemented with fish meal have negatively affected the flavor of chicken meat (Poste, 1990). *Post-mortem* aging results in many chemical flavor compounds including sugars, organic acids, peptides, free amino acids (Yano et al., 1995; Spanier et al., 2004) and thereby leads to increased flavor. Cooking plays a vital role in flavor development and it affects the acceptability and volatile flavor components of poultry meat (Sanudo et al., 2000). However, roasting, grilling, frying, and pressure cooking generates many pyrazines, pyridines, pyrroles and thiazoles compared to boiling of chicken meat (Shi and Ho, 1994).

5.2 Technological quality: Water Holding Capacity (WHC) and pH

Water-holding capacity (WHC) of fresh meat determines the visual acceptability thus influencing the consumers' willingness to purchase the product. WHC is defined as the ability of meat and meat products to bind water (Pearce et al., 2011) during slicing, mincing, and pressing and also during transport, storage, processing, and cooking (Hamm, 1986). The ability of meat to bind water is a complex trait that is influenced by structural and biochemical changes that occur during the transformation of muscle to meat by the process of *rigor mortis* (Bowker and Zhuang, 2015). Fresh muscle is approximately 75% water by weight. Water in muscle is classified as "bound", "immobilized" or "entrapped", and "free water" (Offer and Knight, 1988). Bound water has reduced mobility and is resistant to freezing and heating (Fennema, 1985). Immobilized water makes up to 80% of the water in muscle and is held within the myofibrillar structure,

between the myofibrils, and between the myofibrils and the sarcolemma (Offer and Cousins, 1992). This water does not easily leave the structure but can be removed by drying and be lost during the *rigor* process and by changes in the physical protein structure, e.g., through protein degradation or denaturation. Due to changes in muscle structure and pH that occur during the transformation of muscle to meat, immobilized water can escape from the muscle along with the free water as drip loss (Offer and Knight, 1988). Free water is water whose flow from the tissue is unimpeded. Weak surface forces mainly hold this fraction of water in meat. Free water is not readily seen in pre-*rigor* meat but can develop as conditions change that allow the entrapped water to move from the structures where it is found (Huff-Lonergan, 2005).

It has been found that predominantly, WHC varies with water in this extramyofibrillar fraction, as well as through the loss of intramyofibrillar water through contraction. The extramyofibrillar water, and a small fraction of the intramyofibrillar water, is easily mobilized such as by myofibrils and cells shrinking during the *rigor* process, but this water does not flow freely in pre-*rigor* or high ultimate pH meat (Pearce et al., 2011).

The main determinants of WHC in meat are the muscle PH and the protein denaturation (Offer and Knight, 1988). As muscle pH decreases with the progression of *post-mortem* metabolism, the net charges of the muscle proteins are reduced. This decrease in net protein charge results in diminished WHC due to the availability of fewer charged protein sites for binding water and because the lack of repulsive charges allows muscle proteins to become more closely packed, which forces more of the immobilized water into the free water compartment, with consequent loss of water from the meat as drip, exudate, or purge. Meat of high ultimate pH does not undergo shrinkage in the myofibrils and muscle cells *post-mortem*. In contrast, meat of low ultimate pH with denatured proteins has excessive shrinkage in the myofibrils and muscle cells. The isoelectric point, defined as the point of minimum charge, of the myofibrillar proteins occurs at pH 5.0-5.2. As the ultimate or final pH approaches the

isoelectric point of the muscle proteins, the WHC of the muscle reaches a minimum. Meat that reaches a low pH of 5.2-5.3 (i.e., large extent of muscle pH fall *post-mortem*), will lose more fluid and have lower WHC. Conversely meat that only has a small drop in pH (low extent of pH fall, pH does not drop very far *post-mortem*) will lose less fluid due to the high ultimate pH.

pH has a direct bearing on the meat quality attributes such as tenderness, water-holding capacity, colour, juiciness and shelf life. The pH of broiler meat is the function of amount of glycogen in the muscle prior to slaughter and the rate of glycogen conversion into lactic acid after slaughter. Identification of colour is an easy way to determine the pH of meat. If the meat is very dark, it will have a high pH and if it is very light, it will have a low pH (Anadon 2002). The variations in breast meat colour, mainly due to pH effects, were shown to affect shelf life, odour development, moisture pick up in marination, drip loss, water holding capacity and cooking loss (Allen et al. 1998).

5.3 Chemical composition and nutritional content of poultry meat

Meat is a concentrated nutrient source, previously considered essential to optimal human growth and development (Higgs, 2000). Although some epidemiological data has revealed a possible association between its consumption and increased risk of several forms of cancer, cardiovascular and metabolic diseases, meat consumption has been important in human species evolution, especially the brain and intellectual development (Pereira and Vincente, 2013). Consumers demand a protein supply that is wholesome, nutritious and affordable. Poultry meat is economical and quick and easy to prepare and serve. It also has a number of desirable nutritional properties. In general, animal source foods can provide a variety of micronutrients that are difficult to obtain in adequate quantities from plant source foods alone. Animal meat is a particular rich source of micronutrients such as vitamin A, vitamin B12, riboflavin, calcium, iron, and zinc. Negative health outcomes associated with

inadequate intake of these nutrients include anemia, poor growth, rickets, impaired cognitive performance, blindness and neuromuscular deficits. Nutritionally, people eat poultry meat for its high content of high-quality protein. Chicken meat is slightly higher in protein and slightly lower in fat than beef and other red meats. Additionally, protein is a rich source of all the essential amino acids. However, eating chicken with the skin on doubles the amount of fat and saturated fat in the dish. For this reason, chicken should best be skinned before cooking. Chicken consumption is increasing as people look for alternative ways to reduce fat such as cholesterol in their diets. Chicken also provides vitamins B6 and B12, iron, zinc, and phosphorus.

5.3.1 Protein

The nutritional value of proteins is determined first by their content of essential amino acids and their digestibility. Currently, the amino acid composition of a protein as determined by chemical analysis compared with that of a reference amino acid pattern. The score obtained from this comparison is corrected for protein digestibility. Animal foods in general are considered to be foods with high protein qualities. The human body needs 20 different amino acids, nine of which are called *essential* because the body cannot make them and must get them in the diet. Essential amino acids for adults are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Additionally, children need arginine. Food proteins that supply all the essential amino acids in the proportions needed by the body are called *complete*. Animal foods are considered to have high protein qualities, although their qualities are not always similar because of differences in essential amino acids. The higher quality of animal protein is due to the high lysine and methionine content (Edmonson and Graham, 1975; Jenkins and Mitchell, 1989). Even if the non-essential amino acids can be produced by the human body, it is mandatory to have all the raw materials necessary for their production. The nutritional value of

each food can be determined by the quantity and the quality of the several amino acids present or absent.

The low content of collagen is another favorable characteristics of poultry meat, and high levels of this protein in muscular meat are associated with a lower percentage of digested product per unit of time (Marangoni et al. 2015).

5.3.2 Fats

Meat is generally high in fat particularly saturated fatty acids and its consumption therefore is associated with an increased risk of diseases incidence. The dietary target for fats in the general healthy population ranges from 25 to 35% of total energy. When consumed in appropriate quantities, compatible with a healthy balanced diet, fat plays a number of important roles: it provides 'essential fatty acids' (such as linoleic and alpha-linolenic acids) and lipophilic vitamins (A, D, E, and K); it represents a major source of energy; it promotes a sense of satiety due to slowing effects on gastric emptying; it reduces, for the same reason, the bioavailability of carbohydrates (and, hence, the glycemic response); and it enhances the taste, smell, and texture of foods. Lipid intake associated with poultry meat is variable and dependent on the cut considered. Fats are mainly found in skin. The lipid content of chicken is around 1% in the leanest cuts, such as chicken breast, and around 17% in cooked chicken wings with skin.

From a nutritional point of view, the composition of poultry fat is favorable: it includes significant amounts of monounsaturated fatty acids and, in comparison with bovine, ovine, or pig meat, substantial amounts of polyunsaturated fats, especially the omega-6 or n-6 linoleic acid (18:2 n-6) and arachidonic acid (20:4n-6), which can be found mostly in the skin (Hibbeln et al., 2006).

5.3.3 Fatty acids composition

Dietary guidelines have recommended avoiding saturated fat in order to prevent cardiovascular disease (Krauss et al., 2000) leading to a significant decrease in

the consumption of animal products especially meat. The mechanisms through which saturated fat exert pejorative effects in cardiovascular and general metabolic health are diverse. Kennedy et al. (2009) have proposed that an excessive consumption of saturated fatty acids could promote white adipose tissue expansion and hypertrophy leading to apoptosis. These phenomena would promote the release of inflammatory proteins like cytokines and chemokines inducing inflammation and insulin resistance, thus increasing the risk of cardiovascular disease and metabolic syndrome (Willerson and Ridker, 2004). Considering the important role of meat in the human diet and the estimated consumption growth through the years, and the concerns related to its role in human health, several studies have focused on ways of improving meat fatty acid composition. Kishowar et al. (2004) have studied the fatty acid composition in chicken breast from different production regimes and have found that the dominant saturated fatty acids were palmitic acid (C16:0; 21 to 24%) and stearic acid (C18:0; 15 to 17%). Myristic acid (C14:0) contents ranged from 0.40 to 1.02%. Among the monounsaturated fatty acids, the dominant fatty acid was oleic acid (C18:1), then palmitoleic acid (C16:1) and gadoleic acid (C20:1). With regard to polyunsaturated fatty acids (PUFA), linoleic acid (C18:2 *n*-6 LA) was dominant, at 16.1%. Arachidonic acid (C20:4 *n*-6 AA) was found over the range 1.5 to 5.6%. The dominant *n*-3 fatty acid was found to be α-linolenic (C18:3 *n*-3 LNA), with a range of 1.15 to 2.51%. The least abundant *n*-3 fatty acid was eicosapentaenoic acid (EPA) (C20:5 *n*-3), comprising 0.24 to 0.96%. The contents of docosahexanoic acid (DHA) (C22:6 *n*-3) varied from 0.67 to 3.35%. The *n*-6/*n*-3 ratios varied from 3.37 to 11.35. The consumption of chicken breast contributes to a healthy diet through its low fat content (1.15%), cholesterol (245 to 627 mg/kg), the atherogenic myristic acid (C14:0), and greater contents of total PUFAs, particularly the beneficial *n*-3 PUFAs, notably EPA and DHA. The importance of a relatively high intake of (PUFAs) in human nutrition is now generally accepted; PUFA should constitute 7% of total energy consumed (Ralph, 2000). The dietary fatty acid modification has proved to be a feasible method of

adding value to poultry products for the health-conscious consumer (Hargis et al., 1993). Those fatty acids that are deemed to be essential for human consumption can be found in PUFA. Because of the association with a decreased risk of coronary heart disease, recent dietary fat studies have centered on the manipulation of specific PUFA. The quantitatively and qualitatively most important metabolites of LA and LNA are arachidonic acid, eicosapentaenoic acid, and docosahexanoic acid. DHA and AA are the major PUFAs in the membranes of brain and retinal cells and have an impact on neuronal functions (Alessandri et al., 2004). As a dietary staple, chicken muscle should ideally provide the essential fatty acids (Kishowar et al., 2004).

Besides, the anti-inflammatory, antithrombotic, antiarrhythmic, and immuno-modulating properties of EPA and DHA can be helpful in the prevention of atherosclerosis (Moreno and Mitjavila, 2003), coronary heart diseases (Yaqoob, 2004), hypertension, inflammatory (Calder, 2001) and autoimmune disorders (Zamaria, 2004), cancers (Terry et al., 2004), and diabetes (Nettleton and Katz, 2005). Both *n*-3 and *n*-6 fatty acids make powerful substances in the body that play key roles in the structure and function of every cell and, ultimately, in health and well-being (Harbige, 2003).

5.3.4 Cholesterol

In addition to fatty acids, cholesterol is a nutritionally important component of meat. Knowledge of cholesterol content is important especially in poultry because the consumption of poultry is currently increasing based on recommendations regarding healthy nutrition. Cholesterol content of raw and cooked meat and poultry products ranges from 40 to 90 mg/100 g (Bragagnolo, 2009). Therefore, a serving of 100 g of poultry meat contributes 18 to 28% of dietary cholesterol limit per day (Komprda et al., 2003). It is difficult to compare cholesterol content of poultry to that of beef and pork because poultry products sometimes contain skin, which is high in cholesterol (approximately 80 to more than 100 mg/100 g; Bragagnolo, 2009). In general, raw poultry meat has

approximately 27 to 90 mg cholesterol/100 g and cooked poultry meat contains around 59 to 154 mg/100 g (Bragagnolo, 2009). Tavaniello et al. (2018) reported that the muscular cholesterol content ranging from 46.74 to 49.57 mg/100 g. A significant factor affecting cholesterol content of poultry is type of retail cut because of the difference between dark and white chicken meat and the presence of skin in many retail cuts. Poultry skin has the greatest cholesterol concentration compared with poultry meat or poultry fat. Cholesterol content of visible fat and breast meat is similar to or lower than that of dark meat (Komprda and al., 2003). Moreover, the difference in cholesterol content between white and dark poultry meat is more pronounced than that between white (predominantly glycolytic) and red (predominantly oxidative) muscles in beef and pork (Browning et al., 1990).

Cholesterol, cholesterol metabolites, and immediate biosynthetic precursors of cholesterol play essential roles in cellular membrane physiology, dietary nutrient absorption, reproductive biology, stress responses, salt and water balance, and calcium metabolism. Cholesterol is a precursor for the synthesis of steroid hormones, bile acids, and vitamin D (Russell et al., 1992). The human body manufactures around 1 g of cholesterol each day and approximately 20-25% of total daily cholesterol production occurs in the liver (Lewis et al., 2006). Synthesis of cholesterol is a series process and starts with acetyl CoA and acetoacetyl-CoA, which are hydrated to form 3-hydroxy-3- methylglutaryl CoA (HMG-CoA). This molecule is subsequently reduced to mevalonate by the enzyme HMG-CoA reductase (Hampton et al., 1996). This is the regulated, rate-limiting, and irreversible step in cholesterol biosynthesis and is the target of action for statin drugs (HMGCoA reductase competitive inhibitors) (Barrios-Gonzales et al., 2010). Both dietary cholesterol and synthesized *de novo* are transported by lipoprotein particles through the circulatory system. The four major types of lipoproteins are chylomicron, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Chylomicrons and VLDL deliver triacylglycerol to cells in the body, whereas LDL delivers cholesterol to cells in the body.

Meanwhile, HDL is involved in reverse cholesterol transport. The synthesis and utilization of cholesterol must be tightly regulated in order to prevent over-accumulation and abnormal depositing within the body. There are two manifestations of cholesterol disorders, hyperlipidemia and hypolipidemia. The reasons for cholesterol disorders include dietary issues, genetic disorders, and other diseases (Khosla et al., 1993; Porter et al., 2011; Pollin et al.,2013). The level of cholesterol in the body being too high or too low may cause varies symptoms, syndromes, or diseases. Excessive cholesterol is associated with several cardiovascular diseases and such levels are easily attained with an unhealthy diet. In fact, it should be noted that it is not essential for cholesterol to be obtained from one's diet as it is easily synthesized in the body. Whereas, low cholesterol is associated with mental disorders, neuropsychiatric diseases, and mortality in elderly (Martinez- Carpio 2009).

However, there is little doubt that the disease process responsible for the leading cause of death in the industrialized world, atherosclerosis, is a disorder in which an excess of cholesterol is a major culprit. The relationship between plasma cholesterol concentration and atherosclerosis was already demonstrated in rabbits in 1913 (Vance and van den Bosch, 2000), however, it is questionable whether the relationship between dietary cholesterol and heart disease is significant, since the endogenous cholesterol synthesis in liver is three times higher than amounts usually consumed (McNamara, 1995), which lead to weakening of the importance of the dietary cholesterol and increasing interest in total dietary energy intake, saturates, monounsaturated and polyunsaturated fatty acid intake and the PUFA n-6/n-3 ratio in foods.

5.3.5 Vitamins

Meat represents an excellent source of the majority of hydrophilic vitamins. Vitamins, a heterogeneous group of substances, are vital nutrients that must be obtained from the diet. With the exception of vitamin D, they cannot be produced by the body. Thirteen substances, divided into two categories, are

recognized as vitamins: the fat-soluble vitamins, of which there are four (vitamins A, D, E, and K) and the water-soluble vitamins, of which there are nine (vitamins C, B1, B2, B6, B12, niacin, pantothenic acid, and biotin). The water-soluble vitamin group contains eight vitamins collectively known as the B-complex vitamins, plus vitamin C. Poultry provides vitamins B2, B6, and niacin (Borenstein, 1981). Riboflavin (Vitamin B2) is the most widely distributed of all the vitamins and is found in all plant and animal cells, although there are relatively few rich food sources. It is present naturally in foods in two bound forms as coenzymes: riboflavin mononucleotide and flavin adenine dinucleotide. These coenzymes participate in numerous metabolic pathways, including the citric acid cycle and the β-oxidation pathway, which breaks down fatty acids. Chicken meat is a moderately good source of riboflavin and thiamine. Differences have been found in the thiamine and riboflavin contents in breast and thigh from broiler meat according to age, sex, and the part being examined (Singh and Essary, 1971).

