UNIVERSITY OF MOLISE
Department of Agricultural, Environmental and Food Sciences

INTERNATIONAL Ph.D. in
"WELFARE, BIOTECHNOLOGY AND QUALITY OF ANIMAL PRODUCTION"
(XXIV CYCLE)

Related disciplinary scientific section: 07/G1 (Scienze e Tecnologie Animali)

General Coordinator: Prof. Giuseppe Maiorano

Doctorate Thesis Title:

Effect of prebiotic and synbiotic injected *in ovo* on performance, meat quality and hystopathological changes in muscle of broiler chickens

PhD Candidate:
Dr. Daniela Cianciullo

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To my beloved husband and son, and my parents.

Their true love and happiness are giving me strength and motivation. Thank you for everything that you have done for me. With all my love, this is for you.

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I owe million thanks and a debt of gratitude to many people who made this doctoral dissertation possible.

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Abstract

In poultry farming, the intestinal microbiota and the "gut health" are topical subjects, especially since the EU has banned the use of auxinic antibiotics to avoid the onset of antibiotic resistance and safeguard the consumer health. As a consequence of their prohibition, a higher incidence of enteric diseases is observed in poultry farming with loss of productivity and increased mortality. In the post-antibiotics era, probiotics and prebiotics are proposed as a solution to the intestinal problems of poultry. Studies carried on these bioactives, administered in feed or water, show conflicting results due to the different environmental conditions (experimental and field conditions) and the way of use. The "*in ovo*" injection of pre-/pro-biotics and their combination (synbiotic), an emergent and original technique, shows promising results similar to those of auxinic antibiotics. The work aimed to evaluate the effects of these substances, "*in ovo*" administered, on growth performance, meat quality traits (cholesterol content, intramuscular collagen properties, fiber measurements), and the presence of histopathological changes in the pectoral muscle (PS) of Ross 308 broiler chickens. On d 12 of incubation, 480 eggs were randomly divided into five experimental groups treated with different bioactives, *in ovo* injected: C, control with physiological saline solution; T1 with 1.9 mg of Raffinose Family Oligosaccharides (RFOs); T2 and T3 with 1.9 mg of RFOs enriched with two different homemade probiotic bacteria (from Microbiological Bank of Institute of Biochemistry and Biophysics, Warsaw, Poland), specifically 1,000 cfu of *Lactococcus lactis* ssp. *lactis* SL1 and *Lactococcus lactis* ssp. *cremoris* IBB SC1, respectively; T4 with commercially available synbiotic Duolac, containing 500 cfu of both *Lactobacillus acidophilus* and *Streptococcus faecium* with the addition of lactose (0.001 mg/embryo). Among the hatched chickens, sixty males were randomly chosen (12 birds for each group) and reared according to the animal welfare recommendations of European Union directive 86/609/EEC in an experimental poultry house that provided good husbandry conditions. Birds were grown up to 42 d in collective cages (n = 3 birds in each 4 cages: replications for experimental groups). Broilers were fed *ad libitum* commercial diets according to their age and water was provided *ad libitum*. Amounts of feed offered to each cage were recorded, and uneaten feed in each cage was weighed daily (from 1 to 42 d). Cumulative feed intake and feed conversion ratio (FCR) were calculated on a cage basis. At 42 d of age, broilers were weighed individually (after a fasting period of 12 h) and then were electrically stunned and slaughtered at a commercial poultry slaughterhouse. At slaughter, hot carcass weight was recorded, and carcass yield percentage was calculated. Abdominal fat was removed, measured and its percentage was calculated based on hot carcass weight. The pectoral muscle was removed from all carcasses (n = 60) and its percentage was calculated based on hot carcass weight. In addition, pectoral muscle pH was recorded at 45 min (pH_{45}), 12 h (pH_{12}), and 24 h (pH_{24}) *postmortem*. Samples of the right pectoral muscle of 40 animals, 8 birds from each experimental group, were taken and frozen in liquid nitrogen (-196ºC) for histological and histopathological analyses. The left pectoral muscle was vacuum packaged and stored frozen (-40°C) until intramuscular collagen (IMC) and cholesterol analyses. To verify significant differences in relation to the treatments, the data were evaluated by using 1-way ANOVA and means were separated by Scheffe's battery of pairwise tests (SPSS Inc.,

2010). *In ovo* prebiotic and synbiotics administration had a low effect on investigated traits, but depend on the kind of bioactives administered. Commercial synbiotic treatment (T4) reduced carcass yield percentage, and the feed conversion ratio was higher in T3 and T4 groups compared with other groups. The abdominal fat, the ultimate pH, and cholesterol of the PS were not affected by treatment. Broiler chickens of treated groups with both slightly greater PS and fiber diameter had a significantly lower amount of collagen. The greater thickness of muscle fibers (not significant) and the lower fiber density (statistically significant), observed in treated birds in comparison with those of C group, are not associated with histopathological changes in the PS of broilers. The incidence of histopathological changes in broiler chickens from examined groups was low, which did not affect the deterioration of meat quality obtained from these birds.

Overall, the results obtained at the end of this work have asserted how the *in ovo* administration, in showing greater effectiveness in terms of uniformity of application, dose used and duration of treatment, as well as homogeneity of the study population (age, weight), may represent a valid alternative to the traditional and well-established methods of post-hatching administration (feed and water) in order to minimize all those variables that could affect the effectiveness of bioactive. Moreover, the study has provided information for an effective application of these natural agents to be used in the future in breeding industry, with significant and positive impact on animal welfare and public health.

Riassunto

Nel pollame, il microbiota intestinale e la "gut health" sono temi di attualità, soprattutto da quando l'UE ha vietato l'uso di antibiotici auxinici per evitare l'insorgenza dell'antibiotico-resistenza e salvaguardare, così, la salute del consumatore. Come conseguenza della loro proibizione, in avicoltura si è osservata una maggiore incidenza di malattie enteriche che hanno determinato perdite di produttività ed un aumento della mortalità. Nell'era post-antibiotici, i probiotici ed i prebiotici sono proposti come soluzione dei problemi intestinali dei polli. Gli studi condotti su tali composti bioattivi, somministrati direttamente nel cibo o in acqua, mostrano risultati contrastanti a causa sia delle diverse condizioni ambientali (condizioni sperimentali e di campo) che del modo di utilizzo. L'iniezione *in ovo* di pre-/pro-biotici e loro combinazione (simbiotici), tecnica emergente ed innovativa, mostra risultati promettenti simili a quelli degli antibiotici auxinici. Il presente lavoro ha avuto come scopo quello di valutare gli effetti di prebiotici, utilizzati da soli od in combinazione con batteri probiotici rigorosamente selezionati e caratterizzati (simbiotici; Bardowski and Kozak, 1981;. Boguslawska et al, 2009), somministrati "*in ovo*", sulle performance di crescita, sulla qualità della carne (contenuto di colesterolo, proprietà del collagene intramuscolare, misure delle fibre muscolari), nonché sull'incidenza di alterazioni istopatologiche a carico del muscolo pettorale di polli da carne linea Ross 308. Al 12° giorno di incubazione, 480 uova sono state divise a *random* in cinque gruppi sperimentali, cui sono stati somministrati, mediante tecnica di iniezione *in ovo*, differenti bioattivi: C, gruppo controllo iniettato con soluzione fisiologica; gruppo T1 iniettato con 1,9 mg di prebiotico (oligosaccaride della famiglia del raffinosio, RFOs); gruppi T2 e T3 iniettati con due formulazioni simbiotiche contenenti 1,9 mg di RFOs arricchiti con due diversi batteri probiotici appartenenti alla banca microbiologica dell'Istituto di Ricerca "Biochemistry and Biophysics" (IBB) di Varsavia (Polonia), nello specifico 1,000 ufc di *Lactococcus lactis* ssp. *lactis* SL1 e *Lactococcus lactis* ssp. *cremoris* IBB SC1, rispettivamente; gruppo T4 iniettato con un probiotico disponibile in commercio quale Duolac, contenente 500 ufc di *Lactobacillus acidophilus* e 500 ufc di *Streptococcus faecium* con aggiunta di lattosio (0.001 mg/embrione). Dopo la schiusa, sessanta pulcini maschi sono stati scelti a *random* (12 pulcini per ogni gruppo) e allevati in azienda sperimentale in buone condizioni di allevamento secondo le raccomandazioni previste dall'Unione europea in materia di benessere animale (direttiva 86/609/CEE). I polli sono stati allevati fino a 42 giorni di età in gabbie collettive (n = 3 uccelli per gabbia: 4 repliche per ciascun gruppo sperimentale), sono stati alimentati *ad libitum* con diete commerciali formulate in funzione della loro età e hanno potuto usufruire di acqua *ad libitum*. E' stata registrata la quantità di mangime offerto per gabbia, ed il cibo non consumato è stato pesato giornalmente per ciascuna gabbia (1-42 giorni). L'assunzione di cibo e l'indice di conversione alimentare (FCR) sono stati calcolati per gabbia. A 42 giorni di età, i polli da carne sono stati pesati individualmente (dopo un periodo di digiuno di 12 ore) e sono stati, successivamente, storditi elettricamente e macellati presso un macello commerciale. Al momento della macellazione, è stato registrato il peso della carcassa e calcolata la resa. Il grasso addominale ed il muscolo pettorale sono stati rimossi da tutte le carcasse (n = 60), pesati e le rispettive percentuali sono state calcolate in funzione del peso della carcassa. Inoltre, è