Vitamin B6 (pyridoxine) activity is shown by three compounds, pyridoxol, pyridoxal, and pyridoxamine, and these are often considered together as pyridoxine. Vitamin B6 is found in red meat, liver, poultry, milk, and green vegetables. The vitamin B6 coenzyme pyridoxal phosphate supports different enzymes involved in reactions that include the transfer of amino groups (NH_2), carboxyl groups (COO^- or COOH), or water (as H and OH). These enzymes support protein metabolism, blood cell synthesis, carbohydrate metabolism, and neurotransmitter synthesis. The term *niacin* is generic for both nicotinic acid and nicotinamide (niacinamide) in foods. The two forms have equal vitamin activity; they are present in a variety of foods and are also available as commercial isolates. Niacin occurs naturally in poultry, meat, and liver of hoofed animals. The niacin precursor tryptophan is found in protein-rich animal foods. To convert tryptophan to niacin in the body, it needs riboflavin, pyridoxine, and iron. A deficiency of any one of these nutrients decreases tryptophan to niacin; a deficiency of any one of these nutrients decreases tryptophan conversion. The

niacin coenzymes play key roles in oxidation–reduction reactions. Many metabolic pathways that promote the synthesis of new compounds, such as fatty acids, rely on reduced nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is concentrated in cells (such as liver cells) that make up large amounts of fatty acids.

5.3.6 Minerals

Poultry meat also provides several minerals. Minerals are inorganic substances, present in all body tissues and fluids and their presence is necessary for the maintenance of some physicochemical processes which are essential to life. They play important roles in many activities in the body (Malhotra, 1998;). Minerals may be broadly classified as macro (major) or micro (trace) elements and as ultra-trace elements. Minerals act in the body in three many ways: as structural components (e.g., calcium, phosphate, and magnesium in bones and teeth); in organic combinations as physiologically important compounds (e.g., phosphorus in nucleotides, zinc in enzymes such as carbonic anhydrase, iodine in thyroid hormone) and in solution in body fluids to maintain pH, helping to conduct nerve impulses and control muscle contraction (e.g., sodium and potassium in blood and intracellular fluids). Mineral elements play important roles in health and disease states of humans. For example, iron insufficiency is probably the most common nutritional deficiency in the world. Even among the inhabitants of well-fed developed countries, it continues to be common, especially in women (Looker et al., 1997). Iron has two major roles in human physiology. As a component of hemoglobin, the pigment of blood and myoglobin in muscle, iron atoms combine reversibly with oxygen to act as its carrier from the lungs to the tissues. In a variety of enzymes (e.g., the cytochromes), iron atoms, present in the ferrous and ferric states, interchange with gain or loss of an electron, as part of the electron chain responsible for the redox reactions necessary for release of energy in cellular catabolism and the synthesis of large molecules (Brock et al., 1994). In addition to its major functions in oxygen transport and as a cofactor in

many enzymes, iron also plays an important role in the immune system. Although the mechanisms involved are complex, there is good evidence that an abnormal iron nutritional status can lead to impaired immune function, with serious consequences for health (Walter et al., 1997). One of the richest sources of dietary iron is animal offal, especially liver. Other animal products, in particular red meat, are also rich in iron. This iron is organically bound heme iron, which is easily absorbed. Trace elements of significance to people are zinc and selenium. Zinc is ubiquitous within cells, the role of zinc in biology can be grouped into three general functional classes, namely catalytic, structural and regulatory functions (Cousins et al., 1996). Zinc content in mixed dark and white chicken meat, reported by Bou et al. (2004, 2005), falls within a range of 8.5 to 9.0 mg/kg of the edible portion, and 100 g provides, roughly 6% of the daily value. Selenium is a component of several seleno-proteins with essential biological functions (Van Cauwenbergh et al., 2004). This element acts as a cofactor of the GPx family of enzymes which protect against oxidative stress. Specifically, Selenium dependent GPx enzyme recycles glutathione, reducing lipid peroxidation by catalyzing the reduction of peroxides, including hydrogen peroxide. Selenium is widely distributed, but normally at levels of less than 1 mg/kg, in most foods. The richest sources are organ meat, such as liver (0.05 to 1.33 mg/kg), muscle meat (0.06 to 0.42 mg/kg), and fish (0.05 to 0.54 mg/kg) (Jorge Soriano Santos, 2010).

Chapter 6

6. Poultry meat quality defects

Throughout the world, poultry meat consumption continues to grow, both in developed and in the developing countries. By 2020 this trend is expected to continue to grow, so that poultry meat will become the consumers' first choice (Bilgili, 2002). Such good assessment of poultry meat is influenced by many factors, such as short fattening duration, excellent space utilization, high reproductive ability of poultry, excellent feed conversion, satisfactory nutritional value of poultry meat and relatively low sales prices. Nowadays poultry is fattened in an intensive way and they have been subjected to intense genetic selection for rapid lean muscle growth. The selection traits used are often related to economic importance, rather than to biophysical and biochemical significance. The selection practices to achieve maximal growth performance often results in stress syndromes and myopathic conditions (Solomon et al., 1998; Petracci et al., 2010). It is generally recognized that this selection for muscle growth has resulted in poorer water-holding capacity of breast muscles during processing and storage (Dransfield and Sosnicki, 1998). These have also resulted in the production of animals that are much more susceptible to stress and consequently the development of meat quality defects such as Pale Soft Exudative (PSE- like) and Dark Firm Dry (DFD- like) meats. At the present, a high increase of quality issue such as white striping (WS) (Kuttappan et al., 2012 a; Petracci et al., 2013), spaghetti meat (SM) (Petracci and Cavani, 2012), wooden breast (WB) (Chatterjee et al., 2016) and Oregon disease has been observed at the poultry-industry level.

6.1 Pale soft exudative (PSE-like)breast meat

Increased stress susceptibility often gives rise to pale soft exudative (PSE) muscle (Figure 7). It is defined in connection with the pH of meat at a specific time after slaughter. PSE is said to have occurred when the pH of meat is < 6 at 45 minutes

after slaughter. The term PSE was originally used to define pork meat characterized by light color, flaccid texture, poor water-holding capacity and substantially reduced cooking yield. In swine, a genetic single mutation in the ryanodine receptor of the sarcoplasmic reticulum involved in calcium release has been identified and it has been associated with animals that are stress-susceptible and prone to developing PSE meat (Barbut et al., 2005; Picard et al., 2010). Acute or short term stress that can lead to PSE include the use of electric goads, fighting among animal just before sticking, beating of animals prior to slaughter and overcrowding in the lairage. Acidification occurs in muscles *post-mortem* due to the breakdown of glycogen to lactic acid. In PSE meats, the rate of acidification after slaughter is stimulated faster than normal and lower pH values are reached in the muscle when the temperature of the carcass is still high. The combination of low pH and high temperature in PSE meat causes the denaturation of some muscle proteins leading to reduction in their water holding capacity. This happens because the myofibrillar components expel the resulting fluid into the extracellular space which increases in volume (Warriss, 2000). When such meat is cut the fluid is released resulting in the exudates. A large amount of exudates reflects poor water holding capacity as found in PSE meats. Warriss (2000) explained that, light scattering from meat surface is probably due to differences in refractive indices of the sarcoplasm and myofibrils. The larger the difference, the higher the scattering and the paler the meat appears. The shrinkage of the myofilament increases the amount of light reflected from the meat. At high scattering the amount of absorbed light is low and the haem pigments selectively absorbed green light, thus reducing the normal red color. This makes PSE meat less red and more yellow. The low pH in PSE also promotes the oxidation of haem pigments from purple or red myoglobin (Mb) and oxymyoglobin (MbO2) to brown metmyoglobin (met Mb).

In literature the existence of PSE also in poultry meat has been reported (Barbut, 1998; Sosnicki, 1998). Several studies have been conducted to define the main causes of PSE-like meat condition. These studies show that the modern rapidly

growing strains of meat poultry exhibit an elevated incidence of spontaneous or idiopathic myopathy and an increased susceptibility to stress-induced myopathy (Mitchell, 1999; Sandercock et al., 2006). Le Bihan-Duval et al. (2001) found correlations for meat quality and body traits in broilers (Le Bihan-Duval et al., 2001). They found that selection for growth and muscle development did not alter the pH of the meat but weakly modified its color by decreasing the redness (a*) and yellowness (b*). Berri et al. (2007) suggested that selection for increased muscle yields and against fat deposition exerted cumulative effects on muscle metabolism, decreasing glycogen storage and thereby reducing the extent of *post-mortem* acidification. As a consequence of higher ultimate pH, the WHC and processing ability of the meat was improved (Berri et al., 2007). Other authors explain that these pathologies are attributable to alterations in intracellular calcium homeostasis (Sandercock and Mitchell, 2003; Sandercock et al., 2006) and consequent changes in sarcolemmal membrane integrity and may result from excessive myofiber hypertrophy and inadequate development of support tissues and vascular supply (MacRae et al., 2006). According to Petracci et al. (2004), also environmental factors may induce PSE-like meat occurrence, they studied the effect of heat stress during the end of the growing phase, considering 3 different seasons. The authors stated that the incidence of PSE-like meat is higher in hot than in cold seasons (Petracci et al., 2004). During the summer, the broiler breast muscle fillets exhibited significant higher lightness values (53.1) in respect with those obtained during autumn (52.8) and winter (51.3). Moreover, considering an L* cut-off value of 56, PSE-like incidences were 15.5, 11.3, and 2.7% in summer, autumn, and winter, respectively. In order to reduce the incidence of PSE like meat, pre-slaughter stress should be reduced, minimizing the transport time and improving capture operations. Diet could also play a key role by providing small amounts of key nutrients (e.g. Vitamins, minerals, amino acids) before slaughter (Petracci et al., 2009).

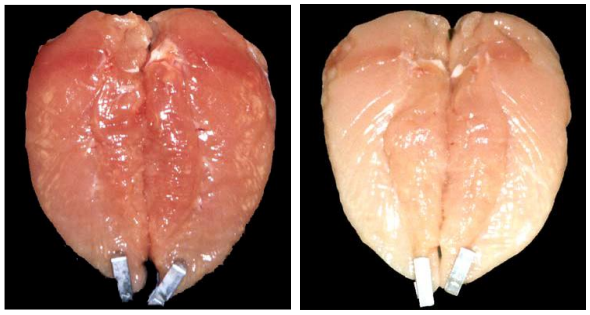

Figure 7. Pale, soft and exudative (PSE)-like broiler breast meat. (Source: Petracci et Cavani 2012).

6.2 *Dark firm dry (DFD-like)breast meat*

This condition occurs in all species depending on how animals are handled pre-slaughter: the most common factor leading to DFD in meat is stress *ante-mortem.* DFD (also known as dark cutting in beef) is when the ultimate pH *post mortem* measured after 12-48 hours is ≥ 6. Red, oxidative muscle fibers have relatively low concentration of glycogen which can easily be depleted *post mortem*. This makes them prone to DFD. When animals are exposed to chronic or long term stress before slaughtering DFD meats can occur. Examples of chronic stress are transportation animals over long distances, long hours of starvation, and overcrowding of animals in the lairage over a long period of time. Chronic stress prior to slaughter leads to the depletion of stored glycogen, thus less glycogen is available *post mortem* affecting the normal process of acidification and leaving the pH of meat high, this condition is referred to as DFD. Warriss (2000) explained that, in DFD condition the high pH results in relatively little denaturation of proteins, water is tightly bound and little or no exudates is formed. This is because there is little or no shrinkage of the myofilament lattice and the differences in refractive index of the myofibrils and sarcoplasm are reduced. The muscles absorbed light making the meat appear darker. Oxygen penetration is reduced by the closed structure and any oxygen reaching the interior is used up by the high cytochrome activity encouraged by the high pH. This results in a thin surface layer of bright red oxygenated myoglobin (MbO_2)

allowing the purple color of the underlying reduced myoglobin (Mb) to show through (Warriss 2000). The prevention of PSE and DFD in meats will rely mainly on measures to avoid stress in animals prior to slaughter. These stresses include removal from their home environment, loading and unloading onto vehicles, feed and water deprivation during transportation, holding in unfamiliar surroundings, mixing with strange animals, lousy odor, high temperature and noise produced by moving vehicles (Warriss, 2000).

6.3 White striping (WS) defect

White striping sarcolemmal integrity is characterized by white striations parallel to muscle fibers on the surface of *Pectoralis major* muscle (Figure 8), due to fiber degenerations with fat infiltration (lipidosis) and connective tissues (fibrosis) (Kuttapan 2013a). White striations appear in the cranial part of the fillet near the wing attachment and may, or may not, extend along the muscle to the caudal region (Ferreira et al., 2014). Kuttappan et al. (2012a) developed a classification system for WS based on visual appearance: normal, moderate and severe. Breasts with a moderate degree present striations with a thickness of less than 1 mm, while the severely affected case have striations with a thickness greater than 1 mm; both easily visible (Kuttappan et al., 2012a). It is possible classified these fillets also using the three-point scale of severity proposed by Bailey et al. (2005): 1 (mild, focal appearance of stripes covering part of the breast), 2 (moderate, stripes extensively covering the breast surface), 3 (severe, very thick stripes with extensive coverage over the breast surface) (Bailey et al. 2015).

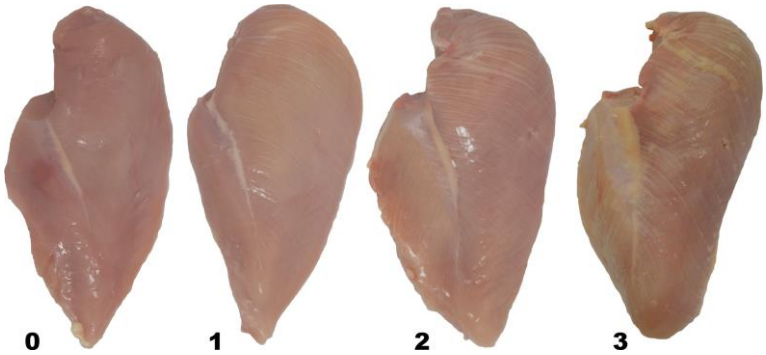

Figure 8. White striping defect in broiler breast meat. Normal breast =0; mild WS breast =1; moderate WS breast =2; severe WS breast =3.(Source Kuttapan et al., 2016)

The causes of WS are still unknown and there are not found sign of systemic infections, inflammatory, or stress conditions, but WS muscle breasts had increased levels of serum creatine kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) (Kuttapan et al., 2013 a). Kuttapan et al. (2013 a) reported that when the damages are acute or continuous, and the attempts to repair the damages fail, satellite cells of the muscle tissues become fibroblasts or adipocytes and lead to fibrosis and lipidosis (Kuttapan et al., 2013 a). The histopathological changes associated with WS are necrosis of fibers, degenerating and regenerating fibers of variable size, loss of cross striations, mineralization of nuclei, hyalinization, replacement of muscle fibers with fibrous connective tissues (fibrosis), accumulation of adipose tissues (lipidosis), and lymphocytes and macrophages infiltration (Kuttappan et al., 2013b,c; Ferreira et al., 2014; Sihvo et al., 2014). In a recent review, Petracci et al. (2019) stated that selection for fast-growing and high-breast development in modern broiler hybrids, achieved through fibers' hypertrophy, resulted in compromised blood and oxygen supply to the muscle tissue leading to the development of hypoxia. The notable development of the *pectoralis major* muscle could compress the pectoral artery reducing oxygenation and nutrients transportation to the muscle. This hypothesis is supported by the evidence that the severity of the presence of WS decreases from the skin-facing surface towards the inner section of the *pectoralis major* (Petracci et al., 2019).

Chicken breasts affected by WS manifest also difference in chemical composition: higher fat and collagen content and lower protein content, higher levels of monounsaturated fatty acids and lower levels of saturated EPA, DPA, DHA fatty acids, compared to normal breasts (Baldi et al., 2019). Moreover, the occurrence of WS on the surface of chicken breasts impaired visual appearance and reduced the consume willingness to buy this type of meat (Kuttapan et al., 2012). The perception of increased fat deposits in the breast fillet is unfavorable for consumers as it gives the impression that the breast fillet is "unhealthy" (Kuttapan et al., 2012). Besides, when the fillets are marketed as tray packs and present different degrees of WS, the whole tray pack could be rejected by the consumer. For these reasons, fillets showing severe WS are downgraded in commercial plants and they are used for manufacturing further processed products, causing severe economic loss to poultry industry (Kuttapan et al., 2012).

6.4 Wooden breast (WB) defect

Wooden breast (Figure 9) is a defect characterized by bulged, hard and rigid muscles with a surface hemorrhage and the presence of a light-yellow viscous exudate on the muscle surface (Mazzoni et al., 2015; Mudalal et al., 2015). It is possible classified the severity of WB in four categories: mild (focally diffused and light firmness), moderate (focally diffused with extensive firmness of the breast), severe (>75% of the breast being extremely firm and with diffuse coverage) and extremely severe (firm breast) (Papah et al., 2017; Sihvo et al., 2017; Petracci et al., 2019).

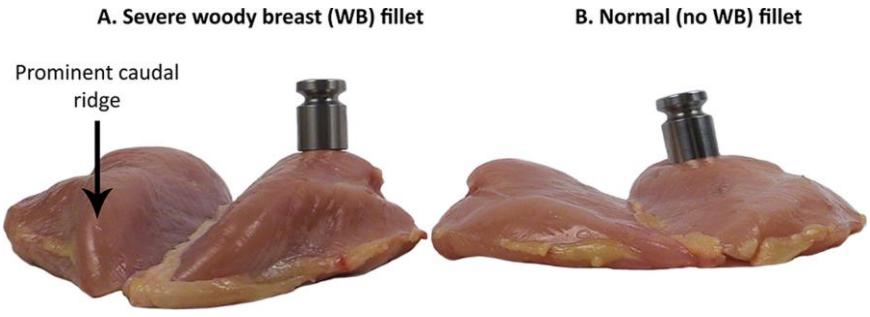

Figure 9. Wooden breast defect in broiler breast meat (Source: Kuttapan et al., 2016)

WB results in the necrosis of muscle fibers with macrophage infiltration. In response to the necrosis, fibrosis takes place, leading to connective tissue synthesis and the replacement of muscle-specific proteins with highly cross-linked collagen. The highly cross-linked collagen gives the muscle its wooden or very stiff phenotype (Velleman, 2015). WB muscle has a larger cross-sectional area, and higher intramuscular collagen and higher ultimate *post-mortem* pH in comparison with normal breast muscle (Dalla Zotte et al., 2014; Petracci et al., 2015; Chatterjee et al., 2016; Clark and Velleman, 2016; Soglia et al., 2016). Histologically, WB is characterized with increased degenerative and atrophic fibers, variability in fiber size, floccular/vacuolar degeneration and lysis of fibers, hyalinization, lipidosis, extensive fibrillar collagen deposition (fibrosis), and macrophage infiltration (Sihvo et al., 2014; Velleman and Clark, 2015; Soglia et al., 2016). The birds with high growth rate, feed efficiency, and breast muscle yield are more likely to develop myopathies including WB (Griffin et al., 2018) because the muscle tissues outgrow the supporting systems such as connective tissues and capillaries and change the structure and metabolism of the muscle (Sandercock et al., 2009; Petracci and Cavani, 2012). Zambonelli et al. (2016) reported that WB muscle showed different genetic expression for glycolysis, oxidation, calcium signaling pathways, and proteoglycan and polysaccharide synthesis from normal muscles. The RNA-seq analysis and the microscopic and biochemical studies indicated that the localized hypoxia, increased muscle degradation, reduced glucose utilization, increased intracellular calcium and muscle fiber-type switching are the critical features of myopathic muscles (Mutryn et al., 2015; Petracci et al., 2015; Abasht et al., 2016). After the initial degeneration, the damages to the sarcoplasmic reticulum surrounding muscle fibers increase calcium influx and activate calcium-dependent protease and initiate necrosis. WB exhibited a significantly higher amount of free calcium. The increase of sodium and calcium content changes the intracellular ion homeostasis, and the increase of glycolytic activity lowers the amount of

glycogen reserve in the muscle, which leads to an increase of ultimate pH in abnormal muscles (Soglia et al., 2016).

Regarding the chemical composition different studies in literature show that in chicken breast with WB there is a significant reduction in protein and ash contents and increase in moisture and lipid levels (Wold et al., 2017; Cai et al., 2018). Soglia et al., (2016) reported that WB breasts showed no difference in saturated, monounsaturated, polyunsaturated and DHA fatty acids if compared to normal breasts, but breasts with WB showed more susceptibility to oxidation, with a higher levels of TBARS content (Soglia et al., 2016).

WB defect also has an adverse effect on the poultry industry, which is facing substantial economic losses due to customer complaints regarding fillets affected by these myopathies.

6.5 Spaghetti Meat (SM) defect

Spaghetti meat (Figure 10) is characterized by poor muscle cohesiveness due to the immature intramuscular connective tissues (Bowker and Zhuang, 2016; Radaelli et al., 2017; Sihvo et al., 2017).

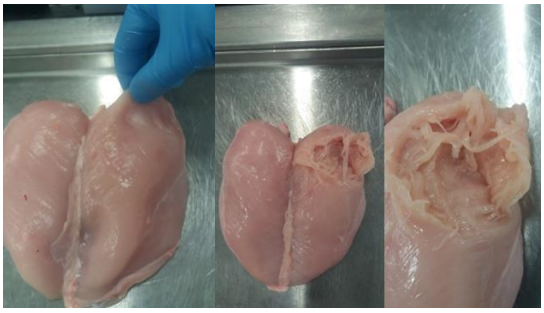

Figure 10. Spaghetti meat defect in broiler breast meat.

Collagen maintains the structural integrity of skeletal muscle (McCormick, 2009; Purslow, 2005): intramuscular connective tissues are composed of three layers called endomysium, perimysium, and epimysium, which surrounds individual muscle fibers, the bundles of muscle fibers, and the whole muscle, respectively.

In SM, the density of connective tissues in endomysium and perimysium progressively decreases, and thus the muscle fiber bundles become easily disintegrated or mushy (look like spaghetti) (Puolanne and Voutila, 2009). Muscles with SM conditions showed severe histological lesions with increased adipose tissue infiltration in the endo- and perimysial spaces and high intraand extra-myofibrillar water content (Radaelli et al., 2017). Using NMR, Baldi et al. (2018) found a movement of water from intra-myofibrillar to extra-myofibrillar area in SM. A higher proportion of extra myofibrillar water in the superficial section of SM led to a reduction of WHC of the meat. They showed also that in SM breast there were a higher concentrations of oxidized proteins than normal breast muscle, supporting a likely influence of the chemical state of meat proteins on their functionality (Baldi et al., 2018). SM had more pronounced adverse effects on meat quality than the WS (Baldi et al., 2018).

6.6 Oregon disease (OD)

Oregon disease (Figure 11), also known as deep pectoral disease, or green muscle disease, was first described in 1968 as “degenerative myopathy” in turkeys (Dikinson et al., 1969) and it was subsequently studied more extensively by Harper and his collaborators at Oregon State University (Harper et al., 1969, 1971, 1975; Harper and Heifer, 1972).

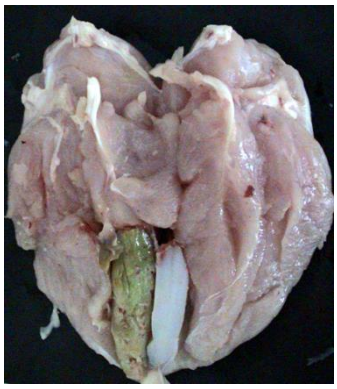

Figure 11. Oregon disease. (Source: Petracci and Cavani, 2012)

In chronic cases, incision of the *superficial pectoral* muscle reveals the partially atrophied supracoracoid muscle with its green, necrotic middle portion enclosed in a thick fibrous capsule (Siller and Wight, 1978). The cut surface of the necrotic muscle is dry and friable, with a characteristic pale green color. The cranial part of the supracoracoid is often normal, and the part caudal to the necrotic portion is pale and distinctly atrophied. OD occurs exclusively in birds that have been selected for breast muscle development (Siller, 1985). It can be both unilateral or bilateral, affecting just one or both pectoralis minor muscles, respectively (Petracci and Cavani. 2012). The cause and pathogenesis of Oregon disease are now well undeerstood, it has been estimated that, in turkeys and broilers, the supracoracoid increases in weight by about 20% during activity for the huge blood flow into the muscle (Petracci and Cavani, 2012). The increased size of the muscle is so marked in the heavy breeds that the muscle becomes strangulated and ischemic, because the increased pressure within the muscle occludes the blood vessels and causes a necrosis of the muscle (Petracci and Cavani, 2012). Upon gross examination and under the microscope, there is no evidence of inflammation, and the green necrotic lesions show the characteristic features of discoid necrosis, interpreted as evidence of ischemia (Siller, 1985). The muscle fibers are split into their individual sarcomeres, because of loss of the Z-bands, and the nuclei are either lost altogether or appear only as faint, so-called "ghost" nuclei (Siller and Wight, 1978). There are no obvious blood vessel lesions, except that the small vessels are filled with lysed red blood cells. Some of the larger arteries within the necrotic lesions may show evidence of aneurysm, but these do not appear to be of causal significance. Under the thick fibrous capsule, which sequestrates the necrotic tissue, the dead muscle is gradually being removed by phagocytosis. The pale atrophic tissue caudal to the necrotic lesion is distinctly different. The muscle fibers are clearly atrophic and sparse, being largely replaced by adipose tissue. Such lesions are known as denervation atrophy and considered to be typical of a loss in nerve supply. Based on histological appearance, the "green necrosis" is due to a circulatory failure, while the

atrophy of the caudal portion results from an interruption of nerve impulse transmission. The lesion does not impair the general health of birds, neither public health significance is associated to OD myopathy, but it is aesthetically undesirable. The necrotic part is removed while the rest of the breast is intended for human consumption, this operation causes the downgrading of the products and produces an economic loss for the industry.

Chapter 7

7. Effect of heat stress on poultry

Climate change, particularly global warming, strongly affects production performances of farm animals and impact worldwide on livestock production (Nienaber et al., 1999). High ambient temperature has been recognized as one major environment factor influencing poultry production (Lin et al., 2006). The optimal temperature for performance is 19 to 22°C for laying hens and 18 to 22°C for growing broilers (Lin et al., 2006). When ambient temperature is higher than the thermo-neutral temperature, heat stress may occur. Heat stress, one of the most important environmental stressors for the animals, is a major source of production loss in the poultry and beef industry and whereas new knowledge about animal responses to the environment continues to be developed, managing animals to reduce the impact of climate remains a challenge (Hahn, 1995, 1999; Sprott et al., 2001; Hahn et al., 2003). According to Selye (1976), "*stress is the nonspecific response of the body to any demand*", whereas stressor can be defined as "*an agent that produces stress at any time*". Therefore, stress represents the reaction of the animal organism (i.e., a biological response) to stimuli that disturb its normal physiological equilibrium or homeostasis (Lara and Rostagno, 2013). The damaging effects of heat stress on broilers range from reduced growth to decreased quality of broiler products. Selection for rapid growth has been associated with increased susceptibility of broilers to heat stress (Cahaner et al., 1995; Berong and Washburn, 1998). The poultry industry is improving in meat production through the intensive genetic selection for the growth rate and the improvement in nutrition and management of broilers, but the rapid rate of muscle growth is not followed by the development of thermoregulatory systems (Havenstein et al., 2003), causing the inability of modern hybrids to regulate body heat with variable environmental temperatures and high metabolic rates (Settar et al., 1999; Deeb et al., 2002). Moreover, birds are more sensitive to high temperatures than other monogastric animals

because of feather coverage and the absence of sweat glands (Loyau et al., 2013). Temperatures >30 °C are conditions for heat-stress for birds (Ensminger et al., 1990). Especially in the hot regions, heat stress is of major concern for the poultry industry because it causes high mortality of broiler chickens (De Basilio and Picard, 2001), reduction in feed intake, body weight, carcass weight, carcass protein and muscle calorie content (Tankson et al., 2001). Feng et al. (2008) observed significant decrease of initial pH and increased L*, drip loss, and shear force of breast muscle in heat-stressed broilers. The expression of heat stress in poultry production can be described as 'acute' or 'chronic'; acute heat stress refers to short and sudden periods of extremely high temperature, whereas chronic heat stress refers to extended periods of elevated temperature. The majority of heat stress studies have investigated the effect of heat stress on physiological response and on meat quality of broilers.

7.1 Physiological response to heat stress

Under high temperature conditions, birds alter their behavior and physiological homeostasis seeking thermoregulation, thereby decreasing body temperature. Birds subjected to heat stress conditions spend less time feeding, more time drinking and panting, as well as more time with their wings elevated, less time moving or walking, and more time resting (Mack et al., 2013). Generally, when subjected to high environmental temperatures, animals for maintaining thermoregulation utilize convective and evaporative heat loss by vasodilatation and perspiration (Mustaf et al., 2009). Birds to promote heat exchange between their body and the environment utilize the air sacs. Air sacs, during panting promote air circulation on surfaces contributing to increase gas exchanges with the air, and consequently, the evaporative loss of heat (Fedde et al., 1998). Increased panting under heat stress conditions leads to an increase in carbon dioxide levels and a higher blood pH, a condition referred as respiratory alkalosis, which in turn hinders the availability of bicarbonate of blood by reducing the

levels of free calcium in the blood. These changes accelerate the rate of metabolism and energy expenditure and the depletion of ATP (Korte et al., 1999; Yahav et al., 2005). Glycolysis and creatine kinase (CK) activity are stimulated under heat stress conditions and are the key for ATP generation in muscle (Mitchell and Sandercock, 2004; Zhang et al., 2012). The metabolic acidosis converts more pyruvate to lactate (anaerobic metabolism) and induces higher dependency to anaerobic metabolism to generate energy in the muscle, which will continue during the early stage of *post-mortem*. So, in heat-stressed chickens, the rate of anaerobic glycolysis to generate energy (ATP) by breaking down muscle glycogen is faster than normal birds. The accelerated lactic acid production induces rapid pH decline while the body temperature is high, which results in PSE-like conditions in meat (Warriss and Brown, 1987; Fernandez et al., 1994; Santos et al., 1994; Wang et al., 2017). The combination of accelerated *post-mortem* glycolysis, which results in a rapid *post-mortem* decline in pH, and the high carcass temperature results in protein denaturation of the muscle that leads to pale meat color, decreased WHC, and poor texture (Alvarado and Sams, 2002; Woelfel et al., 2002; Wilhelm and Maganhini, 2010). The increased activity in the plasma of CK is an indicator of muscle damage (myopathy) and the malfunction of sarcolemma in muscle cells (Ostrowski- Meissner, 1981), which could be associated with the disturbance of intracellular Ca^{2+} homeostasis in muscle. The hyperthermia-associated myopathy is characterized by an increase in the plasma activity of the skeletal muscle derived isoenzyme creatine kinase (CK) and reflects Ca^{2+} mediated alterations in muscle membrane integrity (Mitchell and Sandercock, 1997). The *ante-mortem* alterations induced by heat stress in the permeability of the muscular membrane and the concomitant changes in muscle metabolism in broiler chickens may influence the quality of *post-mortem* meat (Wang et al., 2017). Increase in panting is also associated with a high release of corticosteroid hormones (Zaboli et al., 2019). High concentration of corticosterone hormone (glucocorticoid) increases fat accumulation in abdominal, cervical, and thigh adipose tissues (Cai et al.,

2009;Wang et al., 2012a,b), but stimulates protein degradation and breakdown of skeletal muscle (Scanes, 2016), possibly through the expression of fatty acid transport protein I and insulin receptor in the pectoralis major (Wang et al., 2012b). Song et al. (2011) reported that corticosterone hormones consistently increases the concentration of urate/uric acid in the blood, indicating increased protein catabolism. However, a decrease in thyroid hormone (T3) during heat stress is involved in reduced basal metabolic and physical activities, and redirecting available energy for growth (Zaboli et al., 2017). This extra energy is primarily stored as abdominal and subcutaneous fats in chickens (Ain Baziz et al., 1996). Furthermore, corticosteroid hormones can accelerate production of reactive oxygen species (ROS) (Sato et al., 2010), which are involved in the incidence of PSE-like meat. The changes in body composition due to the increased corticoid hormones can negatively influence the processing quality, which includes higher drip loss, lower marinade uptake, lower protein solubility, higher shear force, and lower cooking yields, of broiler meat (Van Laack et al., 2000; Barbut et al., 2005). Heat stress leads also to oxidative stress, reflected by an increased production of reactive oxygen molecules and decreased concentrations of serum vitamins and minerals that play a role in the defense system (Halliwell and Gutteridge, 1989; Sahin and Kucuk, 2003; Sahin et al., 2009). Oxidative stress in the body occurs when the oxidants exceed the antioxidants, including superoxide dismutase, catalase, glutathione peroxidase, ascorbate, and vitamin E in cells (Star et al., 2009). ROS can alter the functions of muscle components such as enzymes, cause aging and loss of protein functions, and inactivate protein, deoxyribonucleic acid, and ribonucleic acid (Zablocka and Janusz, 2008). In living animals, oxidation is typically initiated by ROS, which are generated by cellular metabolism as well as external sources, including feed that contain oxidized fats and lipids (Cadenas and Davies, 2000). The primary source of ROS in broiler muscles is the leakage of electrons from the respiratory chain in mitochondria during the conversion of molecular oxygen to water (Mujahid et al., 2007). Oxidative stress impairs cell membrane and mitochondrial integrity

and causes cell damage through lipid peroxidation (Halliwell and Gutteridge, 1989; Meng et al., 2008). Malondialdehyde (MDA) and protein carbonyls are compounds derived from lipid and protein oxidation. The amount of these compounds corresponds to the extent of lipid and protein oxidation (Srinivasan et al., 1996). Broilers exposed to acute heat stress exhibited more than two-fold increases of MDA as a marker for lipid peroxidation in the skeletal muscle (Mujahid et al., 2009; Wang et al., 2009). Mujahid et al. (2009) suggested that elevated MDA levels in the skeletal muscle of acute heat-stressed birds were caused by increased substrate oxidation and membrane potential of mitochondria that is associated with a decrease in uncoupling protein. The effects of heat stress on oxidative damage of muscle proteins were showed by Feng et al. (2006), they studied the effects of daily cycling high temperature on breast muscle mitochondria and discovered that it induced oxidative stress of the breast muscle. Wang et al. (2009) reported that heat exposure increased the oxidation of sarcoplasmic and myofibrillar proteins from *pectoralis major* of broiler chickens to different extents, whereby the muscle with heat exposure for three hours had the highest lipid and protein oxidative value (Wang et al., 2009). Oxidation of thiol groups in the ryanodine receptor can change the calcium sensitivity of calcium channel (Zissimopoulos and Lai, 2006). Klebl et al. (1998) demonstrated that sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA), an enzyme that removes calcium from sarcoplasm (Adachi et al., 2002), is not able to adjust the sarcoplasmic calcium concentration when the enzyme was damaged by ROS. As a result, the calcium levels increased in the sarcoplasm, which induced uncontrollable muscle contraction. Consequently, the pH of muscle dropped and the WHC of meat decreased. pH decline could accelerate ROS production and result in protein oxidation (Srinivasan et al., 1996; Estévez, 2011). Other parameters related to WHC, such as drip loss and cooking loss, are also negatively affected especially under PSE conditions. Furthermore, protein oxidation lowers their solubility and ability to bind water, resulting in increased drip and cooking losses (Wang et al., 2009). In addition, a variety of changes have

been observed in the gastrointestinal tract including an impairment of intestinal barrier integrity (Lambert et al., 2002). These authors suggested that these changes allow the translocation of luminal antigens and pathogens through the intestinal epithelium and facilitate the response of the innate immune system and leading to the development of intestinal inflammation and damage (Lambert et al., 2002).