stato registrato il pH del muscolo pettorale a 45 min (pH_{45}), 12 ore (pH_{12}), e 24 h (pH_{24}) *post-mortem*. I campioni di muscolo pettorale destro di 40 animali, 8 volatili per ciascun gruppo sperimentale, sono stati prelevati e congelati in azoto liquido (-196°C) per le analisi istologiche ed istopatologiche. Il muscolo pettorale sinistro è stato imballato sottovuoto e congelato (-40°C) fino al momento delle analisi del collagene intramuscolare (IMC) e del contenuto di colesterolo. Al fine di verificare le differenze significative rispetto al trattamento applicato, i dati sono stati valutati utilizzando l'ANOVA ad una via. Le differenze tra le medie sono state valutate mediante il test di Scheffé (SPSS Inc., 2010). La somministrazione *in ovo* di prebiotico e simbiotico ha avuto un impatto limitato sulle caratteristiche prese in esame, ciò in dipendenza del tipo di bioattivo impiegato. Il trattamento con simbiotico commerciale (T4) ha determinato una riduzione della resa alla macellazione, e l'indice di conversione alimentare è risultato maggiore nei gruppi T3 e T4 rispetto agli altri gruppi. Il grasso addominale, il pH_{24}, ed il contenuto di colesterolo del muscolo pettorale non sono stati influenzati dal trattamento. I polli da carne dei gruppi trattati, con un'incidenza lievemente maggiore del muscolo pettorale ed un diametro delle fibre leggermente più grande, presentavano una quantità di collagene intramuscolare significativamente inferiore. Il maggiore spessore delle fibre muscolari (non significativa) e la minore densità delle fibre (statisticamente significativa), osservata nei polli trattati rispetto a quelli del gruppo controllo, non sono stati associati alle alterazioni istopatologiche del muscolo pettorale. L'incidenza delle istopatologie nei polli da carne dei gruppi esaminati è risultata bassa e non ha influenzato il deterioramento, in termini di qualità, della carne ottenuta da questi polli. Nel complesso, i risultati ottenuti da questo lavoro hanno consentito di affermare come la somministrazione *in ovo*, presentando maggiore efficacia in termini di uniformità di applicazione, di dose impiegata e di durata del trattamento nonché di omogeneità della popolazione di studio (età, peso), può rappresentare una valida alternativa ai tradizionali e già consolidati metodi di somministrazione post-schiusa (mangime e acqua) per ridurre tutte quelle variabili che possono inficiare l'efficacia dei bioattivi. Inoltre, hanno fornito informazioni per un uso efficace di questi agenti naturali da impiegare, in futuro, in allevamento industriale, con importanti e positive ricadute sulla salute animale e pubblica.

PART 1: INTRODUCTION

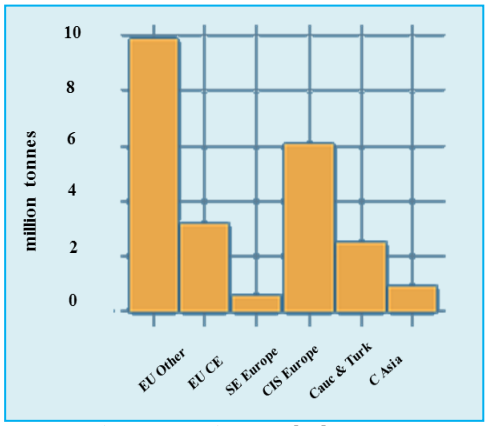
Chapter 1. Poultry production overview

1.1 Historic advances in poultry industry

Poultry meat and eggs during the last 50 years have become among the most affordable food staple for people throughout the world. As technological development advanced during the twentieth century, the world's population became more urbanized (Lobo, 2006) and dependent upon concentrated large-scale food production that can be easily and efficiently transported to metropolitan areas. The poultry industry led this transformation from subsistence agriculture to integrated production to feed this growing urbanized society, elevating the consumption of poultry products ahead of other animal products as the least expensive and most popular animal protein source (Hammerstedt, 1999). Chickens were imported to America from Europe and Asia during the 1800's, where they were raised for eggs and meat in the backyards of most homes. Indeed, the most profitable part of the poultry business until the early 1920's was for competitive exhibitions rather than for food (Winter and Funk, 1960; Hanke et al., 1972). But then the profit on poultry production shifted from the exhibitions fancy feather breeds to the egg and meat production poultry (Winter and Funk, 1960; Hanke et al., 1972). Emphasis on poultry meat and egg production accelerated after Herbert Hoover's 1928 presidential campaign promise of "A chicken in every pot…and a car in every garage." The outcome was so successful that since 1992 poultry meat has been the most consumed meat in the US, surpassing beef and pork, based in the retail weight series (Smith, 2006a). The growth of the poultry industry has been increasing over the time. In Italy were raised 115 million broiler chickens in 2008, 120 million in 2009 and 130 million in 2010 (Faostat, 2012). The consumption of chicken meat in Italy was 19 kg per capita in 2009 (Eurostat, 2012).

The poultry meat output is 17.6 million tonnes, which is 29.3 percent of the global total. After EU (Europe Union) other and EFTA (European Fair Trade Association), and CIS (Commonwealth of Independent States) Europe, EU Central and Eastern is the most important subregion in terms of poultry production, which for 14 percent of total regional production in 2010 (Figure 1.1).

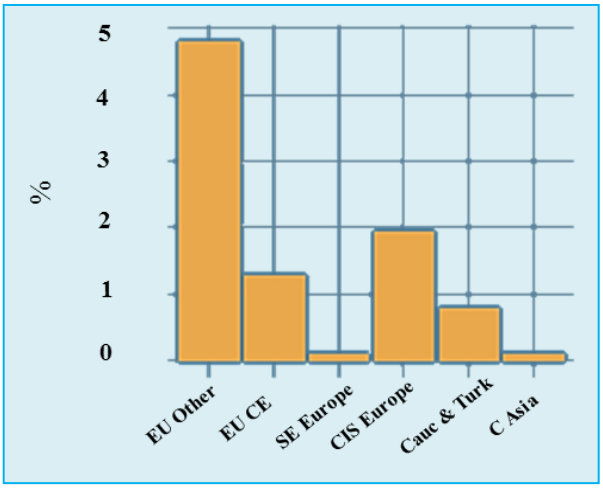
Figure 1.1. Poultry meat production (2010)



Source: Statistics Division (FAOSTAT). Metalink: P3.REU.FAO.ESS.POULSC, p.111.

The poultry sector is one of the most rapidly growing subsectors of the livestock industry. Production is becoming more intensive and vertically integrated due to technological advancements. Global demand is expected to continue growing, and Asia, prominently China, is playing a main role in this trend. In 2010 there were more than 21 billion heads of poultry in the world, and 11 percent of this global stock was in this region. The EU other and EFTA accounted for five percent of the global stock, CIS Europe for three percent, EU Central and Eastern for two percent. The other sub-regions of Europe and Central Asia had lower shares. More than 83 percent of the 2.5 billion heads of poultry in this region (2010 data) was in CIS Europe, and the EU and EFTA sub-regions. The Caucasus and Turkey accounted for another 11 percent. Nearly 404 million heads of poultry was kept in the Russian Federation, 234 million in Turkey and 190 million in Ukraine. There are further significant stocks in countries like France, the United Kingdom, Italy and Spain. In 2010, the average number of poultry in the world was 422 thousand per hundred hectares. Generally, intensity tends to decrease from west to east, which can be explained by the general economic conditions and technological gaps between the countries (Figure 1.2).