7.2 Meat quality effects of heat stress in broilers

Heat stress has also been related to changes in meat quality traits in broilers. Carcass and meat quality traits, such as tenderness and color, are critical for consumer acceptance. Feng et al. (2006) investigated the effects of daily circular high temperature on meat quality effects of heat stress in broilers, revealing that cyclic high temperature induced lower pH, denatured muscle protein and increased drip loss, L* value and shear force. Petracci et al. (2004) observed higher L* and lower redness (a*****) and yellowness (b*****) for the breast muscle fillets of broilers reared in the summer than those reared in winter. The results of decreased a* value indicated that there is more oxidized myoglobin in the heat-exposed birds' muscle (Mancini and Hunt, 2005). Another important chemical indicator that influences meat quality is the pH. Heat exposure increased the release of hormones, accelerated the decomposition of glycogen and increased the rate of muscle glycolysis, leading to the pH decline observed. Wang et al. (2009) reported a significant decrease in pH_{30min} and pH_{24h} of *pectoralis major* from one to five hours of heat exposure, compared with the control group. They theorized that the decrease of pH value during aging was mainly due to lactic acid accumulation in glycolysis and H^+ from ATP hydrolysation. The rapid drop in pH can be associated with low redness, high drip and cooking losses in chicken breast meat (Hao and Gu., 2014). WHC is one of the important sensory qualities of meat. Heat exposure increases the drip and cooking losses to various extents, which suggests the WHC of proteins is susceptible to oxidative damage (Wang et al., 2009). Oxidation could impair water-binding in meat (Morzel et al., 2006).

Water-holding properties are of great importance, because retention and gains or losses of water affect the weight and thus the economic value of chicken products. Additionally, the content and distribution of water within muscles may affect the visual appearance as well as tenderness and juiciness of meat. Heat stress caused a high metabolic rate *rigor mortis*, resulting in pronounced protein denaturation (Channon et al., 2000; Deng et al., 2002). A loss of water can be caused also by an increased lipid oxidation, as it may change membrane structures and functions, and membrane osmosis.

Northcutt et al. (1994) reported that broilers raised under acute or short duration hot conditions produced PSE-like *post-mortem* muscle. McKee and Sams (1997) indicated that seasonal heat stress accelerated ATP depletion and increased the percentage of bird muscles that would be classified as PSE-like. High ambient temperature significantly decreased body protein content, protein gain, and protein retained: protein intake in broilers, suggesting that chronic heat stress changes protein metabolism, decreases protein synthesis, and increases catabolic rate (Geraert et al., 1996). It is reported that high environmental temperature accelerated protein breakdown (Yunianto et al., 1997). In finishing broiler chickens, chronic heat exposure induced a marked decrease in protein synthesis rates, as measured directly in different muscles; muscle protein turnover was lower under hot conditions than at thermo-neutrality. At 32°C, protein synthesis was more susceptible than proteolysis, at least in the *pectoralis major* and the gastrocnemius muscles, which thereby reduced muscle protein deposition (Temim et al., 2000). It has been demonstrated that heat stress lowers protein synthesis by changing ribosomal gene transcription (Jacob, 1995; Temim et al., 1998). The high environmental temperature can lower the ribosomal capacity, lead to decreased rate of protein synthesis, and result in the reduction of the protein deposition. Moreover, environment is an important factor influencing fat deposition in birds (Sands and Smith, 1999). Excessive fat deposition in broilers is a worldwide concern. Chronic exposure of broilers to a high ambient temperature is associated with enhanced

abdominal, subcutaneous, and intramuscular fat deposits (Ain Baziz et al., 1996; Geraert et al., 1996). Zhang et al. (2012) reported that broilers exposed to constant high temperature had significantly higher fat deposition in breast muscle compared with control standard group (Zhang et al., 2012).

Aim of the thesis

Until 20 years ago, most poultry feeds contained antibiotic growth promoters (AGPs) used as a tool for the control of pathogenic diseases and for the efficient livestock production. AGPs act against pathogen bacteria, which are associated with animals' poorer health performance. However, this approach had significant and unwanted side effects, such as development of antibiotic-resistant pathogens and carryover of the antibiotic residues to poultry products, such as meat and eggs. Therefore, the role of AGPs in the emergence of antibiotic resistance in humans has been questioned, and based on the "precautionary principle" (Turndige, 2004) the European Commission decided to ban AGPs. The ban of antibiotics at sub-therapeutic level contributed to increased incidence of enteric diseases in farms, with serious economic consequences. Many alternatives have been investigated to replace antimicrobials without any loss of productivity or negative influence on health. Probiotics, prebiotics and synbiotics are one of the proposed solutions, as alternatives to AGPs, to prevent enteric disease and increase performance in poultry. As aforementioned, alternative for AGPs are of practical significance, when they improve animal performance at levels comparable to AGPs. There are different ways to deliver these bioactives into avian gastrointestinal tract. Conventionally, in-feed or in-water supplementation has been used at first hours/days post-hatching. This approach relies on amount of feed and/or water intake, the quality of water (chlorinated) and other experimental factors (Bednarczyk et al., 2016). Consequently, consumed dose of prebiotics varies in the first hours/days after hatching. Furthermore, during early post-hatching period, infection of chicks by detrimental bacteria is also possible. Therefore, to be effective, these compounds must be administered to the animals under fully controlled conditions and as early as possible. In fact, some recent research tends to exclude the unwanted effects of several factors that may affect the action of supplements. *In ovo* technology enables delivery of sustainable bioactives, such as pre-probiotics and synbiotics, directly into the egg air chamber at day 12 of

embryonic incubation; it allows for a precise delivery of the bioactive to all embryos, which equalizes the effects across the flock and assures proper development of the gut microflora in all chicks. This could have important positive consequences in terms of improving animal welfare, reducing public health risks through the prevention and control of animal diseases, as well as reducing breeding and management costs. Furthermore, several studied suggests that these compounds, in particular prebiotics, can help chickens adapt and respond positively to situations of thermal stress. Heat stress is one of the major environmental stressors in poultry production since it adversely affects behavior, immune response, intestinal integrity and meat quality of chickens (Lara and Rostagno 2013). According to Deeb and Cahaner (2002), today the modern poultry genotypes are more susceptible to heat stress because of continuous selection for fast growth rate and improved feed efficiency.

At the same time, the increasing growth rate and body size of modern hybrid birds recently caused the appearance of breast muscle abnormalities such as Oregon disease (OD), white striping (WS), wooden breast (WB) and spaghetti meat (SM). Because of impaired appearance, poultry plants tend to downgrade abnormal breasts and potentially divert the meat into processed products with considerable economic losses. Therefore, poultry industry reveals a growing interest to understand how this new muscle abnormalities can affect meat quality and, more importantly, how to deal with this significant problem.

Because of these evidences, the aim of these study is:

1) to estimate the incidence of three emerging abnormalities of poultry meat in commercial conditions and to evaluate the effect of some *ante- mortem* factors (genetic type; sex; diet; average live weight of the lot; age at slaughter; transport time; duration of the pre-slaughtering stop) on the incidence of the above-mentioned muscle defects;

2) to investigate the effect of GOS delivered *in ovo* on meat quality traits of fast broiler chickens exposed to heat stress.

Chapter 8

Research N.1: Analysis of abnormalities in broiler breast muscle in commercial conditions

8.1 Introduction and investigations' aim

In recent decades, global consumption of poultry meat has increased significantly and is expected to become the first type of meat produced in the world in the coming years. The main reasons that are driving the success of poultry meat in developed and developing countries are mainly the low cost and healthier nutritional profile than red meats. Furthermore, the absence of religious ties, the relative ease of culinary preparation, together with the great availability of processed products, play a positive role in promoting the expansion of its consumption.

As regards the nutritional aspects, poultry meat adapts well to the modern consumer demand for meat with a high degree of long-chain polyunsaturated fatty acids, high levels of vitamins and antioxidants and low in fat, sodium and cholesterol. Therefore, there is a strong need for agri-food companies to increase production efficiency and, at the same time, guarantee consumers an excellent quality product. However, poultry meat nowadays contains a higher lipid content than that produced a few years ago. In addition, chicken breast anomalies such as white striping, wooden breast and spaghetti meat have been on the rise for several years, which represents a critical point for consumer preferences in choosing the product. To date, the cause of these anomalies is not yet well known, it is hypothesized that it is due to genetic selection programs based on improving the quality and quantitative characteristics of broilers, such as the speed of growth of the body, the increase in the muscle size, especially for cuts with greater commercial interest such as the breast and lean muscle mass at the expense of fatty tissue and intramuscular fat. Due to the appearance of the chicken breast, given by the presence of these anomalies, poultry farms tend to downgrade the cuts of meat that present these defects into processed products, with significant economic losses.

For the poultry industry to succeed, it is necessary to meet the growing needs of consumers in both quality and quantity. Therefore, the poultry industry reveals a growing interest in understanding how these new muscle abnormalities can affect the quality of meat and, above all, how to deal with this significant problem.

This investigation aims to assess the incidence of chicken breast myopathies (OD, WS, WB and SM) and to evaluate the possible effect of some *ante mortem* production factors, as:

- genetic type;
- sex;
- diet (exclusively vegetable or standard, with animal fats);
- average live weight of the lot;
- age at slaughter;
- transport time (breeding-slaughterhouse);
- duration of the pre-slaughtering stop.

8.2 Materials and methods

The investigation and data collection took place at the slaughtering and processing plants for poultry meat. A total of 90 production lots were examined. These lots consisted of broilers belonging to commercial hybrids (Ross 308), bred according to a conventional intensive system and slaughtered in industrial conditions. Each lot consisted of animals from the same farm, the same sex, the same age and belonging to the same genetic type. The age of slaughter of broilers depended on the market demand for poultry products and on the production and sale policies established by the poultry farm. The broilers included in the slaughter program were captured and placed in transport crates by workers of the affiliated poultry farms. The crates were then loaded into company-owned vehicles and transported to the processing plants by road. The surface of the floor of the crates was designed to allow ventilation during transport, to minimize the leakage of feces and to be non-slip.The birds were

weighed on the vehicles in the reception section at the entrance of the processing plant and stopped on the slaughter line for a period that depended on the slaughter order. During the stop at the slaughterhouse, the animal rest area was illuminated by a blue light. The combination of partially dark atmosphere with the use of blue light reduced the excitement of the birds and maked them immobile, preventing fractures and bruises. In addition, the rest area was equipped with fans and nebulizers that ensured the well-being of the animals.

The *ante-mortem* variables that have been recorded for each sampling were: the genetic type; sex; diet (exclusively vegetable or standard, with animal fats); average live weight of the lot; age at slaughter; transport time (breeding-slaughterhouse) and the duration of the pre-slaughtering stop.

The evaluation was carried out on a total of 200 breasts directly on the processing line, in correspondence with the sectioning area, about 3 hours *post-mortem* of the animals. A visual evaluation of the pectoral muscles (*Pectoralis major* and *Pectoralis minor*) was performed on each single breast in order to define the presence/absence of the white striping (WS), wooden breast (WB), spaghetti meat (SM) and Oregon disease (OD) defects and simultaneously indicate their level (absent, moderate and severe). In addition, weight was determined on each breast.

For the white striping defect, the distinction, absent, moderate and serious, was based on the assessment of the extent and size of the white striations as proposed by Kuttappan et al. (2012): Normal (breasts that had no white striations on the surface); moderate (breasts with superficial striations localized above all in the cranial part of the *P. major* muscle and in any case less than 1 mm thick); severe (breasts with diffuse superficial striations and a thickness greater than 1 mm). For the wooden breast defect, the evaluation was carried out on the basis of the consistency of the pectoral muscle: normal (breasts that did not have hardening of the pectoral muscle); moderate (breasts moderately hard to the touch, characterized by a visible " hard bulge" in the caudal part,

possible presence of pale and exudative areas); severe (breasts that were hard or extremely hard to the touch along the entire surface of the muscle, evident pale areas and extremely exudative tissue). For spaghetti meat, the assessment was based on the integrity of the pectoral muscle fibers: normal (breasts that no had a separation of the bundles of muscle fibers); moderate (breasts with poor fiber cohesion); severe (breasts that were completely unstructured in the muscle fibers). For Oregon disease, the classification was performed by evaluating the presence of signs of necrosis in the deep pectoral muscles: normal (breasts that did not have necrotic areas); moderate (breasts that presented the first stage of the anomaly characterized by pale edematous pink areas); severe (breasts that present the advanced stage of the anomaly, clearly visible areas of green necrosis).

The criterion used for assessing the degree of severity of the anomalies follows a score scale from zero to two (0 = absence of anomaly, 1 = moderate anomaly, 2 = severe anomaly).

8.3 Statistical analysis

The data collected during the investigation for the evaluation of the incidence of the WS, WB, SM and OD anomalies were included in a database subsequently used for statistical-descriptive analysis (average, standard error of the average, minimum and maximum). Following this analysis, 11 lots were highlighted which presented data that differed excessively from the others and therefore were eliminated from the database and subsequent analyzes were conducted on 78 lots. In addition to the data relating to the incidence of each anomaly, it was assessed on the whole the distribution of normal samples (which did not present any anomaly), affected by anomalies of moderate level (which presented at least one anomaly in the moderate stage) and serious (which presented at least one anomaly in the serious stage). Data were analyzed using the one-way ANOVA, considering the *ante-mortem* factors.

First of all, it was assessed the influence exercised by the commercial category defined as follows: Medium broilers, consisting of lots of female and male chickens with a live weight of less than 3 kg (n = 45); Heavy broilers, made up of lots of male chickens with a live weight of more than 3 kg (n = 34).

After that, within each commercial category, the production factors reported in Tables 2 and 3 respectively were assessed.

The limits for continuous variables were identified using the mean and the standard deviation in order to obtain classes of homogeneous numerosity.

Table 2. Classification criteria for lots belonging to the commercial category "medium broilers"

Factors analyzed considering all lots (n. 45)			
Sex	Females n° 28 lots	Male n° 17 lots	
Growth rate	Low (v < 58.5 g/die) n. 17 lots	Med (58.5 < v > 61.7 g/die) n. 14 lots	High (v > 61.7 g/die) n. 14 lots
Transport time	Short (t < 120 min) n. 21	Long (t > 120 min) n. 24 lots	
Stop at the slaughterhouse	Short (t < 225 min) n. 24 lots	Long (t > 225 min) n. 21 lots	
Factors analyzed in the lots consisting of female broilers (n .28)			
Diet	Standard n. 16 lots	Vegetable n. 12 lots	

Table 3. Classification criteria for lots belonging to the commercial category "Heavy broilers"

Factors analyzed considering all lots (n. 34)			
Growth rate	Low (v < 69.7 g/die) n. 12 lots	Med (69.7 < v > 72.1 g/die) n. 11 lots	High (v > 72.1 g/die) n. 11 lots
Transport time	Short (t < 120 min) n. 17 lots	Long (t > 120 min) n. 17 lots	
Stop at the slaughterhouse	Short (t < 180min) n. 14 lots	Long (t > 180 min) n. 20 lots	

However, they were considered only the factors for which there was no consistent interaction with the other variables or there was a high inhomogeneity in the number of classes (Table 4).

Table 4. Variables for which it was assessed the influence on chicken breast anomalies and for which any problems were identified that made it impossible to use the results.

Factor	**Medium Broilers**	**Heavy Broliers**
Sex (F; M)	OK	Not evaluable
Growth Rate (Low; Medium; High)	OK	OK
Diet (Standard; Vegetable)	OK	Not evaluable
Transport time (Short; Long)	OK	OK
Stop at the slaughterhouse (Short; Long)	OK	OK

8.4 *Results and discussion*

8.4.1 Overall incidence of chicken breast abnormalities and frequency distribution of breasts' weight

Figure 12 shows the distribution of all the breasts analyzed (n = 15,800) as a function of the presence and severity of the meat anomalies (WS, WB and SM). In particular, the samples were grouped into five categories regardless of the type of defect present. The breasts that showed no defect were 34%, while those who were affected by at least one of the 3 anomalies tested at the moderate stage represented 43%. Therefore, the percentage of samples with at least one of the 3 severe stage anomalies was 23%. Of these, 16% had only one anomaly in the severe stage, 7% showed two in the severe stage and in 0.2% of cases all defects in the most serious stage were observed.