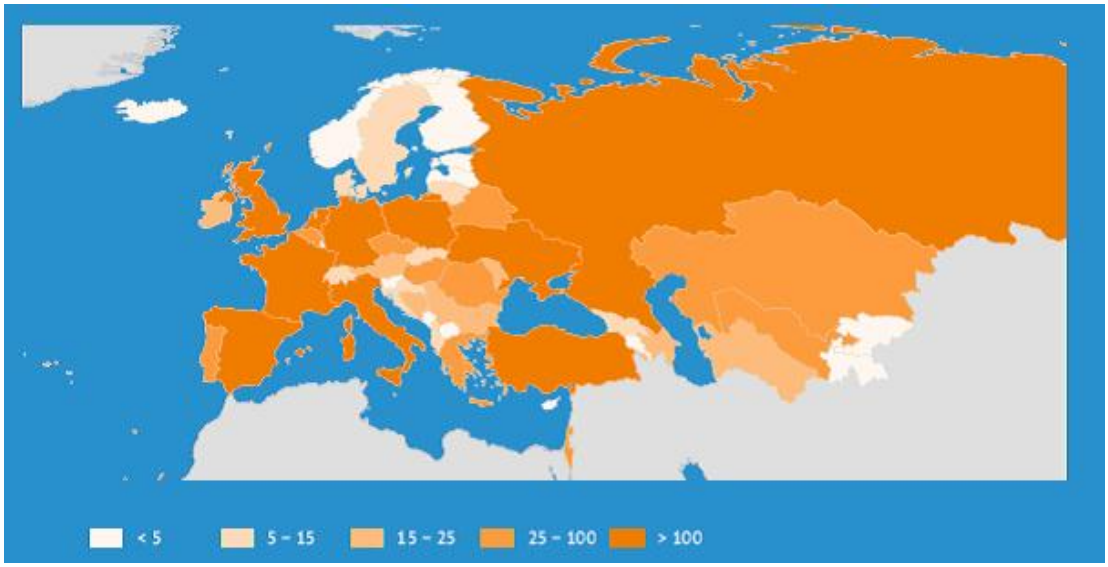
Figure 1.2. Stock of poultry, share of world total (2010)



Source: Statistics Division (FAOSTAT). Metalink: P3.REU.FAO.ESS.POULSC, p.111.

In EU other and EFTA the indicator was 744,000 heads per capita, while in EU Central and Eastern the per capita figure was 662,000 and 226,000 in CIS Europe. Globally, poultry numbers have increased by two thirds over the last two decades (Figure 1.3). In line with this, there has been significant growth in South Eastern Europe, the Caucasus and Turkey and in EU Central and Eastern, while contrary to this global trend, in CIS Europe and Central Asia the poultry population has fallen considerably (Faostat, 2012).

Figure 1.3. Mapping of poultry (million heads, 2010)



Source: Statistics Division (FAOSTAT).Metalink: P3.REU.FAO.ESS.POULSC, p.111.

There are several reasons for the success of the poultry industry and the phenomenal growth in consumer demand for its products, including rapid application of scientific discoveries, specialization into integrated production systems, and free acceptance in world market. Since its' beginning, the poultry industry has being strongly dependent on research (Smith, 2006a). Large scale production was only possible after the discovery of vitamins, improvement of artificial incubation, introduction of disease test kits, vaccination, and breeder genetic selection application (Winter and Funk, 1960; Hanke et al., 1972; Morris, 1980). This continuous industry progress is based on the application of scientifically-proven technologies that help poultry growers in improving production yield, bird health and nutrition, housing, genetics and reproduction, food quality, safety, and efficiency (Smith, 2006a). The best example of dramatic advances can be seen in animal performance, especially improvements in body weights at market. A typical 1957 chicken strain reached 539 g of body weight at 42 days of age, while a 2001 commercial broiler chicken strain reached 2,672 g at the same 42 days of age, which constitutes remarkable progress (Havenstein et al., 2003). This improvement in growth performance and meat yield was driven by costumer preference for white breast meat products, and the industry's focus on greater meat yields per unit of input costs (Smith, 2006b).

Today, most of the broiler carcasses are deboned and further processed and the amount of poultry meat produced are sustained by heavier birds, even when the number of birds decreases (Smith, 2006b). Such accomplishment could only be reached by constant research and innovation to overcome challenges, and there is still more room for improvement. Another reason why the poultry industry has grown so much is attributed to having a refined and specialized segmentation. A few breeding companies provide elite genetic stock, while production companies grow out hybrid crosses to produce the final product (Hammerstedt, 1999). The grower receives a technology package and mainly concentrates on managing the birds, while genetic companies concentrate in producing a more efficient bird every generation. Finally, the phenomenal growth of the poultry industry would not have occurred if not for few social and trade barriers. There are no

barriers against poultry meat, like religious or cultural impediments that exist to other meats. This allied to attractive prices, led exports to boom worldwide, providing to the industry an even greater incentive for growth. In other words, poultry is the perfect animal product to feed a globalized world.

1.2 Origins of domestic fowl

Modern chickens were domesticated from the Red Jungle Fowl (Gallus gallus) over the last four or five thousand years for eggs and meat, for game and for exhibition (Winter and Funk, 1960; Roberts, 1998; Klasing, 2005) and they are scientifically classified as the same species (Wong et al. 2004). As such it can and does freely interbreed with populations of red jungle fowl (Wong et al. 2004). The traditional poultry farming view is stated in Encyclopædia Britannica: "Humans first domesticated chickens of Indian origin for the purpose of cockfighting in Asia, Africa, and Europe. Very little formal attention was given to egg or meat production... " (Garrigus, 2007). In the last decade there have been a number of genetic studies. According to one study, a single domestication event occurring in the region of modern Thailand created the modern chicken with minor transitions separating the modern breeds (Fumihito et al., 1994). However, that study was later found to be based on incomplete data, and recent studies point to multiple maternal origins, with the clade found in the Americas, Europe, Middle East, and Africa, originating from the Indian subcontinent, where a large number of unique haplotypes occur (Liu et al., 2006; Zeder et al., 2006). It has been claimed (based on paleoclimatic assumptions) that chickens were domesticated in Southern China in 6000 BC (West and Zhou, 1988). However, according to a recent study (Al-Nasser et al., 2007), "it is not known whether these birds made much contribution to the modern domestic fowl. Chickens from the Harappan culture of the Indus Valley (2500-2100 BC), in what today is Pakistan, may have been the main source of diffusion throughout the world."

A northern road spread chicken to the Tarim basin of central Asia. The chicken reached Europe (Romania, Turkey, Greece, Ukraine) about 3000 BC (Kiple and Ornelas, 2000). Introduction into Western Europe came far later, about the 1st millennium BC. Phoenicians spread chickens along the Mediterranean coasts, to Iberia. Breeding increased under the Roman Empire, and was reduced in the Middle Ages (Kiple and Ornelas, 2000). Middle East traces of chicken go back to a little earlier than 2000 BC, in Syria; chicken went southward only in the 1st millennium BC. The chicken reached Egypt for purposes of cock fighting about 1400 BC, and became widely bred only in Ptolemaic Egypt (about 300 BC) (Kiple and Ornelas, 2000). Little is known about the chicken's introduction into Africa. Three possible ways of introduction in about the early first millennium AD could have been through the Egyptian Nile Valley, the East Africa Roman-Greek or Indian trade, or from Carthage and the Berbers, across the Sahara. The earliest known remains are from Mali, Nubia, East Coast, and South Africa and date back to the middle of the first millennium AD. Domestic chicken in the Americas before Western conquest is still an ongoing discussion, but blue-egged chicken, found only in the Americas and Asia, suggest an Asian origin for early American chickens. A lack of data from Thailand, Russia, the Indian subcontinent, Southeast Asia and Sub-Saharan Africa makes it difficult to lay out a clear map of the spread of chickens in these areas; better description and genetic analysis

of local breeds threatened by extinction may also help with research into this area (Kiple and Ornelas, 2000).

1.3 Broiler chickens

Broilers are chickens (*Gallus gallus domesticus*) bred and raised specifically for meat production.(Kruchten, 2002). Chickens are one of the most common and widespread domestic animals, and although the global population has decreased from more than 24 billion in 2003 (Perrins, 2003) to 19 billion in 2011 (Faostat, 2012), there are more chickens in the world than any other species of bird.