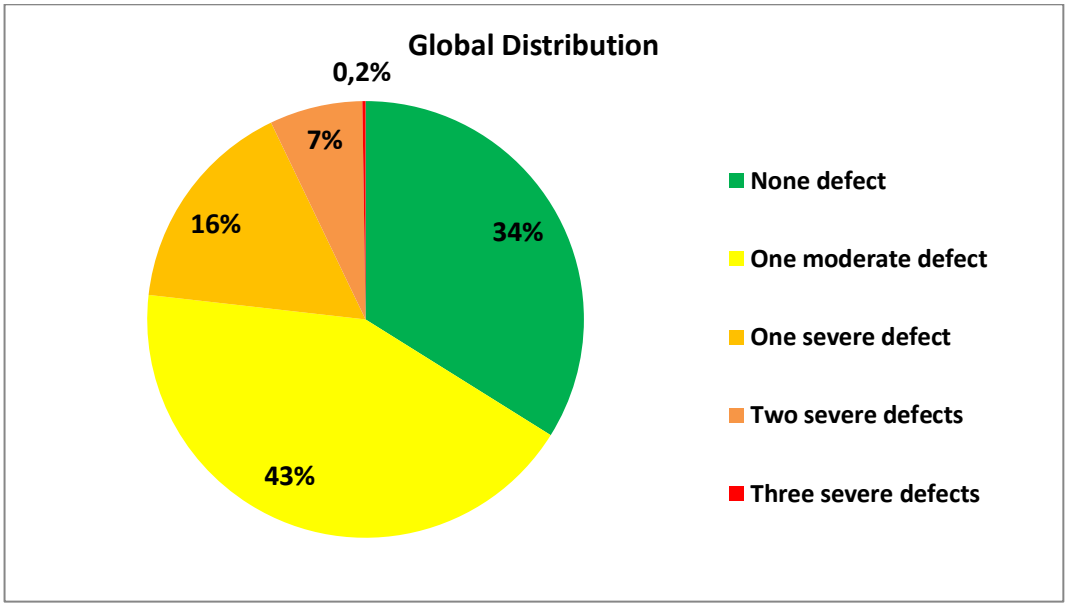

Figure 12. Global distribution of breast defects.

The frequency distribution of breast weight was then calculated (Figure 13). The average weight was between 300 and 1300 grams and the average value was around 715 grams.

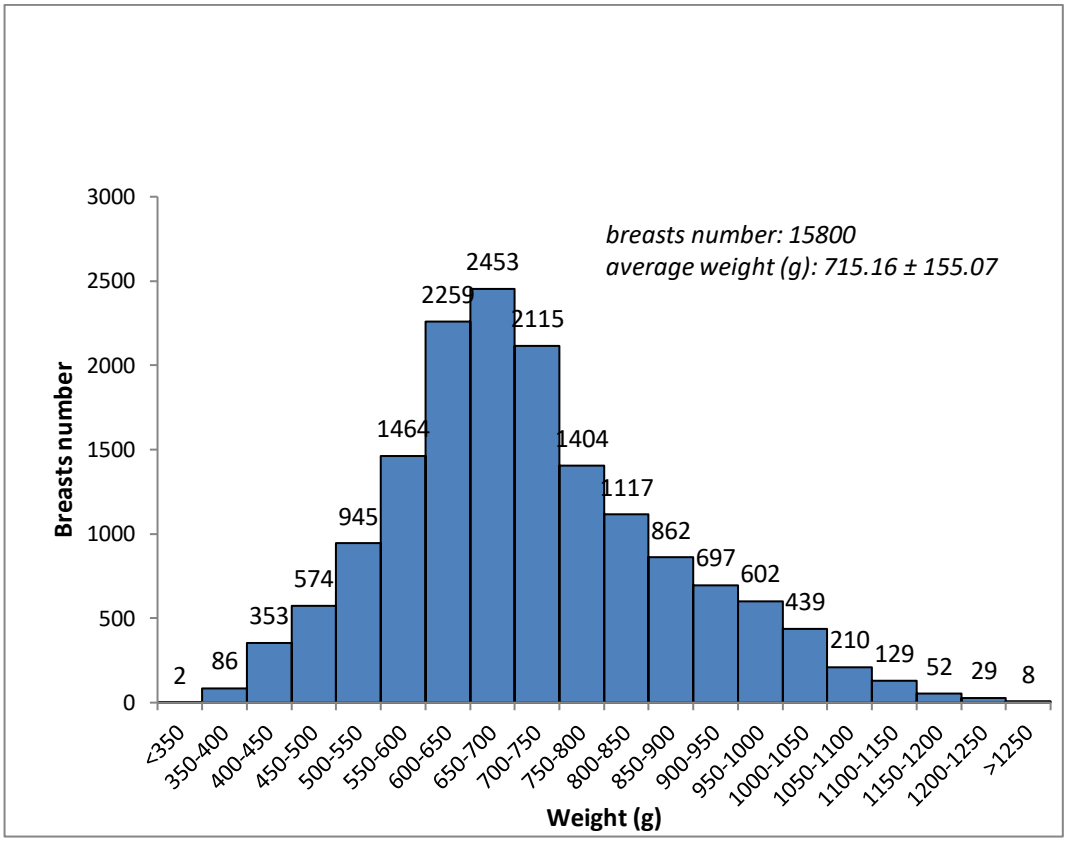

Figure 13. Frequency distribution of breasts' weight

8.4.2 Incidence of individual defect

The Table 5 shows the incidences for the individual anomalies.

The WB anomaly had the highest incidence, with a total percentage of 60.6%, of which 42.5% in the moderate stage and 18.1% in the severe stage. As for WS, the incidence was 30.9% overall, of which 21.6% in moderate form and 9.3% in severe form. In 21% of cases, the SM defect was detected, of which 17.4% in the moderate stage and 3.6% in the severe stage. Finally, the incidence of OD was very low (moderate: 0.12%; Severe: 0.27%). To date, the information on the incidence of breast defects, in different countries, is limited and the comparison is difficult because the classification criteria can vary among investigations. These results are not in accordance with those reported by Petracci et al. (2019) who reviewed that in Italy, France, Spain and Brazil WS defect affects the 50% of chickens breasts. Kuttapan et al. (2017) reported that in United States around

the 85% of the breasts from birds at 9 weeks were affected by WB. In the present study the animals were around 6-7 weeks of age; however, Sihvo at al. (2017) reported that WB begin to develop at 2 weeks of age and as the age increases, the defect tends to aggravate.

Table 5. General overview of the incidence of chicken breast anomalies (total lots n = 79)

	Mean	Minimum	Maximum	Std. Dev.	ESM
Live weight (g)	3101.7	2319.0	4000.0	414.8	46.7
Slaughter age (*die*)	47.9	40.0	56.0	3.5	0.4
Growth rate (g/d)	64.8	53.1	81.6	6.9	0.8
Transport (min)	174.4	18.0	389.0	88.1	9.9
Stop (min)	204.5	10.0	589.0	125.2	14.1
➢ ***White Striping***					
Normal	69.2	24.0	95.0	17.7	2.0
Moderate	21.6	4.5	50.0	9.4	1.1
Severe	9.3	0.0	53.5	12.8	1.4
➢ ***Wooden Breast***					
Normal	39.7	5.5	80.5	19.3	2.2
Moderate	42.5	10.0	69.5	11.5	1.3
Severe	18.1	1.0	48.5	12.1	1.4
➢ ***Spaghetti Meat***					
Normal	79.0	33.5	98.0	15.5	1.7
Moderate	17.4	1.0	60.5	14.1	1.6
Severe	3.6	0.0	10.5	3.1	0.3
➢ ***Oregon Disease***					
Normal	99.61	97.50	100.00	0.58	0.06
Moderate	0.12	0.00	1.00	0.27	0.03
Severe	0.27	0.00	2.00	0.48	0.05

8.4.3 Evaluation of production factors

The second purpose of this investigation was to verify whether *ante-mortem* production factors could influence the incidence of myopathies examined. In this regard, the production factors were assessed individually as shown in the following tables.

8.4.3.1 Commercial category effect

It should be emphasized that the definition of the commercial categories used in this work does not fully correspond to that used in the company where the data was collected. In fact, in the "medium broilers" category, lots of animals slaughtered at a weight of less than 3 kg were included, while those of a higher weight were all included in the "heavy broilers" category unlike in commercial practice.

Comparing the values obtained in the "medium broilers" *versus* "heavy broilers" category, the WS and WB anomalies presented different percentages. In particular, the incidence of defects is higher in heavy broilers than medium ones, especially for the severe stage (16.2 % for WS; 27.9 % for WB; $P < 0.001$). While, SM and OD defects do not present big differences in the categories examined ($P > 0.05$) (Table 6). These results confirm that the occurrence of breast anomalies is associated with the high weight of birds reached in few weeks and, as suggested by Sihvo et al. (2018), it could be due to the modern selection of birds for a rapid growth rate and high muscle yield, which cause insufficient vascularization and oxidative stress which can lead to tissue degeneration. These findings agree with Mazzoni et al. (2015) who observed in heavy broilers, produced under intensive farming system, a higher incidence of myodegeneration. Petracci et al. (2013), with lighter birds slaughtered from 45 to 54 d of age (average live weight: 2.75 kg), found 12% of WS breast fillets (8.9% moderate and 3.1% with severe). The impact of the defects on poultry meat quality result in serious economic loss. In particular, the severity of WS on the surface of the breasts has negative effects on appearance and consumers

purchases. Moderate WS breasts are usually marketed as such to consumers, while severe stage of WS and WB breasts are downgraded and used in the manufacturing of further-processed products.

Table 6. Effect of the commercial category (Medium *vs* Heavy)

	Medium Weight <3 kg	Heavy Weight >3kg	Esm	Prob.
Lots	45	34		
Live weight (g)	2785.5	3520.4	414.8	<0.001
Slaughter age (die)	46.5	49.6	3.5	0.001
Growth rate(g/d)	60.1	70.9	6.9	0.001
Transport (min)	135.0	226.5	88.1	0.001
Stop (min)	170.3	249.6	125.2	0.005
➢ ***White Striping***				
Normal	78.8	56.5	17.7	0.001
Moderate	17.1	27.5	9.4	0.001
Severe	4.1	16.2	12.8	0.001
➢ ***Wooden Breast***				
Normal	50.3	25.7	19.3	0.001
Moderate	39.1	47.0	11.5	0.002
Severe	10.8	27.9	12.1	0.001
➢ ***Spaghetti Meat***				
Normal	81.4	75.8	15.5	0.112
Moderate	15.0	20.7	14.1	0.076
Severe	3.6	3.5	3.1	0.924
➢ ***Oregon Disease***				
Normal	99.69	99.50	0.58	0.150
Moderate	0.12	0.12	0.27	0.941
Severe	0.19	0.38	0.48	0.075

8.4.3.2 The gender effect in the medium broilers category

Within the medium broilers category, the incidences of myopathies between the male and female sexes were compared (Table 7).

This division was only possible with regard to “medium broilers”, while it was not carried out in the category of “heavy broilers”, represented only by male specimens. No significant differences emerged for WS, WB and OD anomalies. In a similar study, Trocino et al. (2015) found differences on the occurrence of WB, in fact it was doubled from females to males (8.0% *vs* 16.3%; P <0.05). Kuttappan et al. (2013) reported no significant effect of sex on WS occurrence, but they observed that females had a higher rate of normal breasts whereas males showed a higher rate of severe WS breasts. These effects were attributed to differences in bird weight.

On the contrary, SM defect in the severe stage shows a higher (P=0.000) incidence in the lots of female broilers, this result agrees with Soglia et al. (2019) that reported SM condition is more prevalent in female animals. To our knowledge, to date, no published data are available on the occurrence rate of SM according to sex.

Table 7. Gender effect in the medium broilers category.

	Females	Male	Esm	Probability
Lots	28	17		
Live weight (g)	2780.8	2793.2	27.20	0.828
Slaughter age (die)	47.4	45.0	0.54	0.031
Growth rate (g/d)	58.7	62.4	0.71	0.009
Transport (min)	98.9	194.5	13.64	0.000
Stop (min)	169.1	172.4	14.20	0.913
➢ ***White Striping***				
Normal	77.9	80.3	1.65	0.495
Moderate	17.9	15.9	1.14	0.402
Severe	4.2	3.9	0.69	0.810
➢ ***Wooden Breast***				
Normal	51.8	47.7	2.49	0.433
Moderate	38.4	40.4	1.79	0.595
Severe	10.1	11.9	1.05	0.416
➢ ***Spaghetti Meat***				
Normal	80.1	83.6	2.30	0.456
Moderate	15.1	14.8	2.08	0.955
Severe	4.9	1.5	0.47	0.000
➢ ***Oregon Disease***				
Normal	99.68	99.71	0.07	0.859
Moderate	0.11	0.15	0.04	0.629
Severe	0.21	0.15	0.05	0.548

8.4.3.3 Effect of the diet in the medium broilers category

The effect of the diet was assessed only in the category of medium female broilers because male chickens do not follow a vegetable and / or antibiotic-free diet. The antibiotic free food plan was also included in the lots of chicken fed with a plant-based diet.

As can be seen in Table 8, the influence of this factor was absent or limited. Trocino et al. (2015) evaluated whether feeding birds *ad libitum* or at a restricted rate during the first growth period (from 13 to 21 d of age) may affect the occurrence of WS and WB in broilers. They found that the occurrence of WS (moderate and severe) was lower in broilers always fed *ad libitum* compared to those submitted to feed restriction (P = 0.07).

Feeding strategies may be used to control the occurrence of myopathies in broiler chickens: low-energy diets reduce both growth rate and the occurrence of white striping (Kuttappan et al., 2012). Similarly, feed restriction may reduce the incidence of myopathy by controlling the early growth and the occurrence of metabolic diseases (De Jong et al., 2012; Sahraei, 2012; Butzen et al., 2013).

Table 8. Effect of feeding on the category of medium female broilers

	Standard	Vegetable	Esm	Prob.
Lots	16	12		
Live weight (g)	2750.0	2821.8	37.30	0.350
Slaughter age (die)	47.2	47.8	0.60	0.652
Growth rate (g/d)	58.4	59.1	0.63	0.610
Transport (min)	129.8	57.8	17.16	0.035
Stop (min)	156.1	186.5	15.52	0.341
➢ ***White Striping***				
Normal	79.1	76.4	2.32	0.577
Moderate	16.7	19.5	1.62	0.403
Severe	4.3	4.2	0.91	0.952
➢ ***Wooden Breast***				
Normal	49.4	55.0	3.08	0.376
Moderate	39.5	36.9	2.21	0.571
Severe	11.1	8.8	1.21	0.350
➢ ***Spaghetti Meat***				
Normal	81.2	78.5	2.76	0.644
Moderate	13.8	16.8	2.39	0.553
Severe	5.0	4.7	0.59	0.812
➢ ***Oregon Disease***				
Normal	99.5	99.9	0.10	0.037
Moderate	0.2	0.0	0.05	0.084
Severe	0.3	0.1	0.07	0.131

8.4.3.4 Effect of growth rate, transport time and stop at the slaughterhouse in the medium and heavy broilers categories.

The following tables (Tables 9-14) show, for the medium and heavy broilers categories, the effect of the growth rate of the animals, the transport time of the animals from the breeding to the slaughterhouse and the dwell time at the slaughterhouse.

The growth rate “v”, and the times “t”, have been defined as "short", "medium" and "long", these time intervals have been calculated using the formulas: mean ± (DS / 3) and mean ± (DS / 2) (DS= standard deviation).

In the category “medium broilers” the growth rate and transport time factors affect the occurrence of OD and WS defects respectively, while, the other factors do not have a significant effect on the incidence of myopathies. The table 9 shows that OD has a higher percentage in severe stage (0.35% P=0.038) in lots with a low growth rate, this result can be overlooked because the total severe OD samples observed in this investigation were 3552 on 18000 breasts observed. In Table 10 it can be observed that transport time had a significant effect on the occurrence of moderate WS (19.8% P=0.025) even if the transport time is short. Several studies were carried out to correlate pre-slaughter stress to meat quality. Fletcher (2002) asserted that poultry meat quality is highly dependent on pre-slaughter management. The transport could be a source of stress for animals, due to the fear, to the low ventilation during the transport and so could accelerate *post-mortem* glycolysis, which is detrimental to the quality of breast meat.

In the category “heavy broilers”, the stop at the slaughterhouse is the only factor that has a significant effect on incidence on the occurrence of severe WS (P= 0.037) and moderate SM (P= 0.012). Also, this factor is a source of stress for animals: temperature, humidity, light intensity in the room are all changes that the animals experiences. In the table 14 is possible observe that for the factor “long time” the incidence of severe WS and moderate SM are lower, probably it

is due to the fact that the animals have more times to settle into the new conditions.

However, also for these factors, to our knowledge, no published data are available on the occurrence rate of incidence of myopathies.

Table 9. Effect of growth rate of animals on the category medium broilers

	LOW v ≤58.5 g/die	MEDIUM 58.5 ≤ v ≥ 61.7 g/die	HIGH v ≥61.7 g/die	Esm	Prob.
Lots	17	14	14		
Live weight (g)	2716.4	2777.0	2877.9	27.20	0.045
Slaughter age (die)	48.7	46.3	44.1	0.54	0.001
Growth rate (g/d)	55.8	59.9	65.5	0.71	0.000
Transport (min)	134.0	117.6	153.8	13.64	0.588
Stop (min)	194.8	164.6	146.4	14.20	0.367
➢ ***White Striping***					
Normal	76.7	80.6	79.6	1.65	0.605
Moderate	18.0	15.5	17.5	1.14	0.655
Severe	5.3	3.9	2.9	0.69	0.370
➢ ***Wooden Breast***					
Normal	46.3	56.6	48.8	2.49	0.222
Moderate	42.5	35.9	38.2	1.79	0.310
Severe	11.2	8.1	13.0	1.05	0.179
➢ ***Spaghetti Meat***					
Normal	75.4	83.8	86.3	2.30	0.119
Moderate	20.5	12.8	10.5	2.08	0.109
Severe	4.1	3.4	3.3	0.47	0.739
➢ ***Oregon Disease***					
Normal	99.59	99.86	99.64	0.07	0.297
Moderate	0.06	0.11	0.21	0.04	0.262
Severe	0.35	0.04	0.14	0.05	0.038

Table 10. Transport time effect on the medium broilers category

	SHORT t ≤ 120 min	LONG t ≥ 120 min	Esm	Prob.
Lots	21	24		
Live weight (g)	2778.1	2791.9	27.20	0.803
Slaughter age (die)	46.6	46.4	0.54	0.838
Growth rate (g/d)	59.5	60.6	0.71	0.476
Transport (min)	48.0	211.3	13.64	0.000
Stop (min)	174.6	166.6	14.20	0.784
➢ ***White Striping***				
Normal	75.9	81.3	1.65	0.101
Moderate	19.8	14.7	1.14	0.025
Severe	4.3	3.9	0.69	0.805
➢ ***Wooden Breast***				
Normal	52.2	48.6	2.49	0.485
Moderate	37.4	40.6	1.79	0.379
Severe	10.8	10.8	1.05	0.977
➢ ***Spaghetti Meat***				
Normal	82.0	80.9	2.30	0.814
Moderate	13.6	16.2	2.08	0.541
Severe	4.4	2.9	0.47	0.119
➢ ***Oregon Disease***				
Normal	99.8	99.6	0.07	0.221
Moderate	0.1	0.2	0.04	0.232
Severe	0.1	0.2	0.05	0.426

Table 11. Effect of the stop at the slaughterhouse on the medium broilers category

	SHORT t ≤ 225 min	LONG t ≥ 225 min	Esm	Prob.
Lots	24	21		
Live weight (g)	2773.8	2798.8	27.20	0.652
Slaughter age (die)	45.4	47.9	0.54	0.020
Growth rate (g/d)	61.5	58.5	0.71	0.038
Transport (min)	152.5	115.1	13.64	0.174
Stop (min)	94.9	256.5	14.20	0.000
➢ ***White Striping***				
Normal	77.4	80.4	1.65	0.361
Moderate	18.4	15.7	1.14	0.245
Severe	4.3	3.9	0.69	0.796
➢ ***Wooden Breast***				
Normal	49.0	51.7	2.49	0.596
Moderate	39.7	38.4	1.79	0.717
Severe	11.3	10.3	1.05	0.652
➢ ***Spaghetti Meat***				
Normal	83.5	79.1	2.30	0.348
Moderate	13.2	17.0	2.08	0.376
Severe	3.3	4.0	0.47	0.507
➢ ***Oregon Disease***				
Normal	99.67	99.71	0.07	0.750
Moderate	0.19	0.05	0.04	0.076
Severe	0.15	0.24	0.05	0.395

Table 12. Growth rate effect on heavy chicken category

	Low v ≤ 69.7 g/die	Medium 69.7 ≤ v ≥ 72.1 g/die	High v ≥ 72.1 g/die	Esm	Prob.
Lots n= 34	12	11	11		
Live weight (g)	3376.7	3530.0	3667.5	36.54	0.002
Slaughter age (die)	50.0	49.9	49.0	0.41	0.565
Growth rate (g/d)	67.6	70.7	74.9	0.60	0.000
Transport (min)	224.7	205.6	249.5	8.19	0.094
Stop (min)	237.0	274.7	238.4	24.99	0.796
➢ ***White Striping***					
Normal	57.2	55.9	56.5	2.89	0.983
Moderate	24.2	32.0	26.7	1.38	0.059
Severe	18.6	12.2	17.7	2.81	0.617
➢ ***Wooden Breast***					
Normal	26.9	23.0	27.1	2.10	0.676
Moderate	46.1	50.6	44.5	1.55	0.266
Severe	28.7	26.5	28.4	1.80	0.871
➢ ***Spaghetti Meat***					
Normal	74.2	73.8	79.5	2.62	0.626
Moderate	21.8	23.4	16.7	2.36	0.507
Severe	4.1	2.8	3.7	0.51	0.563
➢ ***Oregon Disease***					
Normal	99.3	99.6	99.6	0.11	0.571
Moderate	0.1	0.2	0.0	0.05	0.524
Severe	0.5	0.2	0.4	0.10	0.454

Table 13. Effect of transport time on heavy broilers category

	Short t < 120 min	**Long t > 120 min**	**Esm**	**Prob.**
Lots	17	17		
Live weight (g)	3492.1	3548.6	36.54	0.447
Slaughter age (die)	49.5	49.8	0.41	0.675
Growth rate (g/d)	70.6	71.3	0.60	0.582
Transport (min)	191.9	261.2	8.19	0.000
Stop (min)	270.2	229.1	24.99	0.418
➢ ***White Striping***				
Normal	62.4	50.7	2.89	0.043
Moderate	26.0	29.0	1.38	0.285
Severe	12.2	20.3	2.81	0.152
➢ ***Wooden Breast***				
Normal	28.7	22.7	2.10	0.152
Moderate	46.6	47.5	1.55	0.773
Severe	25.9	29.9	1.80	0.274
➢ ***Spaghetti Meat***				
Normal	76.3	75.3	2.62	0.844
Moderate	20.7	20.6	2.36	0.995
Severe	3.0	4.1	0.51	0.288
➢ ***Oregon Disease***				
Normal	99.71	99.29	0.11	0.069
Moderate	0.03	0.21	0.05	0.062
Severe	0.26	0.50	0.10	0.252

Table 14. Effect of the stop at the slaughterhouse on the heavy broilers category

	Short t < 180 min	**Long t > 180 min**	**Esm**	**Prob.**
Lots	14	20		
Live weight (g)	3427.1	3585.6	36.54	0.031
Slaughter age (die)	48.4	50.6	0.41	0.007
Growth rate (g/d)	70.9	71.0	0.60	0.925
Transport (min)	213.1	235.9	8.19	0.175
Stop (min)	120.6	340.0	24.99	0.000
➢ ***White Striping***				
Normal	50.2	61.0	2.89	0.066
Moderate	26.6	28.1	1.38	0.597
Severe	23.2	11.4	2.81	0.037
➢ ***Wooden Breast***				
Normal	25.3	26.0	2.10	0.870
Moderate	45.9	47.9	1.55	0.542
Severe	28.8	27.2	1.80	0.660
➢ ***Spaghetti Meat***				
Normal	68.6	80.8	2.62	0.020
Moderate	27.6	15.8	2.36	0.012
Severe	3.8	3.4	0.51	0.695
➢ ***Oregon Disease***				
Normal	99.36	99.60	0.11	0.300
Moderate	0.11	0.13	0.05	0.856
Severe	0.54	0.28	0.10	0.210

8.4.4 Incidence of the severe forms of wooden breast, white striping and spaghetti meat in function of breast weight

Figures 14 and 15 show the relation of the incidence of severe forms of WB, WS and in function of breasts weight. An exponential increase in the incidence of WS condition was observed when the weight of breasts was higher. An even more drastic increase was observed in the WB condition related with the increased breast weight, while no relationship with the SM condition emerged. Although these results are similar to those found in the literature, it is important to underline their relevance. The entity of these defects (normal, moderate and/or severe) can only be assessed after the animals die. These graphs, above all for WB and WS, show that it is possible to predict the onset of myopathy at a certain weight value. Therefore, the researchers can use these data to study the myopathies development related to the weight in order to improve the animals health. At the same time, they can be used by producers in poultry industry to avoid the downgrading of the meat retails with consequent loss of money.

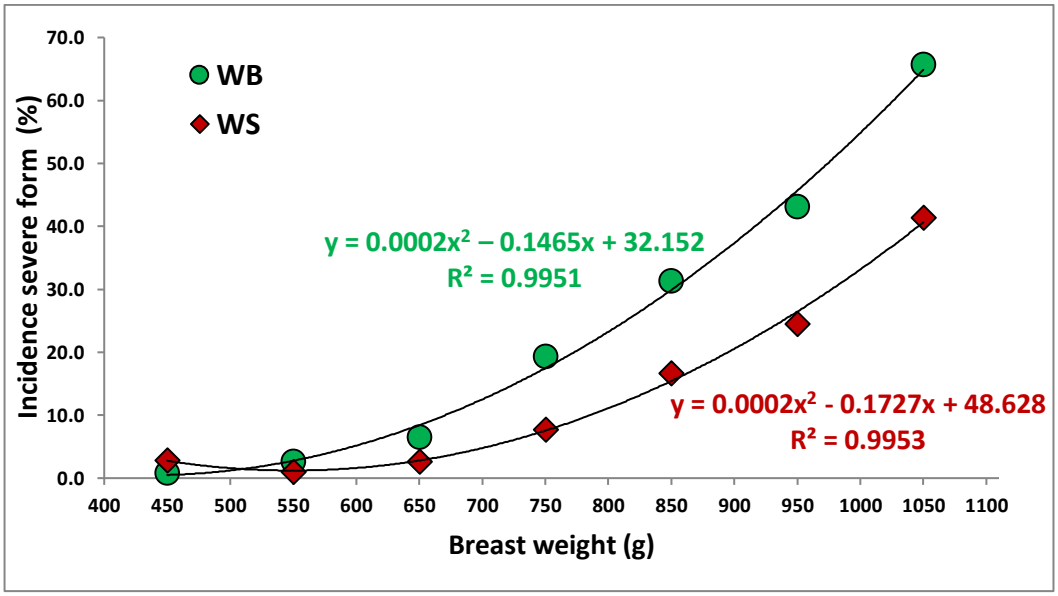

Figure 14. Incidence of severe form of WS and WB in relation to breast weight

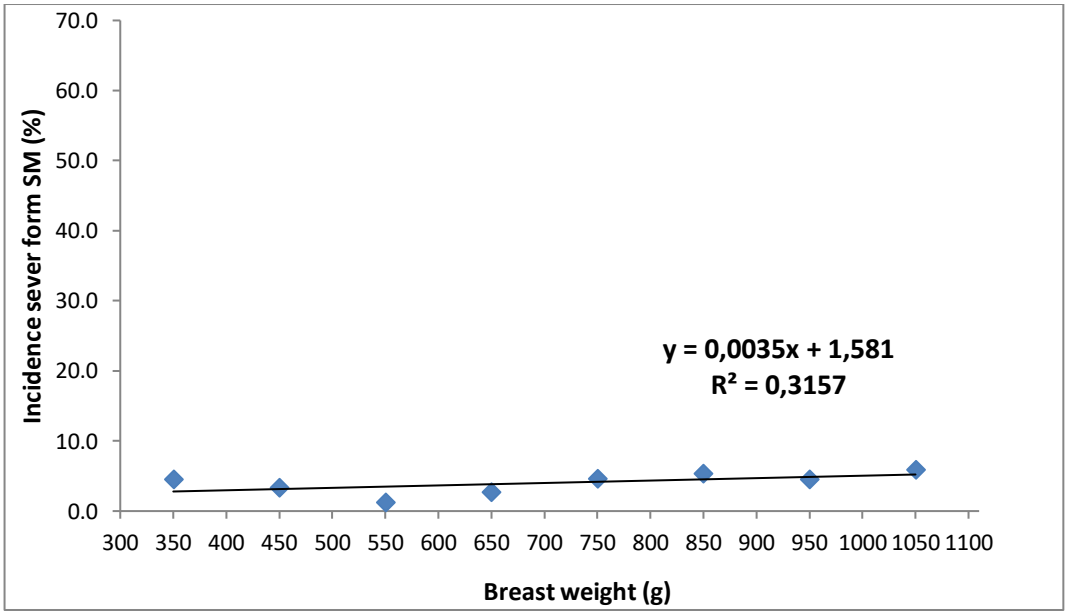

Figure 15. Incidence of severe form of SM in relation to breast weight

8.5 Conclusions

This investigation has allowed to estimate the incidence in commercial conditions of the three emerging anomalies of poultry meat (White Striping, Wooden Breast, Spaghetti Meat, and Oregon disease) and has also allowed to evaluate the effect exerted by some production factors.

From the overall examination of the results of this investigation, it can be highlighted that:

- 43% of the analyzed breasts showed at least one of the moderate stage defects and 23% of the breasts had at least one severe defect;
- among the defects taken into consideration, the Wooden Breast anomaly presented the highest incidence, with a total percentage of 60% (42% moderate and 18% severe); the incidence was higher in lots of heavy broilers;
- the incidence of breasts with White Striping defect was equal to 31% (22% moderate and 9% severe);
- 21% of breasts presented Spaghetti Meat defect (17% moderate and 4% severe) and it was more pronounced in females;
- An exponential increase in the incidence of White Striping condition was observed when the weight of breasts was higher. An even more drastic increase was observed in the Wooden Breast condition related with the increased breast weight, while no relationship with the SM condition emerged;
- Oregon disease does not seem to have a relevant effect, even if slightly higher in heavy broilers category.

In conclusion, *ante mortem* factors, such as transport time and arrest at the slaughterhouse, do not play an important role in the appearance of breast abnormalities. On the contrary, the growth rate of animals seems to be a possible cause especially for the Wooden Breast and Spaghetti Meat, which are the most common defects in heavy chickens. Besides, it is hypothesized that another critical factor is the genetic selection of birds as a consequence of the

demand of the poultry industry to produce heavier birds, this has led to an increased pressure on muscle development rate and on the appearance of breast abnormalities. Various studies have shown that the incidence of these modern myopathies have been increased over the past few years. Moreover, in the future if the growth rate in bird will continue to increase, it will lead not only to economic loss, but also it will result as a welfare issue of live animals. Therefore, more research is needed to expand knowledge regarding the effects of the factors considered in this study on the incidence of breast myopathies in chickens bred in commercial conditions.

Chapter 9

Research N. 2: Effect of galactooligosaccharides delivered *in ovo* on meat quality traits of broilers chickens exposed to heat stress

9.1 Introduction and investigations' aim

As aforementioned, continuous selection for fast growth and improved feed conversion efficiency has made modern poultry genotypes more susceptible to heat stress than ever before (Deeb and Cahaner, 2002). Heat stress is one of the major environmental stressors in poultry production since it adversely affects behavior, immune response, intestinal integrity, productivity and meat quality of chickens (Lara and Rostagno, 2013). Either acute or chronic heat stress could lead to meat quality decline due to: increase in *ante* and *post mortem* glycolytic metabolisms; decrease in protein synthesis and turnover; enhanced fat deposition; overproduction of reactive oxygen species (Temim at al., 2000; Lu et al., 2007; Zhang et al., 2012; Zaboli et al., 2019). Exposure of broilers to high temperatures can induce a lower ultimate pH with variation in meat color, water holding capacity and tenderness of meat (Berri et al., 2005; Aksit et al., 2006; Zhang et al., 2012; Wang et al., 2017), resulting in a lower consumer acceptability. In recent years, poultry industry has made several efforts to contrast and prevent the negative effects of heat stress on poultry production, in order to reduce economic losses. Feed additives, such as probiotics, prebiotics and synbiotics has been proposed as a strategy to improve resilience of animals against heat stress. Currently, there is a growing evidence that supplementation with prebiotics can be effective in alleviating detrimental effects of heat stress in chickens (reviewed in Varasteh et al., 2015). Prebiotics such as fructooligosaccharides, galactooligosaccharides (GOS), and mannanoligosaccharides are considered preventative agents since they can select for a gastrointestinal microbiota which not only benefits the host but can serve as a barrier to pathogen colonization (Ricke, 2018). In the poultry practice, prebiotics and probiotics are conventionally added to feed and/or water at first

hours/days post-hatching. The main concern about the use of these bioactives is their efficient administration under fully controlled conditions. Moreover, during early post-hatching period, the possible infection of chicks by harmful bacteria cannot be overlooked. Thus, to ensure that the chicks' intestine is protected over that time, the natural promoters of the beneficial microflora such as prebiotics, probiotics, and synbiotics should be applied before hatching. The main concept of *in ovo* technology is to apply bioactives long before the bird hatches (day 12 of egg incubation) to stimulate native egg microflora and helps to program lifelong phenotypes (e.g., immunity, gut microbiome, performance, adaptive) already during the embryonic phase (Siwek et al., 2018). Several studies suggest the gut health-promoting effect of dietary GOS. Varasteh et al. (2015) found that GOS could not mitigate the alterations in the ileum, but successfully prevented all heat-stress induced changes in the jejunum. The aim of this study was to evaluate the efficacy of a commercial prebiotic (Bi^{2}tos, a trans-galactooligosaccharides - GOS) delivered *in ovo* on performance and meat quality traits in fast-growing broiler chickens subjected to heat stress (HS).

9.2 Materials and methods

9.2.1 Birds and experimental design

To evaluate the efficacy of prebiotics delivered *in ovo* on productive and meat quality traits under HS conditions, fertilized eggs obtained from the same breeder flock (Ross 308) were incubated in a commercial hatchery. At the day 12th of incubation, eggs were candled and 3,000 eggs with viable embryos were randomly divided equally into 3 experimental groups: prebiotic group (GOS) injected with a single dose of 3.5 mg GOS/egg suspended in 0.2 mL of physiological saline (0.9 % NaCl); saline group (S) injected with 0.2 mL of pure physiological saline; control group (C) remained uninjected. Saline and GOS solution were injected into air chamber and the hole was sealed with organic glue.