1.3.1 General biology

Modern commercial broilers are specially bred for large scale, efficient meat production and although they are the same species, grow much faster than egg laying hens or traditional dual purpose breeds. They are noted for having very fast growth rates, a high feed conversion ratio, and low levels of activity. Broilers often reach a slaughter weight of four to five pounds (dressed) in only five weeks (Damerow, 1995), although more slow growing free-range and organic strains reach slaughter weight at 12 to 16 weeks of age. As a consequence, their behaviour and physiology are those of immature birds rather than adults. Typical broilers have white feathers and yellowish skin. Recent genetic analysis has revealed that at least the gene for yellow skin was incorporated into domestic birds through hybridization with the Grey Junglefowl (*Gallus sonneratii*) (Eriksson et al., 2008). Modern crosses are also favorable for meat production because they lack the typical "hair" which many breeds have that necessitates singeing after plucking. Both male and female broilers are reared for their meat.

1.3.2 Behaviour

Because broiler chickens are the same species as egg laying hens, their behavioural repertoires are initially similar, and also similar to those of other gallinaceous birds. However, broiler behaviour is modified by the environment and alters as the broilers' age and bodyweight rapidly increase. For example, the activity of broilers reared outdoors is initially greater than broilers reared indoors, but from six weeks of age, decreases to comparable levels in all groups (Weeks et al., 1994). The same study shows that in the outdoors group, surprisingly little use is made of the extra space and facilities such as perches – it was proposed that the main reason for this was leg weakness as 80 per cent of the birds had a detectable gait abnormality at seven weeks of age. There is no evidence of reduced motivation to extend the behavioural repertoire, as, for example, ground pecking remained at significantly higher levels in the outdoor groups because this behaviour could also be performed from a lying posture rather than standing.

Broiler breeders, i.e. males and females reared to fertilise and lay the eggs of the offspring reared for food, perform similar mating behaviour to other chicken types. They exhibit male-male aggression, male-hen aggression, hen-hen aggression, male waltzes, hen crouches, attempted hen mounts, completed hen mounts, attempted hen matings, and completed hen matings. These behaviours are seen less often and may not be exhibited as vigorously as observed in other chicken types (Moyle et al., 2010). The frequency of all

sexual behaviour shows a large decrease with age, suggestive of a decline in libido. The decline in libido is not enough to account for reduced fertility in heavy cocks at 58 weeks and is probably a consequence of the large bulk or the conformation of the males at this age interfering in some way with the transfer of semen during copulations which otherwise look normal (Duncan et al., 1990).

1.3.3 Broiler breeder (Parent Stock) farms

The "primary breeding sector" consists of companies that breed pedigree stock. Pedigree stock ("pure line") is kept on high level biosecure farms. Their eggs are hatched in a special pedigree hatchery and their progeny then goes on to the great grandparent (GGP) and grandparent (GP) generations. These eggs would then go to a special GP hatchery to produce parent stock which passes to the production sector (Elfick, 2012).

Worldwide, the primary sector produced 417 million parent stock per year (Elfick, 2012).

Numerous techniques are used to assess the pedigree stock. For example, birds might be examined with ultrasound or x-rays to study the shape of muscles and bones. The blood oxygen level is measured to determine cardiovascular health. The walking ability of pedigree candidates is observed and scored (Hardiman, 2007).

The need for high levels of Reaserch and Development spending prompted consolidation within the primary breeder industry. By the late 2000s only three sizable breeding groups (Elfick, 2012) remained:

- Aviagen (with the Ross, Arbor Acres, Indian River and Peterson brands);
- Cobb-Vantress (with the Cobb, Avian, Sasso and Hybro brands);
- Groupe Grimaud (with the Hubbard and Grimaud Frere brands).

1.4 Poultry Industry challenges

The challenges that the poultry industry faces are the opportunities for research and development. According to Smith (2006a), the poultry industry must commit and coordinate with research to remain as the leading food animal sector. A global commitment to solving the poultry industry challenges is necessary because multinational poultry production companies now produce a significant share of the worldwide trade poultry products. The main issues forecasted by specialists are disease pandemic, feed stuff contamination, environmental impact, shortage of carbohydrate sources, metabolic diseases, and increasing production costs (Shelton, 2006; Smith, 2006a). To reduce production costs, it is important to improve early growth performance characteristics in poultry. As demands for decreasing market weight for age continue, it will become more important to improve growth and development during the brooding phase (Hulet, 2007). Genetic selection has created a more efficient bird, driving costs down (Hammerstedt, 1999), but that would negated if poults do not survive the first week of life.

Great advances in poultry breeding and genetic selection for valuable production traits have been achieved during the last 50 years. Many have predicted that the genetic limit for growth was reached and slower progress will be seen from then on (Shelton, 2006), but we still see advances every year. The formula for success depends on two main branches of research, genetics and nutrition. The first creates a bird with higher potential, and the second has to find a way to help that bird to achieve as much of that potential as possible.

Although considerable progress was been made in the performance characteristics of breeders and commercial poultry, hatchability rate has improvement little during the last twenty years. Indeed, 20% of chicken hatching eggs do not yield hatchlings with the combined monetary losses of low. Each 1% increase in hatchability would result in $25 million in return, so finding ways to improve hatchability and early survival have a significant economic impact (Schaal and Cherian, 2007). According to Keirs et al. (2007), only a small portion of non-hatched embryos have anatomical abnormalities, so the majority of them should have hatched and survived. Most of the knowledge on embryonic development is based on research conducted over 60 years ago. It is now apparent that more emphasis on optimizing the growth and maturity of the developing embryo is needed to maximize post-hatch growth and development (Hulet, 2007).

Chapter 2. Poultry embryo development

2.1 Embryonic use of egg nutrients

2.1.1 Egg composition

Embryo development apart from the hen allows poultry producers to handle large number of animals, but on the down side all nutrients must be present in the egg to sustain embryonic development by the time the egg is laid (Fasenko, 2007). Composition of a typical chicken egg is presented on Table 2.1. It is noteworthy that there is very little energy reserves as carbohydrates in the egg (Romanoff, 1967), being most of lipids in the yolk fraction. Ninety eight percent of the free carbohydrates are present as 0.5% glucose in the albumen (Davis and Reeves, 2002).

Table 2.1. Approximate composition of a 60g chicken egg

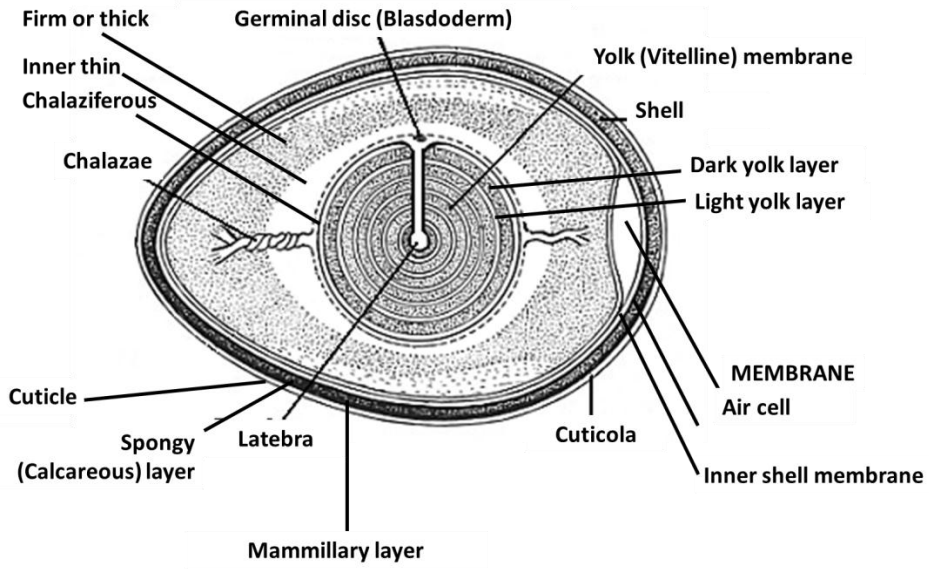
	Egg weight	**Dry Matter**	**Protein**	**Lipids**	**Carbohydrate**	**Minerals**
	%	**%**	**% on dry matter basis**			
Egg yolk	31	49	30	60	1.2	2.2
Egg white	58	12	75	0.08	4.2	1.7
Egg shell	11	99.7	0.7	-	-	98
Total	100					

Adapted from: Davis and Reeves (2002)

2.1.2 Egg structure

A schematic structure of the avian egg before incubation is illustrated by Figure 2.1. These structures simply keep the little embryo alive and protected until incubation starts. Beginning with the outside of the egg moving inward, the egg shell has a porous calcium carbonate structure that allows gas exchange and two membranes. The outer membrane is attached to the shell, while the inner membrane retracts when egg cools down forming the air cell space on the large end of the egg. The next component is the egg white, composed of three layers of protein generally called albumen. The albumen layers are named based on their density as outer thin, dense or thick and inner thin. The central structure is the yolk, composed of multiple layers of lipids enveloped by a membrane. Resting on top of the yolk is the blastodisc where the embryo resides after the egg is fertilized. Concentric yolk layers can be differentiated by the amount of pigment deposited depending whether they were formed during daylight or night darkness. Linking the yolk to each end of the egg are the chalazas that act like bungee cords to keep the yolk horizontally centered in the egg. These rudimentary structures are present at the time of lay but they rapidly change once incubation proceeds.

Figure 2.1. Drawing of a midsagittal section of egg



The layers of albumen are indicated. The layers between thin and thick are not known to be separated by membranes but still have distince boundaries (modified from Kiple and Ornelas, 2000).

2.1.3 Embryo development during incubation

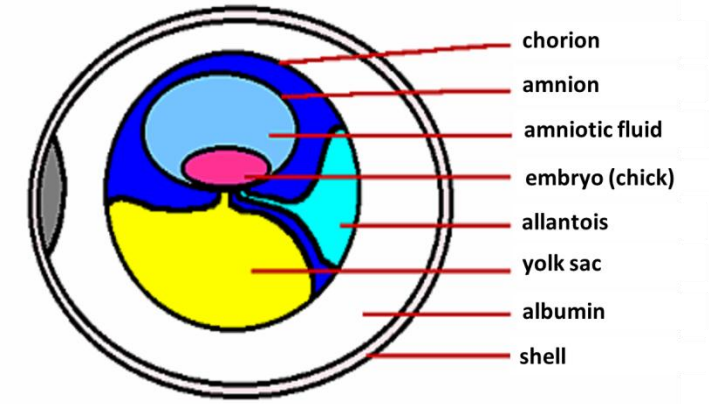
Moran (2007) presented a comprehensive review on the development of the avian embryo and divided embryonic development in three distinct periods:

1) the establishment of the germ;
2) completion of embryonic formation;
3) preparation for emergence.

During the establishment of the germ, the embryo and its sustaining structures resume cell proliferation from the 40,000 to 60,000 cells already present at oviposition (Fasenko, 2007). The energy expended at this time arises by glycolysis of glucose present at the outer thin layer of the albumen. The embryo metabolism is mainly anaerobic, accumulating lactate because of low O_2 diffusion of the primitive hemoglobin. The vascular system is rapidly established and germ invaginations lead to formation of the chorionic sac and the allantoic cavity. The different egg compartments during incubation are illustrated by Figure 2.2. Egg turning is crucial during this period to allow proper formation of egg compartments and to give embryonic access to glucose present in the outer thin. The inner cell layer of the amniotic membrane secretes amniotic fluid in which the embryo floats, keeping the embryo from drying and protecting it from shock. The chorion surrounds all embryonic structures and serves as a protective membrane. The allantois grows larger as the embryo grows, until it fuses with the chorion forming the chorio-allantoic membrane, responsible for exchanging oxygen and carbon dioxide with the environment, and for storing nitrogenous waste (Smith, 2007). The yolk sac membrane selects the nutrients being up taken from yolk sac reserves, which includes lipids, protein, minerals and vitamins. The yolk sac membrane can also modify these nutrients and serve as their short-

term storage. Egg storage and storage conditions prior to incubation can impair formation of a fully functional choriallantois, affecting recovery of nutrients.

Figure 2.2. Schematic representation egg compartmentalization during incubation



From: http://people.eku.edu/ritchisong/avianreproduction.html (viewed September 15th 2012)

The second third of incubation is marked by a fully developed vascular system, with the choriallantois able to assure O_2-CO_2 exchange. The embryo then undergoes the first drastic switch of its metabolism, from carbohydrate to fully dependent on lipid oxidation as the energy source. The embryo grows very fast in size during this phase. Essential fatty acids are preserved for cell membrane synthesis while saturated fatty acids are consumed to sustain the increasing caloric needs of formed tissues (Christensen et al., 1996). The embryo then undergoes another critical period: the transition for emergence.

In preparation for emergence, embryonic size and movements cause rupture of the membrane that separated the albumen and amniotic fluid, causing them to mix. The embryo then orally consumes the amniotic fluid, which passes through the gastrointestinal system. At this stage of intestinal development, enterocytes of the duodenum and jejunum are able to absorb macromolecules of protein, in a process similar to mammalian absorption of colostrum. Such consumption continues until the albumen-amniotic fluid disappears and internal pipping begins. Since embryonic skeletal tissue development is complete at this point, the nutrients absorbed are used for visceral organs maturation and most of it is stored as glycogen. The main protein present in the fluid is called ovomucoid, which has extensive amounts of carbohydrate. Glycogen is deposited in liver and muscle. Some residual albumen-allantoic fluid ingested enters the yolk and continues to express digestive enzymes obtained while passing through gastrointestinal systems. Digestion of nutrients then can occur in the yolk and be absorbed by yolk sac cell villi, especially very low density lipoproteins (VLDL), which accumulates triglycerides in sub-dermal locations of the embryo. Cholesterol accumulates in the liver, causing it to grow in size and become yellow in appearance. At the same time high-density lipoproteins (HDL) aggregates in granules and are covered with calcium from circulation. These granules remain in the yolk sac until pipping. Dissolution of mammary knobs adjacent to the choriallantois-shell membrane interface mobilizes great amounts of calcium, which rises in the circulation favoring calcification of the skeleton, which was mainly cartilage until then. Now the embryo is ready to start pipping the weakened shell and membranes (Moran, 2007).

Emergence starts when the embryo breaks the choriallantois and the inner shell membrane near the air cell, in what is called internal pipping. At this point the embryo must initiate pulmonary respiration, since the outer shell membrane is losing contact with the shell. This is a critical period because limited supply of oxygen suppresses continuing use of lipids as energy, so metabolism switches again to the anaerobic catabolism of glucose from glycogen reserves producing lactate. The remaining yolk sac is retracted into the abdominal cavity, and peripheral blood is recovered into the embryo. A relatively great amount of energy is used to sustain embryonic pipping movements to break the shell, and body rotation. Shell piercing is achieved by the coordination of pipping muscle movements and the egg tooth of the beak. The pipping or hatching muscle is a specialized muscle located in the back of the head. Hatching muscle fibers are exclusively anaerobic and relay on glycogen stored there previously. It also has a special nervous system to coordinate its movements. External air access now provides enough oxygen for oxidation of fatty acids and lactate recovery in the liver. At this point the choriallantois cannot longer extract calcium from the shell, so calcium mobilization begins from HDL granules. The embryo continues breaking the shell, rotating and using the feet to push until it is free from the shell (Moran, 2007). The blood vessels linking the navel to the shell membrane are detached, and the hatching process is over. Although yolk sac lipoproteins continue to be an important nutrient source at this time, fatty acid recovery from preformed body depots appears to dominate. Extensive hepatic cholesterol in place at hatch rapidly dissipates along with the depots. Cholesterol, together with depot essential fatty acids, enables continued membrane formation and growth. A high concurrent demand for glucose appears to reside with its need to support growth of glycolytic muscle. In the intestine, cell proliferation is stimulated by feed intake to replace embryonic enterocytes, able to absorb macromolecules, by mature ones, able to produce digestive enzymes and to absorb external feed nutrients. The complete transition may take up to two weeks post-hatch, and is delayed if feeding is delayed (Moran, 2007).