GOS prebiotic used in this study (trade name: Bi^{2}tos, Clasado Biosciences Ltd., Jersey, UK) is manufactured by enzymatic transgalactosylation of the milk lactose by the whole cells of *Bifidobacterium bifidum* 41171 (Tzortzis et al., 2005). The GOS product obtained this way is a dry powder containing a mixture (wt: wt) of the following oligosaccharides: 45% lactose, 9.9% disaccharides [Gal (*β* 1–3)-Glc; Gal (*β* 1–3)- Gal; Gal (*β* 1–6)-Gal; Gal (*α* 1–6)- Gal], 23.1% trisaccharides [Gal (*β* 1–6)-Gal (*β* 1–4)- Glc; Gal (*β* 1–3)- Gal (*β* 1–4)- Glc], 11.55% tetrasaccharides [Gal (*β* 1–6)- Gal (*β* 1–6)- Gal (*β* 1–4)- Glc], and 10.45% pentasaccharides [Gal (*β* 1–6)- Gal (*β* 1–6)- Gal (*β* 1–6)- Gal (*β* 1–4)- Glc]. The injection dose used was already tested by Bednarczyk et al. (2016) based on the criterion of egg hatchability and intestinal bacteria abundance in 1-day-old chicks.

After hatching, all the chicks were vaccinated according to the current commercial practice (coccidiosis, Infectious Bronchitis Virus, Marek's disease virus, Newcastle and Gumboro disease). A total of 900 male chicks (300 chicks/treatment) were divided into 6 groups (150 birds/group) and reared in floor pens: 3 groups (6 pens/group, 25 birds/pen) were reared in thermoneutral condition (TN) and 3 groups (6 pens/group, 25 birds/pen) were reared under HS condition, induced on day 32 by increasing environmental temperature to 30°C and lasted for 10 consecutive days to mimic a chronic HS.

Animals were fed *ad libitum* with commercial diets (Table 15) according to their age and had free access to water.

Table 15. Composition of the diets supplied to the birds of all the experimental groups

Item	STARTER (0-10 d)	GROWER 1 (11-25 d)	Finisher (26-42 d)
CORN	42.17	34.96	12.73
WHITE CORN	0.00	0.00	15.00
WHEAT	10.00	20.00	25.01
SORGHUM	0.00	0.00	5.00
SOYBEAN MEAL	23.11	20.63	17.60
EXPANDED SOYBEAN	10.00	10.00	13.00
SUNFLOWER	3.00	3.00	3.00
CORN GLUTEN	4.00	3.00	0.00
SOYBEAN OIL	3.08	4.43	5.48
DICALCIUM PHOSPHATE	1.52	1.20	0.57
CALCIUM CARBONATE	0.91	0.65	0.52
SODIUM BICARBONATE	0.15	0.10	0.15
SALT	0.27	0.27	0.25
COLINE CLORIDE	0.10	0.10	0.10
LYSINE SOLFATE	0.59	0.55	0.46
DL-METHIONINE	0.27	0.29	0.30
THREONINE	0.15	0.14	0.14
ENZYME - ROXAZYME G2G	0.08	0.08	0.08
PHYTASE 0.1%	0.10	0.10	0.10
COCCIDIOSTAT	0	0	0
VIT-MINERAL PREMIX [1]	0.50	0.50	0.50
DRY MATTER,%	88.57	88.65	88.64
PROTEIN,%	22.70	21.49	19.74
LIPID,%	7.06	8.24	9.74
FIBER,%	3.08	3.04	3.07
ASH,%	5.85	5.17	4.49
LYS,%	1.38	1.29	1.21
MET,%	0.67	0.62	0.59
MET+CYS,%	1.03	0.97	0.91
CALCIUM,%	0.91	0.80	0.59
PHOSPHATE,%	0.63	0.57	0.46
METABOLIZABLE ENERGY (kcal/kg)	3.076	3.168	3.264

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

9.2.2 *Slaughter surveys and sampling*

At 42 d of age, 15 randomly chosen birds/treatment/environmental condition were individually weighed (after a fasting period of 12 h) and transported within 1 h (including careful catching and loading) to a commercial poultry slaughterhouse. Birds were electrically stunned and slaughtered. At slaughter, pectoral muscle (PM), including *pectoralis major* and *pectoralis minor*, was removed from the carcass and weighed. On the right PM, pH and color were recorded at 24 h (pH24) *post mortem*. pH was measured using a portable pH meter (FiveGo, Mettler-Toledo, Switzerland) equipped with a penetrating glass electrode.

Tri-stimulus color coordinates (lightness, L*; redness, a*; yellowness, b*) were measured 24 h *post-mortem* on the bone-side surface of left-side breast fillet using a Chroma Meter CR-300 (Minolta Corporation,Italia s.r.l., Milano).

9.2.3 Water Holding Capacity, Cooking loss, and Warner-Blatzler Shear Force Analyses

Water-holding capacity, expressed as expressible juice, was measured on PM 24 hours after chilling using the press method (Grau and Hamm, 1953).

As for cooking loss determination, PM samples were individually weighed, placed in metallic trays, and introduced in the oven. All cooked samples (internal temperature 75°C) were drained from the excess liquid in a plastic net, then again individually weighed. Cooking loss was expressed as g/100 g by weight difference between uncooked and cooked samples.

For the determination of meat tenderness, meat samples were cut into 6 cores with similar sizes; each core was sheared perpendicular to the longitudinal orientation of the muscle fiber using a Warner– Bratzler shear blade with the triangular slot cutting edge mounted on Salter model 235 (Warner–Bratzler meat shear, G-R manufacturing Co. 1317 Collins LN, Manhattan, KS) to determine the peak force (kg) when the samples were sheared. Shear force was determined as the average of the maximum force of the 6 replicates from each sample.

Subsequently, pectoral muscles were vacuum packaged and stored (−20°C) until meat quality analyses.

9.2.4 Proximate composition

Proximate composition (moisture, crude protein, total fat, and crude ash) of PM was determined following standard methods. Moisture content was calculated as the percentage of weight lost after drying 5 g of sample in oven (103 ± 2°C for 16 h) (AOAC, 1990). Crude protein content was assessed according to the Kjeldahl method by using copper sulfate as catalyst (AOAC, 1990), whereas lipids were extracted following the chloroform: methanol extraction procedure (Folch et al., 1957). Crude ash content was assessed by weighing samples after incineration at 525°C (AOAC, 1990).

9.2.5 Cholesterol content

Cholesterol was extracted using the method of Marasciello et al. (1996) and then quantified by HPLC. The breast muscle sample (100 mg) was saponified with 2 ml of 0.5 N KOH in methanol for 1 hour at 80 °C. After cooling, 2 ml of distilled water saturated with NaCl was added. The tubes were vortexed followed by addition of 3 ml ether/hexane (1:1, vol/vol) and centrifuged for 10 min at 3000 g. The upper phase was recovered and the hexane/ether extraction step was repeated twice. The extracts were combined and evaporated to dryness and re-dissolved in 1 ml of acetonitrile/isopropanol (1:1) for HPLC analysis. A Kontron HPLC (Kontron Instruments, Milan, Italy) model 535, equipped with a Kinetex 5µ C18 reverse-phase column (150 x 4.6mm x 5 µm; Phenomenex, Torrance, CA), was used. The HPLC mobile phase consisted of acetonitrile:2-propanol (55:45, vol/vol) at a flow rate of 1.0 ml/min. All solvents used were LC grade. The detection wavelength was 210 nm. The quantitation of muscle cholesterol content was based on the external standard method using a pure cholesterol standard (Sigma, St. Louis, MO). The quantitation of muscle cholesterol content was based on the external standard method using a pure cholesterol standard (Sigma, St. Louis, MO).

9.2.6 Collagen analysis

Approximately 50 g of *Pectoralis superficialis* muscle (wet weight) were thawed at room temperature, trimmed of fat and epimysium, lyophilized for 24 hours, and stored frozen (-20°C) until collagen analysis. The lyophilized muscle tissue was weighed (100 mg), and hydrolyzed in Duran tubes (Schott AG, Mainz, Germany) in 5 ml of 6N HCl at 110°C for 18 to 20 h (Etherington and Sims, 1981) for determination of hydroxyproline (Woessner, 1961) and crosslinking. The hydrolyzate was filtered (Whatman No.1) and diluted with water plus. An aliquot of the hydrolyzate was removed for hydroxyproline determination and the remaining part was subjected to HLP (hydroxylysilpyridinoline) crosslink analysis.

9.2.6.1 Intramuscular collagen concentration analysis

The 4-hydroxyproline (intramuscular collagen concentration) was quantified using the colorimetric procedure of Woessner et al. (1961). The hydroxyproline was oxidated with chloramine T (sodium p-toluenesulfonchloramide) that was then inactivated by adding perchloric acid. Finally, a solution of p-dimethylaminobenzaldehyde solution was added and the tube was placed in a 60°C water bath for 20 minutes. The absorbance of the solution was then determined using a spectrophotometer (V-730, Jasco Co., Ltd., Tokyo, Japan) at 557 nm. The hydroxyproline concentration was determined directly from the standard curve of L-hydroxyproline. Intramuscular collagen concentration was calculated, assuming that collagen weighed 7.25 times the measured hydroxyproline weight (Eastoe and Leach, 1958) and expressed as micrograms of hydroxyproline per milligram of lyophilized tissue.

9.2.6.2 Crosslink concentration analysis

Hydroxylysylpyridinoline (HLP) concentration, the principal non-reducible crosslink of muscle collagen and highly correlated with the thermal stability of collagen (McCormick, 1999), was determined according to the method described by Eyre et al. (1984). Hydrolyzate HLP was concentrated and separated from the

bulk of the other amino acids by elution from a CF1 cellulose column using the procedure described by Skinner (1982). The obtained eluate, added of pyridoxamine as an internal standard, was concentrated (Speed Vac® Plus SC110A, Savant Instruments, Farmingdale, NY), resuspended in 1% (v/v) n-heptafluorobutyric acid (HFBA) and filtrated (Nylon syringe filter 0.45µm, Whatman). Quantitation of the HLP crosslink was performed by reversed phase high performance liquid chromatography (RP-HPLC). A Kontron HPLC (Kontron Instruments, Milan, Italy) model 535, equipped with a Luna C18 column (250 x 4.6 mm x 5 µm; Phenomenex, Torrance, CA), was used. The concentration of HLP residues in the samples was calculated based on the concentration of collagen in each hydrolyzate, assuming that the molecular weight of collagen was 300,000 and the molar fluorescence yield of pyridoxamine (internal standard) was 3.1 times that of HLP (Eyre et al., 1984). Crosslink concentration was expressed as moles of HLP per mole of collagen.

9.2.7 Fatty acids composition

The fatty acid composition of intramuscular fat samples was determined after chloroform-methanol extraction (Folch et al., 1957), and fatty acids were determined as methyl esters (FAME), using a gas chromatograph GC Trace 2000 (ThermoQuest EC Instruments) equipped with a flame ionization detector (260 °C) and a fused silica capillary Column (SGE Forte BP×90, Phenomenex, Torrance, CA, USA) 100 m × 0.25 mm × 0.25 µm film thickness.

Helium was used as the carrier gas at a flow rate of 1.5 mL/min with constant flow compensation. The oven temperature program was 100 °C for 5 min then increasing at 4°C/min up to 240 °C where it was maintained for 20 min. The individual FA peaks were identified by comparison of retention times with those of FAME authentic standards run under the same operating conditions. Results were expressed as percentage of the total FA identified. To assess the nutritional implications, the n-6/n-3 FA ratio and the PUFA/SFA (P/S) ratio were calculated. Moreover, to evaluate the risk of atherosclerosis and the potential aggregation

of blood platelets, respectively the atherogenic index (AI) and the thrombogenic index (TI) were calculated, according to the formulas suggested by Ulbricht and Southgate (1991).

9.3 Statistical analyses

Data were analysed by GLM procedure using the SPSS statistical package (SPSS, 2010), where treatment (GOS, S, C) and temperature (TN, HS) were the main factors. Differences among the means were determined with Scheffe's test.

9.4 Results and discussion

9.4.1 Weight and physicochemical traits of breast muscle

Results of the effect of GOS *in ovo* injected in response to heat stress on weight and physicochemical traits of PM from broiler chickens are presented in Table 16. The broiler PM weight was not influenced ($P > 0.05$) by GOS delivered i*n ovo*. Thermal challenge that was applied for the last 10 days of the rearing cycle significantly reduced (-11.7%) PM weight ($P < 0.01$). These results match those reported in earlier studies (Lu et al., 2007; Zhang et al., 2012; Cramer et al., 2018) which found a lower weight and proportion of the breast muscle in response to heat stress, due to heat-induced suppression of growth. On the other hand, heat stress stimulates the hypothalamic-pituitary-adrenal axis in poultry and increases the concentration of circulating corticosterone hormone (Sapolsky et al., 2000), which increments protein degradation and breakdown of skeletal muscle (Yunianto et al., 1997; Scanes, 2016). No significant interaction ($P > 0.05$) was found between *in ovo* treatment and HS for PM weight.

In accordance with previous researches (Maiorano et al., 2012; Tavaniello et al., 2018), *in ovo* delivery of GOS did not affect ($P > 0.05$) ultimate pH (pH_{24}) of PM; while, heat stress had a significant influence on it. Meat from heat-stressed chickens had a higher ($P < 0.01$) pH compared to chickens reared under conditions TN (6.12 *versus* 5.97, respectively). A possible explanation for this

might be that this stressful conditions could lead to the depletion of muscle glycogen reserves before slaughter and consequently may lead to higher ultimate pH values in meat of heat-stressed chickens. Similarly, Lu et al. (2007) found a higher meat pH in both fast- and slow-growing chicken strains kept at constant high ambient temperature (34 °C, from 5 to 8 weeks of age) compared to pair fed chickens kept at constant optimal ambient temperature. However, several works (Zhang et al., 2012; Cramer et al., 2018; Zaboli et al., 2019) indicated that heat stress could increase the rate of glycolysis in skeletal muscles causing a built-up of lactic acid within the muscle tissue (Zhang et al., 2012), which induces a faster pH decline with a lower ultimate pH.The pH_{24} values observed in this study are within the acceptable range for commercial poultry meats.

Color parameters were affected by both factors (Table 16). Color is an important quality attribute both for the consumer's selection of fresh meat at the retail level, and for the consumer's final evaluation and acceptance of a meat product at time of consumption (Fletcher et al., 2002). Meat from GOS and S chickens were lighter ($P < 0.01$) than that from C group; whereas, meat from this latter group showed a higher ($P < 0.05$) yellowness index (b*) compared to S meat. No significant effect of treatment on redness (a*) was found. The observed color coordinates fit within the range which is accepted for good chicken meat appearance, and even if the lightness was little bit higher than that reported for normal meat ($46 < L^* < 53$; Bianchi et al., 2005) no PSE-like condition were detected. Temperature had also an evident effect on meat color. It has been reported that the acute heat stress can increase lightness (L*) and reduce redness (a*) and yellowness (b*) of breast meat. This could be due to the denaturation of sarcoplasmatic proteins which results in scattering of light (reviewed in Zhang et al., 2012). However, in the present study, meat from HS chickens was darker ($P < 0.05$) with a higher yellowness index ($P < 0.05$), compared with meat of the TN chickens. Redness index (a*) was not affected ($P > 0.05$) by heat stress. Interactions ($P < 0.01$) between treatment and

temperature were found for L* and a* indices: *in ovo* delivery of GOS increased L* and decreased a* indices of meat from HS animals.

The WHC of meat products is another important quality attribute which has an influence on product yield, it is also relevant in terms of eating quality (Cheng and Sun, 2008). Water loss reduces the meat nutritional value because some nutrients may be lost in the exudate, resulting in a meat less tender and worst in flavour (Pelicano et al., 2003). Poor WHC in raw poultry meat results in diminished visual appeal and inferior palatability traits for consumers as well as reduced ingredient retention, protein functionality, and product yields for processors (Bowker and Zhuang, 2015). Neither HS nor GOS delivered *in ovo* had impact ($P > 0.05$) on WHC, cooking loss, and Warner–Bratzler shear force (Table 16). These results are inconsistent with the findings by Lu et al. (2007), who observed an increase in L* value and decrease in the WHC of meat from broilers exposed to heat stress. In a similar study, Cramer et al. (2018) did not found any significant effect of heat stress and probiotic feeding on color characteristics, WHC, and shear force of broiler breast muscle, but observed a lower cooking loss in heat-stressed chickens compared to those reared under thermoneutral conditions.

Table. 16 Weight and physicochemical traits of breast muscle from Ross broiler chickens injected *in ovo* with GOS in response to heat stress.

	Treatment (Tr)[1]			Temperature (T)[2]			Significance		
Traits	C	S	GOS	TN	HS	SEM	Tr	T	Tr x T
Breast muscle weight (g)	615.7	651.8	675.5	687.9	607.4	11.9	NS	**	NS
pH_{24}	6.03	6.08	6.04	5.97	6.12	0.01	NS	**	NS
Color 24 h									
L*	52.42[B]	55.07[A]	54.48[A]	54.55	53.43	0.22	**	*	**
a*	3.07	3.28	3.07	3.26	3.01	0.10	NS	NS	**
b*	5.84[a]	4.72[b]	5.46[ab]	4.92	5.76	0.18	*	*	NS
WHC (%)	12.57	12.70	13.24	13.11	12.57	0.15	NS	NS	NS
Cooking loss (%)	19.70	19.77	19.67	19.70	19.72	0.12	NS	NS	NS
WBSF[3] (Kg/cm^2)	1.19	1.23	1.13	1.19	1.19	0.04	NS	NS	NS

[1]C = Control (untreated); S = *in ovo* injected with physiological saline; GOS = *in ovo* injected with GOS

[2]TN = thermoneutral conditions; HS = heat stress conditions (on days 32 to 42).