Chapter 3. The gastrointestinal tract of poultry

3.1 Anatomy and physiology

The gastrointestinal tract (GIT) of poultry (Figure 3.1) is relatively short and appears particularly well adapted for transforming concentrated diets into nutrients. The extremely rapid rate of passage of digesta, which is around 10 hours, implies highly efficient mechanisms of digestion and absorption. In comparison with mammals, the gastrointestinal tract of birds is distinguished by the following features:

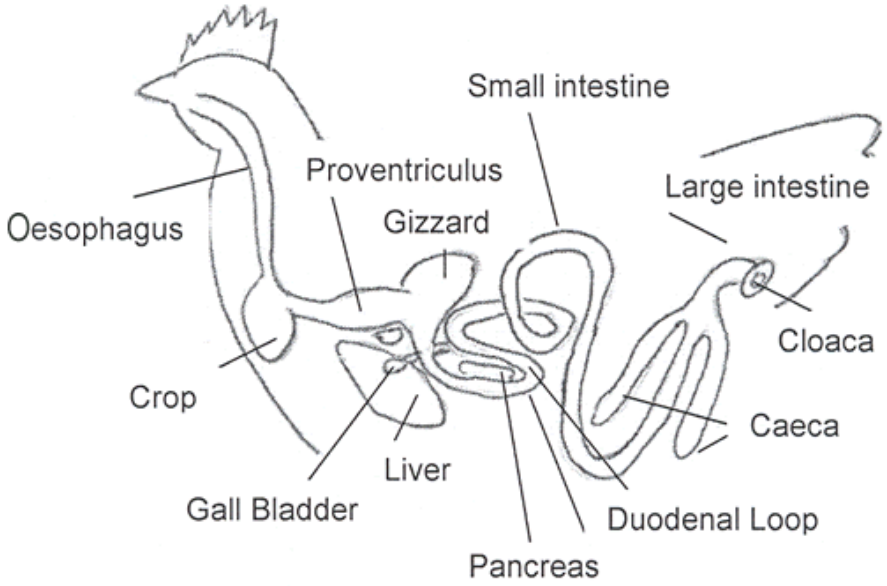
i) replacement of the lips in mammals with beak;

ii) existence of two successive and distinct stomachs. The proventriculus or glandular stomach is the "chemical" stomach. The gizzard, or "mechanical" stomach ensures homogenization and a certain amount of grinding of the food;

iii) the uniqueness of the terminal region of the tract, or cloaca, which acts both as the rectum and the exit for the urino-genital system.

The development of the gastrointestinal tract is very precocious. In the embryo the primordial intestine develops from the second day of incubation. At hatching the tract represents up to 25% of the live weight. This proportion diminishes rapidly and falls to less than 5% in a 8-week old broiler.

Figure 3.1. The digestive system of the chickens - general structure



From the website of the Department of Agriculture, Fisheries and Forestry, Qeensland Government
http://www.daff.qld.gov.au/27_2705.htm (viewed October 10th 2012)

3.2 The gastrointestinal microbiota of poultry

3.2.1 Microbes of the chicken gastrointestinal tract

The gastrointestinal tract of warm-blooded animals is densely populated by bacteria. Composition and density of the microbiota can vary a lot among individuals because it is markedly affected by the bacterial composition of the inoculum received at birth or hatch,

the structure of the host intestinal epithelium and the diet (Apajalahti and Kettunen, 2006; Zhu et al., 2002). Settlement of the microbial population depends on the egg's microbial environment at hatching which determines the order in which animals are exposed to microorganisms, their ability to colonize the intestine and their interactions (Gabriel et al., 2006). Previously, the only way to characterize the microbiota was culturing on selective growth media and subsequent identification of bacteria through biochemical tests, but such methods are laborious and incomplete and, therefore, not suitable for extensive monitoring of the unknown microbiota. Recent developments in the total microbial community analysis by DNA-based methods have brought a new insight into gastrointestinal tract microbiology of chickens and many other animal species (Apajalahti et al., 1998; Gong et al., 2002; van der Wielen et al., 2002; Zhu et al., 2002; Zhu and Joerger, 2003; Apajalahti et al., 2004). The % G+C (guanine + cytosine) profiling and 16S ribosomal RNA sequence analysis of chicken gastrointestinal microbiota underline that only 10% of the gastrointestinal bacteria represents previously known bacterial species. Advances in ribosomal DNA-based molecular techniques have made it possible to obtain new information by identifying different bacterial populations in intestinal contents and mucosal samples as compared with routine culturing methods. These techniques are also helpful for monitoring the effect of diets and other variables on the microbial communities of the GIT under commercial conditions (Apajalahti et al., 1998, 2001; Gong et al., 2002; Knarreborg et al., 2002; Van der Wielen et al., 2002; Amit-Romach et al., 2004). The early stage of the post-hatch period is critical for the establishment of the gut microbial community. This process starts from a sterile gastrointestinal environment at the moment of hatching and continues toward establishing a relatively stable status as the animal ages (Richards et al., 2005; Verstegen et al., 2005). It has been shown that the composition of the microflora changes in relation to the age of the chickens, dietary factors, breed, and geographic location (Salanitro et al., 1974; Apajalahti et al., 2001; Knarreborg et al., 2002; Van der Wielen et al., 2002; Lu et al., 2003). Thirty five percent represents previously unknown species with a known bacterial genus and the remaining 55% represent bacteria for which even the genus is totally unknown. Using this molecular approach 640 different species and 140 different bacterial genera have been found in the chicken gastrointestinal tract (Apajalahti et al., 2004). Bacteria in the gastrointestinal tract have nutritional and spatial requirements, they derive most of their energy for reproduction and growth from dietary compounds which are either resistant to attack by digestive fluids or absorbed so slowly by the host that bacteria can successfully compete for them. Since bacterial species differ from each other in relation to their substrate preferences and growth requirements, the chemical composition and structure of the digesta largely determine the species distribution of the microbial community in the gastrointestinal tract. As a consequence, bacterial community structure is very much dependent upon the diet as the ultimate source of substrates for metabolism. Conversely, the ability of the host digestive system to digest and absorb nutrients is, in part, dependent upon the species distribution and total population of resident microbes. Hence, changes in dietary composition or nutrient density can have dramatic effects on the intestinal microbiota populations, which in turn can influence the ability of the animal to digest and absorb dietary nutrients (Apajalahti et al., 2004; Gabriel et al., 2006). The distribution of indigenous microbiota within the avian GIT

is therefore organized qualitatively and quantitatively along vertical and horizontal regions in the GIT. The vertical distribution refers to the distribution of bacteria from the crop to the caeca. Furthermore, bacteria are distributed horizontally along the GIT and occupy the intestinal lumen, mucous lining, crypt spaces and adhere also to the epithelial cells. Each segment and horizontal layer harbors its own specific bacterial community and this depends, as already indicated on environmental factors such as nutrition, bile salts, oxygen concentration and pH of the different segments (Thomson and Applegate, 2005; van der Wielen et al., 2002).

The diversity of the dominant bacterial community in the intestinal tract increases when broilers grow older. Moreover, it seems that the dominant bacterial community in crop, duodenum and ileum within the same chicken is very similar in 4-day-old broilers. Even the similarity between the dominant bacterial community of the ceca with the other three parts of the intestinal tract is much higher in 4-day-old broilers. This suggests that the environmental conditions along the intestinal tract are rather similar and do not allow niche differentiation. When broilers age, similarity between banding patterns of crop, duodenum, and ileum decrease considerably. This indicates that environmental factors in the intestine change specifically in each compartment. These results can be important for studies related to the manipulation of the intestinal bacterial community in chickens (van der Wielen et al., 2002). The maximum bacterial density is found to be reached in about one week and, after this phase of microbiota development, another one starts that can be called "maturation phase" and it is characterized by:

(i) a low growth rate equal to that of the digesta passage;

(ii) gradual selection of bacteria that most efficiently adapt to the prevailing conditions.

In chicken, the main sites of bacterial activity are the crop and the caeca and, to a lesser extent, the small intestine (Gabriel et al., 2006).

The crop microbiota is mainly composed of lactobacilli attached to the epithelium and forming an almost continuous layer, and enterococci, coliforms and yeasts (Gabriel et al., 2006).

Bacterial density reaches at maturity 10^3-10^5 bacterial cells per gram of digesta in the proximal small intestine (duodenum) because it is characterized by rapid flow of the highly fluid digesta, while the distal small intestine (jejunum and ileum) harbors $>10^9$ bacteria cells per gram of digesta (Gong et al., 2007; Brisbin et al., 2008a). Generally the main genera of bacteria within the chicken small intestine are *Lactobacillus*, *Enterococcus* and *Clostridium*, with some bacteria from the family *Enterobacteriaceae* (Brisbin et al., 2008a). The most predominant *Lactobacillus* species in the upper GIT (gizzard, duodenum, jejunum and ileum) seem to be *L. aviaries* and *L. salivarius* (Gong et al., 2007).

The caeca contain a more diverse community of bacteria, including species of the genera *Bacteroides*, *Bifidobacterium, Clostridium, Enterococcus, Escherichia, Fusobacterium, Lactobacillus, Streptococcus* and *Campylobacter*, and reaching $>10^{11}$ cell/g of digesta (Apajalahti and Kettunen, 2006; Brisbin et al., 2008a). Gong et al. (2007) and Bjerrum et al. (2006) indicated *Faecalibacterium prausnitzii* and butyrateproducing bacteria (mainly *F. prausnitzii, Clostridium* and *Ruminococcus*) as the largest groups in the caeca.

3.2.2 Role of the intestinal microbiota

Microbes of the GIT can be generally divided into potentially pathogenic or beneficial groups. Harmful bacteria may be involved in localized or systemic infections, intestinal putrefaction, and toxin formation. Some intestinal organisms may have beneficial effects such as vitamin production, stimulation of the immune system through nonpathogenic mechanisms, and inhibition of the growth and establishment of harmful microbial groups (Jeurissen et al., 2002). The benefits of normal microbiota such as providing nutrients (Annison et al., 1968) and competitive exclusion (prevention of colonization by pathogenic bacteria) are costly for the host animal (Snoeyenbos et al., 1978; Soerjadi et al., 1982). The cost associated with these benefits may include competition with the host for nutrients, stimulation of rapid turnover of absorptive epithelial cells, secretion of toxic compounds, and induction of an ongoing inflammatory response in the GIT. All these effects occur at the expense of animal production performance (Drew et al., 2003; Richards et al., 2005; Verstegen et al., 2005).

The microbiota of the GIT of mammals can be considered a metabolically active organ with its wide biodiversity in term of species and the high number of cells (Macfarlane and Macfarlane, 2004, Backhed et al., 2005, Murphy et al., 2009). Under normal circumstances, commensal bacteria are an essential health asset with a nutritional function and a protective influence on the intestinal structure and homeostasis. A balanced gut microbiota constitutes an efficient barrier against pathogen colonization; moreover, it produces metabolic substrates (e.g. vitamins and short chain fatty acids) and stimulates the immune system in a non-inflammatory manner. Thus, there is evidence of a correlation between the composition of the colonizing microbiota and variations in immunity (O'Hara and Shanahan, 2006). The gut microbiota, with its metabolic, trophic and protective functions, is able to affect positively the integrity of the intestinal barrier. Loss of the integrity (i.e., intestinal barrier dysfunction) leads to a progressive increase of intestinal permeability, inducing a switch from "physiological" to "pathological" inflammation that is characteristic of diseases such as intestinal bowel disease (IBD) (Frank et al., 2007; Lambert, 2009). Intestinal pathogens produce toxins and other classes of substances i.e. mucinases, adhesins and invasins, which interfere with epithelial metabolism. All together, the pathogenic phenotype is likely to directly trigger uncontrolled pathological inflammation. Increasing evidence indicates that changes in gut microbiota, with an increase of pathogenic bacteria and a decrease of health-promoting bacteria, such as bifidobacteria and lactobacilli, play an important role in promoting and maintaining intestinal inflammation in IBD (Andoh and Fujiyama, 2006).

The intestinal microbiota in fact actively exchanges developmental and regulatory signals with the host that prime and instruct mucosal immunity (O'Hara and Shanahan, 2007; Brisbin et al., 2008a). Physiological and psychological stressors, leading to dysfunction of the intestinal barrier and to the increase of intestinal permeability, have an impact on gut microbial composition and susceptibility to enteric pathogens (Gareau et al., 2009). Moreover, stress situations generally result in a poor growth rate and productivity in livestock and poultry. In poultry production systems, birds are routinely subjected to stressors such as feed withdrawal, temperature fluctuations, and confinement during transportation augmenting disease incidence (St-Pierre et al., 2003; Humphrey, 2006).

3.3 Antibiotic Growth Promoters (AGPs) and animal feed regulations

3.3.1 Antibiotics

Feed additive antibiotics have been used as growth promoters for >50 years in the feed industry all over the world. Antibiotics induce their effect by stabilizing the intestinal microbial flora thereby preventing proliferation of specific intestinal pathogens (Visek, 1978; Shane, 2005). Today, the non-prescription use of antibiotics in poultry feeds has been eliminated or severely limited in many countries because of concerns related to development of antibiotic-resistant human pathogenic bacteria and legislative action to limit their use in probable in many others. Since the proposed total ban on sub-therapeutic feed antibiotics, products such as prebiotics, organic acids and probiotics are receiving considerable attention in animal nutrition because of their non-residual and non-resistant properties (Mellor, 2000; Gill, 2001; Hertrampf, 2001; Kocher, 2005; Plail, 2006).

Antibiotics have been widely used in veterinary medicine for disease treatment and prophylaxis as well as in the character of the growth promoters. Antibiotics growth promoters (AGPs) act by modifying the intestinal microflora, especially against Gram-positive bacteria, which are associated with animals' poorer health performance.The AGPs have been very cost-effective and efficient in improving animals' performance and health, especially in pigs and poultry (de Lange et al., 2010). However, in 2006 the European Union banned the use of antibiotics as growth promoters.

Genetic improvement applied in poultry significantly increased growth parameters of the broilers. At the same time, higher rearing density escalated disease challenges, making birds more susceptible to various pathogens, especially enteropathic microbes. Without addition of the AGPs, pathogenic bacteria, such as *Salmonella* spp., *Escherichia coli*, and *Clostridium perfringens* can proliferate and become dominant compared with other bacteria present in the intestine, allowing for increased incidence of disease and diminished nutritive value of feed consumed (Collier et al., 2003; Niewold, 2007).

Enteric diseases are major economic concern in the poultry industry, which result in loss of productivity and increased mortality in flocks. Besides, as reported by European Food Safety Authority (EFSA), poultry production and poultry products can be considered as a potential major source of human campylobacteriosis and salmonellosis, in the EU. Campylobacteriosis and salmonellosis are now the most frequently reported foodborne illnesses in the EU (40.7 and 38.2 cases per 100,000 population, respectively). Handling, preparation and consumption of broiler meat may account for 20% to 30% of causes of campylobacteriosis in human (EFSA, 2006, 2010).

Modern food animal production depends on the use of large amounts of antibiotics for disease control (Aarestrup, 2002). In 1949, quite by accident, while conducting nutrition studies with poultry, Jukes of Lederle Laboratory and McGinnis of Washington State University obtained startling growth responses from feeding a residue from Aureomycin production. Later experiments revealed that the supplement used by Jukes and McGinnis – the residue of Aureomycin production – supplied the antibiotic chlortetracycline. This was the birth of feeding antibiotics to livestock (Scanes et al., 2004). However, over the past few decades awareness has grown that this application creates favourable conditions for selection, spreading and persistence of antimicrobial-resistant bacteria capable of causing

infections in animals and humans. It has thus become clear that antimicrobial resistance poses a threat to public and animal health and is a reason for serious concern.

In food animal production antimicrobial agents are normally used in one of four different ways, i.e. i) therapy: treatment of infections in clinically affected animals, preferably based on a bacteriological diagnosis, ii) metaphylactics: treatment of clinically healthy animals belonging to the same flock or pen as animals showing clinical signs; in this way infections may be treated before they become clinically apparent and the entire treatment period may thereby be shortened. In fact, in view of the modern production systems, this may often be the only effective approach to treat for instance large broiler flocks through water medication, iii) prophylactics: treatment of healthy animals in a period of stress (e.g. early weaning) to prevent diseases; in such cases the use of antimicrobials is indicative for general management problems, and hence in most countries is either illegal or considered imprudent; and, finally, iv) growth promotion: the continuous inclusion of antimicrobials in animal feed to prevent (subclinical) infections and hence promote growth; such usage is under serious debate (Aarestrup, 2002).

The primary reason for using antibiotics in poultry feeds is for their growth stimulating effect, for which they are generally used in broiler rations. The reason for the beneficial effect of antibiotics still remain obscure, but the best explanation is the disease level theory, based on the fact that antibiotics have failed to show any measurable effect on birds maintained under germ-free conditions (Scanes et al., 2004). The exact mechanisms by which these improvements occur, however, are still not fully understood. Currently, four mechanisms of growth promotion have been proposed by various scientists. Because early researches have indicated that orally dosed antibiotics do not promote growth in germ-free chicks, each of these proposed mechanisms are based on the hypothesis that the presence of bacteria in the intestine reduces animal growth, and include hypotheses that: 1) antibiotics inhibit the occurrence of sub-clinical infections, 2) antibiotics reduce production of growth-depressing microbial metabolites, 3) antibiotics reduce the use of nutrients by intestinal microbes, and 4) antibiotics allow for enhanced uptake of nutrients because they have been shown to reduce the thickness of the intestinal wall. Regardless of the fact that the exact mechanisms of antibiotic-mediated growth promotion are currently incompletely understood, most researchers support the theory that antibiotics reduce the overall numbers or diversity of gut bacteria, which may promote growth (Thompson and Applegate, 2005).