[3]WBSF = Warner-Bratzler shear force.

SEM = standard error means.

Significance: ns = $P > 0.05$; * $P < 0.05$; ** $P < 0.01$.

[A,B] Means within a row lacking a common superscript differ ($P < 0.01$).

[a,b] Means within a row lacking a common superscript differ ($P < 0.05$).

9.4.2 Nutrients content, Cholesterol content, intramuscular collagen properties and fatty acids profile

Regarding meat nutritional properties, there was no significant difference between treatment groups in proximate composition (moisture, protein, lipid, ash), cholesterol content, and intramuscular collagen properties (Table 17). Regarding collagen, Maiorano et al. (2012) found a lower intramuscular collagen content in broiler chickens i*n ovo* injected with prebiotics and synbiotics compared to control group. The authors suggested probably due to a slightly greater PM weight and muscle fiber diameter. Collagen is an abundant connective tissue protein and is a contributing factor to the variation in meat tenderness and texture. Collagen molecules are bound together through intermolecular crosslinks that help to provide structure and strength. These crosslinks are initially reducible, but over time are replaced by mature, thermally stable, and less soluble crosslinks. These mature crosslinks, rather than the total amount of collagen, are the key factors in collagen-related toughness (Weston et al., 2002). Muscle collagen maturation values found in the present study (ranging from 0.039 to 0.042 mol of HLP/mol of collagen) resulted half than those observed by Maiorano et al. (2012) for Ross 308 broiler chickens (ranging from 0.065 to 0.079 mol of HLP/mol of collagen, in control and prebiotic group, respectively), probably due to the immaturity of collagen related to the fast-growing rate of the modern chicken strain used in this trial.

Lipid and cholesterol contents of meat have been of great interest for the researchers for decades. Cholesterol is a nutritionally important component of meat. Cholesterol, cholesterol metabolites, and immediate biosynthetic precursors of cholesterol play essential roles in cellular membrane physiology, dietary nutrient absorption, reproductive biology, stress responses, salt and water balance, and calcium metabolism. Cholesterol content has become an important component in composition studies on meat and poultry products (Dinh et al., 2011). In accordance with our findings, Tavaniello et al. (2018) did

not found any significant effect of different prebiotics, *in ovo* injected, on muscle cholesterol content.

Heat stress only affected the breast muscle content of collagen and lipid (Table 16). Total collagen concentration was lower (-11.7%) in HS group compared to TN one ($P < 0.05$). It can be assumed that the observed reduction could be related to the heat-induced changes in protein metabolism. In fact, it has been reported that high ambient temperature significantly decreased body protein content, protein gain, protein retain and intake, due to a decreased muscle protein synthesis and increased protein catabolism (reviewed by Zhang et al., 2012). Temim et al. (2000) found that protein synthesis is more susceptible than proteolysis to high environmental temperature (32°C); furthermore, it was demonstrated that heat stress determines changes in ribosomal gene transcription lowering the protein synthesis (Jacob, 1995; Temim et al., 1998). Muscle collagen maturation (mol of HLP/mol of collagen) was not affected ($P > 0.05$) by temperature.

As for proximate composition, total lipid content was higher (+ 0.44%) in HS chickens compared to C ones ($P < 0.01$). The obtained results are in accordance with those reported by Zhang et al. (2012). Exposure to high ambient temperature has been recognized as responsible of increased abdominal, subcutaneous, and intermuscular fat deposits (Ain Baziz et al., 1996; Geraert et al., 1996). A significant interaction ($P < 0.01$) between temperature and treatment was found for lipid and ash contents. In particular, in ovo delivery of GOS significantly increased the lipid content in HS animals compared to other groups (C: TN = 2.57%, HS = 2.83%; S: TN = 2.57%, HS = 3.13%; GOS: TN = 2.83%, HS = 3.34%). The increased fat deposition could be related to reduction in basal metabolism and physical activity in order to reduce metabolic heat production and maintain homeothermy (Geraert et al., 1996). Heat stress had no effect on muscle cholesterol content ($P > 0.05$).

Table 17. Proximate composition, cholesterol content and intramuscular collagen properties of breast muscle from Ross broiler chickens injected *in ovo* with GOS in response to heat stress.

	Treatment (Tr)[1]			Temperature (T)[2]			Significance		
	C	S	GOS	TN	HS	SEM	Tr	T	Tr x T
Moisture (%)	74.47	74.66	74.24	74.59	74.32	0.11	NS	NS	NS
Protein (%)	22.32	21.80	22.09	22.16	21.98	0.12	NS	NS	NS
Lipid (%)	2.70	2.85	3.09	2.66	3.10	0.08	NS	**	**
Ash (%)	0.90	0.93	0.90	0.90	0.92	0.01	NS	NS	**
Cholesterol (mg/100g)	38.25	36.98	37.61	36.86	38.87	0.54	NS	NS	NS
Total collagen (μg/mg)[3]	13.85	13.15	12.94	14.14	12.49	0.31	NS	*	NS
HLP[4] (mol/mol of collagen)	0.040	0.039	0.042	0.040	0.041	0.001	NS	NS	NS

[1]C =Control (untreated); S =*in ovo* injected with physiological saline; GOS = *in ovo* injected with GOS.
[2]TN = thermoneutral conditions; HS = heat stress conditions (on days 32 to 42).
[3]liophylized muscle tissue.
[4]HLP = hydroxylysilpiridinoline.
Significance: ns = *P*> 0.05; * *P*< 0.05; ** *P*<0.01.

Results of the effect of GOS *in ovo* injected in response to heat stress on fatty acid (FA) composition of PM from broiler chickens are presented in Table 18.

Considering the important role of meat in the human diet and the estimated consumption growth through the years, and the concerns related to its role in human health, several studies have focused on ways of improving meat fatty acid composition. The importance of a relatively high intake of (PUFAs) in human nutrition is now generally accepted; PUFA should constitute 7% of total energy consumed (Ralph, 2000). Taking into account the general FA profile, total PUFA were the most abundant FA (ranging from 38.50 to 42.81%), followed in descending order by SFA (ranging from 36.95 to 39.27%) and monounsaturated fatty acids (MUFA, ranging from 20.18 to 22.23%). Total SFA content was similar ($P > 0.05$) among treatment groups. Similarly, the concentration of the single SFA showed no significant difference among groups, except for C22:0 that was higher ($P < 0.05$) in GOS group compared with C group, with intermediate value in S

group (P > 0.05). Palmitic (C16:0) and stearic (C18:0) acids were the most abundant SFA, while other detected SFA (C14:0, C15:0, C17:0, C20:0, C22:0, and C24:0) were less than 1% each. SFAs promote apoptosis and increase total cholesterol (Dhayal et al., 2008). Palmitic acid is associated with increase in total serum cholesterol in human, stearic acid on the other hand is generally considered to be a neutral fatty acid because it has been shown to have no net impact on the plasmatic level of either LDL or HDL cholesterol in humans (Yu et al., 1995). Likewise, both the total and the single MUFA amounts were not affected (P > 0.05) by GOS *in ovo* injection. MUFA were mainly in the form of oleic acid (C18:1 n-9), ranging from 18.06 to 19.06%. Treatment significantly affected (P < 0.05) the total PUFA content, which was slightly lower (P = 0.077) in GOS group compared to S one, with intermediate values (P > 0.05) for C group. The same trend was found for n-6 PUFA (P = 0.062). The precursor of the n-6 family, the linoleic acid (C18:2), quantitatively the most concentrated n- 6 PUFA (from 23.34 to 24.40%), was not affected (P > 0.05) by the prebiotic treatment; however, significant differences (P < 0.01) were observed for arachidonic acid (C20:4 n-6), which was higher (P < 0.05) in C and S groups as compared with GOS group. On the contrary, total n-3 PUFA and individual n-3 PUFA were not affected by GOS (P > 0.05). Tavaniello et al. (2018), found that GOS *in ovo* injected increased the content of SFA and PUFA, and reduced MUFA content in breast muscle of chickens. However, it must be taken into account that FA composition of meat greatly depends on diet composition, but also on the production of short-chain Fas and their amount. The nutritional ratios (n6/n3, P/S, AI, and TI) were not affected (P > 0.05) by treatment (Table 19). On the contrary, Tavaniello et al. (2019) found that treatment with GOS increased P/S ratio (0.88 *vs* 0.83) and reduced both n-6/n-3 ratio (2.31 *vs* 4.25, respectively) and AI (0.62 *vs* 0.73), as compared with control group. The n-6/n-3 ratio found in the present study is at a distance from the ideal value of 1 and above the recommended maximum of 4. However, based on cardiovascular considerations, the dietary advice for the adult population should be 250 mg for

eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA) (EFSA, 2017). Considering that the average lipid content of PM found in this study is about 2.9 g/100 g, and the average content of EPA+DHA is about 1.3%, the intake of these long-chain PUFA n-3 per day (38 mg/100 g) is able to satisfy 15% of the daily long-chain PUFA n-3 requirement. The P/S values observed in the present study are favorably high (ranging from 1.03 to 1.17). From a nutritional point of view, a higher P/S ratio is recommended; indeed, it should be increased to above 0.4 (Wood et al., 2004). The AI and TI represent criteria for evaluating the level and interrelation through which some FA may have atherogenic or thrombogenic properties, respectively. The low AI and TI values found in the current study revealed a good nutritional quality of the meat. Heat stress had a marginal effect on FA composition (Table 18). Total contents of SFA, MUFA, and PUFA were similar ($P > 0.05$) between two groups. Also, the composition of the single FA was slightly affected by temperature; in particular, arachidonic acid (C20:4 n-6) was higher ($P < 0.05$) in HS group compared to TN one, as well as other long-chain PUFA of both n-3 (C22:5, C22:6) and n-6 (C22:2, C22:4) series were higher ($P < 0.01$ and 0.05) in HS group; consequently, the total content of n-3 PUFA was also higher ($P < 0.05$). Other statistically significant differences ($P < 0.05$) were found for C14:0, C17:0, C20:1, which were present in very small amount (less than 1%). The nutritional ratios (n6/n3, P/S, AI, and TI) were not affected ($P > 0.05$) by temperature. To our knowledge the information regarding the effect of heat stress on FA composition of chicken meat is limited. In a study conducted on French local broiler chicken, Ain Baziz et al. (1996) found that meat from heat-exposed birds (32°C from 4 to 7 wk old) had the same FA profile than that of control chickens with *ad libitum* feeding, while in pair-feeding conditions, heat-exposed birds showed a higher SFA and lower PUFA contents compared to control chickens. In general, the effect of heat exposure upon FA composition needs more deepen study. Several interactions between treatment and temperature were found for FA composition. In particular, it was found that in ovo delivery of GOS decreased ($P < 0.01$) SFA content (C: TN = 34.26%, HS =

40.33%; S: TN = 35.47%, HS = 38.94%; GOS: TN = 45.70%, HS = 34.13%) and increased ($P < 0.05$) MUFA content (C: TN = 23.95%, HS = 17.74%; S: TN = 21.73%, HS = 18.25%; GOS: TN = 19.26%, HS = 24.31%) in HS animals. Significant interactions ($P < 0.01$ and $P < 0.05$) were also found for all nutritional indices with a positive effect of GOS treatment.

Table 18. Fatty acid composition (% of total fatty acid) and nutritional indices in breast muscle from Ross broiler chickens injected *in ovo* with GOS in response to heat stress.

	Treatment (Tr)[1]			Temperature (T)[2]			Significance		
	C	S	GOS	TN	HS	SEM	Tr	T	TrxT
Fatty acids									
C 14:0	0.40	0.45	0.45	0.37	0.50	0.26	NS	*	NS
C 14:1	0.21	0.13	0.30	0.21	0.22	0.06	NS	NS	*
C 15:0	0.46	0.48	0.53	0.36	0.63	0.08	NS	NS	***
C 16:0	24.59	24.34	27.81	26.49	24.60	1.00	NS	NS	**
C 16:1	1.69	1.72	2.53	2.00	1.95	0.17	NS	NS	***
C 17:0	0.38	0.28	0.22	0.18	0.41	0.05	NS	*	**
C 18:0	10.56	11.02	9.50	9.99	10.77	0.53	NS	NS	NS
C 18:1 n-9	19.05	18.06	19.06	19.38	18.02	0.70	NS	NS	*
C 18:2 n-6	23.34	23.97	24.40	24.94	22.78	0.74	NS	NS	**
C 18:3 n-6	0.22	0.17	0.27	0.26	0.18	0.03	NS	NS	NS
C 18:3 n-3	1.72	1.70	1.79	1.78	1.69	0.17	NS	NS	**
C 20:0	0.20	0.13	0.16	0.15	0.18	0.03	NS	NS	**
C 20:1	0.16	0.13	0.24	0.27	0.07	0.05	NS	*	NS
C 20:2n-6	1.28	1.48	1.17	1.27	1.36	0.07	NS	NS	**
C 20:3n-6	1.10	1.30	1.25	1.15	1.29	0.03	NS	NS	*
C 20:4 n-6	9.63[a]	9.54[a]	5.72[b]	7.12	9.57	0.57	**	*	NS
C 20:5 n-3	0.51	0.51	0.48	0.39	0.62	0.04	NS	NS	NS
C 22:0	0.11[b]	0.13[ab]	0.21[a]	0.16	0.14	0.01	*	NS	**
C 22:1	0.09	0.14	0.10	0.06	0.16	0.03	NS	NS	**
C 22:2n-6	0.45	0.50	0.24	0.15	0.67	0.10	NS	*	
C 22:4n-6	0.78	0.79	0.65	0.57	0.93	0.09	NS	*	***
C 22:5 n-3	1.75	1.71	1.65	1.61	1.80	0.11	NS	**	*
C 22:6 n-3	0.90	0.84	0.67	0.68	0.93	0.06	NS	*	NS
C 24:0	0.26	0.17	0.39	0.26	0.28	0.12	NS	NS	NS
Partial sum									
ΣSFA	36.95	37.01	39.27	37.96	37.52	0.94	NS	NS	**
ΣMUFA	21.19	20.18	22.23	21.92	20.42	0.76	NS	NS	*
ΣPUFA	41.86	42.81	38.50	40.12	42.06	0.73	*	NS	NS
Σn-6	36.81	37.74	33.71	35.46	36.77	0.66	*	NS	NS
Σn-3	6.33	6.54	5.96	5.93	6.65	0.18	NS	*	NS

[1]C =Control (untreated); S =*in ovo* injected with physiological saline; GOS = *in ovo* injected with GOS.
[2]TN = thermoneutral conditions; HS = heat stress conditions (on days 32 to 42).
[3]SFA = saturated fatty Q4 acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
SEM = standard error mean. Significance: ns = *P*> 0.05; * *P*< 0.05; ** *P*<0.01, ** *P*<0.001.
[a,b] Means within a row lacking a common superscript differ (*P* < 0.05).

Table 19. Nutritional indices in breast muscle from Ross broiler chickens injected *in ovo* with GOS in response to heat stress.

	Treatment (Tr)[1]			Temperature (T)[2]			Significance		
	C	S	GOS	TN	HS	SEM	Tr	T	TrxT
Nutritional indices[3]									
n-6/n-3	5.94	5.88	5.69	6.02	5.63	0.14	NS	NS	*
P/S	1.17	1.17	1.03	1.09	1.15	0.04	NS	NS	*
AI	0.42	0.41	0.50	0.46	0.43	0.02	NS	NS	**
TI	0.75	0.74	0.84	0.80	0.75	0.03	NS	NS	*

[3]P/S = PUFA/SFA ratio; [3]AI = atherogenic index; TI = trombogenic index.
SEM = standard error mean.
Significance: ns = *P*> 0.05; * *P*< 0.05; ** *P*<0.01, ** *P*<0.001.
[a,b] Means within a row lacking a common superscript differ (*P* < 0.05).

9.5 Conclusion

The present experiment was conducted to investigate meat quality traits in fast-growing chickens stimulated *in ovo* with trans-galactoolighosaccarides (GOS) and exposed to heat stress. From the overall examination of the results of this study, it can be highlighted that:

- GOS treatment had no effect on PM weight, ultimate pH, water holding capacity, cooking loss and shear force;
- GOS and S birds had lighter PM than C group (L*, $P < 0.01$); while, C group had a higher ($P < 0.05$) yellowness index (b*) compared to S group;
- Proximate composition, cholesterol and intramuscular collagen properties were not affected by GOS treatment;
- As for fatty acid composition only total polyunsaturated fatty acids (PUFA) content and n-6 PUFA were slightly lower ($P = 0.077$ and 0.062, respectively) in GOS group compared to S one;
- Heat stress had a detrimental effect on PM weight ($P<0.01$) and increased meat pH ($P<0.01$);
- PM from HS chickens was darker with a higher b* index ($P<0.05$) and had a higher ($P<0.01$) lipid content and a lower ($P<0.05$) total collagen amount;
- Total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and PUFA were not affected by GOS treatment;
- Significant interactions between factors were found for lightness and yelloness of meat, as well as for lipid, ash and fatty acid composition. In particular, GOS decreased ($P<0.01$) SFA and increased ($P<0.05$) MUFA contents in HS birds.

In conclusion, the results of this investigation show that *in ovo* injection of GOS prebiotic had no negative effect on physicochemical and nutritional properties of meat; furthermore, GOS could contrast the negative effect of heat exposure on FA composition, with positive effect from the nutritional point of view.

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