In addition to their use as growth stimulators, antibiotics are used to increase egg production, hatchability, and shell quality in poultry. They are also added to feed in substantially higher quantities to remedy pathological conditions. Antibiotics are generally fed to poultry at levels of 5 to 50 g per ton of feed, depending upon the particular antibiotic used. Higher levels of antibiotics (100 to 400 g per ton of feed) are used for disease-control purposes. The antibiotics most commonly used in poultry rations are bacitracin, virginiamycin, bambermycin, and lincomycin. High levels of calcium in a laying mash will inhibit assimilation of certain tetracycline-type antibiotics to the bloodstream and reduce their effectiveness. In all probability, antibiotics will always be used as feed additives to control and treat health problems in poultry. But the status of subtherapeutically used antibiotics as production stimulators is, at the present time, tenuous. Pressure from consumer groups and medical people may result in banning many of the antibiotics that are

primarily used for medicinal purposes in humans from the list of approved production promoters. However, in the future, an increasing number of antibiotics will likely be developed specifically for the purpose of improving poultry performance. One example is that of bambermycin. This antibiotic was developed solely for use as a production promoter, serving to increase rate of gain and feed efficiency in chickens and swine. It has no medical applications, and, therefore, poses no health hazard with regards to bacteria becoming resistant to it (Scanes et al., 2004).

As in human medicine, the use of antimicrobial agents in agriculture creates a selective pressure for the emergence and dissemination of antimicrobial-resistant bacteria including animal pathogens, human pathogens which have food animal reservoirs, and other bacteria that are present in food animals. In fact, the use of dietary antibiotics resulted in common problems such as development of drug-resistant bacteria (Sorum and Sunde, 2001), drug residues in the body of the birds (Burgat, 1999), and imbalance of normal microflora (Andremont, 2000). Approximately 8,164,662 kg of antibiotics are used annually in animal farming (70% of which is used for nontherapeutic purposes such as growth promotion and disease prevention) compared with only 1,363,636 kg per year used in human medicine (Roe and Pillai, 2003).

These resistant bacteria may be transferred to humans either through the food supply or by direct contact with animals. The transfer of resistant bacteria from food-producing animals to humans is most evident in human bacterial pathogens which have food animal sources, such as *Campylobacter*, which has a reservoir in chickens and turkeys and Salmonella, which has reservoirs in the cattle, chicken, pig and turkey. Pathogenic bacteria, such as *Campylobacter* and *Salmonella*, are not the only concern when considering antimicrobial resistance in bacteria with food animal reservoirs. Commensal bacteria, which are naturally occurring host microbiota, constitute an enormous potential reservoir of resistance genes for pathogenic bacteria. The prevalence of antibiotic resistance in the commensal bacteria of humans and animals is considered to be a good indicator of the selective pressure of antibiotic usage and reflects the potential for resistance in future infections.

Antibiotic-resistant organisms from animals reenter the human and animal populations through several pathways including natural water, irrigation water, drinking water, vegetables, and foods (Roe and Pillai, 2003). These resistant bacteria are shed in feces, where they can share extra chromosomal antibiotic resistance plasmids (r-plasmids) with native bacteria and may be disseminated to other animals. As the reservoir of resistant commensal bacteria increases, the plasmid reservoir becomes larger and enables more frequent transfer of resistance to pathogenic bacteria including *Salmonella* and *Shigella*. *Escherichia coli*, which is the predominant isolate of aerobic faecal microbiota in humans and most animals, has demonstrated its ability to transfer resistance genes to other species, including pathogenic bacteria (Anderson et al., 2005). Additionally, there is information on cross-talk between pathogens and epithelial tissues, resulting in extensive rearrangement of epithelial cells upon colonization by pathogens (Goosney et al., 2000; Sansonetti, 2001). Hooper et al. (2001) have shown that cross-talk between *Bacteroides thetaiotaomicron* and the epithelium results in epithelial secretion of specific glycans, which are utilized by the bacterium.

3.3.2 Feed additives legislation and antibiotic ban

Feed additives encompass a variety of products. According to the currently applicable legislation (EC 1831/2003, Art. 2 (2a)), "feed additives" means substances, microorganisms or preparations – other than feed materials premixtures – which are intentionally added to feed or water to perform, in particular, one or more of the functions mentioned in Article 5. Article 5 can be summarized as follows: a feed additive should favourably affect one of the following characteristics of feed or animal products; colour of ornamental fish or birds; the environment; animal production, performance or welfare through positive effects at gut level, or satisfy the nutritional needs of animals or have a coccidiostatic or histomonostatic effect (Doeschate and Raine, 2006).

At EU level, Directive 70/524 (23 November 1970) was really the first one that tried to regulate the use of feed additives across the EU Member States; but even though the directive was intended to lead to consistent legislation across the EU, it did not achieve this. Moreover, the fact that the Scandinavian countries banned antibiotic-growth promoters ahead of the rest of Europe resulted in a campaign to get these products banned in the whole of the EU. Directive 70/524 appears complicated, with many annexes and different authorization periods for different products. The industry worked with the Directive and the country-specific implementation of the Directive into law as well as possible. Regulation 1831/2003 thus sets out to review all the rules on additives, with a change of emphasis towards the protection of human health, animal health and the environment, based on precautionary principle. Regulation 1831/2003 applies directly in each Member State, and there should thus be less opportunity for country-specific rules and derogations. All additives authorized under regulation 1831/2003 will be given time-limited authorization to allow technological progress and scientific developments to be taken into account in the review of the product authorization.

The categories of additives identified in 1831/2003 are:

- technological additives: any substances added to feed for a technological purpose;
- sensory additives: any substance, the addition of which improves or changes the organoleptic properties of the feed, or the visual characteristics of the food derived from animals;
- nutritional additives (such as amino acids);
- zootechnical additives: any additive used to affect favourably the performance of animals in good health or used to affect favourably the environment;
- coccidiostats and histomonostats.

Regulation 1831/2003 has at least stated that antibiotics, other than coccidiostats and histomonostats, might be marketed and used as feed additives only until December 31, 2005; as from January 1, 2006, those substances would be deleted from the Community Register of authorized feed additives (Castanon, 2007). The removal of these compounds in animal diet result in changes to the microbial composition of the intestinal tract, which may in turn have consequences for commercial poultry flocks (Knarreborg et al., 2002) and has put tremendous pressure on the livestock and poultry farms, one of the main consequences being a substantial increase in the use of therapeutic antibiotics (Casewell et al., 2003). It has been evidenced that AGP have long been effective in prevention of

necrotic enteritis (NE) in poultry flocks and that the incidence of NE have increased in countries where AGP have been stopped (Van Immerseel et al., 2004).
In other ways, the ban of growth promoters demands the improvement of the hygiene from farms. It was shown that under good production conditions it is possible to reach good and competitive production results for the rearing of poultry without the continuous use of antibiotics in feeds. Moreover, safer nonantimicrobial substances have been studied as alternatives for replacing antibiotics to interact with the intestinal microbiota, including enzymes, prebiotics and probiotics or acidification of diets (Castanon, 2007).

Chapter 4. Alternatives to antibiotics: Probiotics, Prebiotics and Synbiotics

4.1 Probiotics

Many definitions have been proposed for the term probiotic. Probiotics are pure cultures of one or more live bacteria that exhibit a beneficial effect on the health of the host when they are ingested. Improved epithelial cell integrity, increased immune response, well balanced gut microflora, better utilisation and digestion of diet are also additive beneficial effects of dietary probiotics (Jin et al., 1998; Panda et al., 2001).

The most recent one is "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). In this definition it is implicit that a health effect must be demonstrated for the probiotics. The beneficial modes of action include: regulation of intestinal microbial homeostasis, stabilization of the gastrointestinal barrier function (Salminen et al., 1996), expression of bacteriocins (Mazmanian et al., 2008), enzymatic activity inducing absorption and nutrition (Hooper, 2002; Timmerman et al., 2005), immunomodulatory effects (Salzman et al., 2003), inhibition of procarcinogenic enzymes and interference with the ability of pathogens to colonize and infect the mucosa (Gill, 2003). The expected health-promoting characteristics and safety criteria of probiotics are shown in Table 4.1.

Table 4.1. Expected characteristics and safety criteria of probiotics

Non toxic and non pathogenic
Accurate taxonomic identification
Normal inhabitant of the targeted species
Survival, colonization and being metabolically active in the targeted site, which implies:
resistance to gastric juice and bile
persistence in the GIT
adhesion to epithelium or mucus
competition with the resident microbiota
Production of antimicrobial substances
Antagonism towards pathogenic bacteria
Modulation of immune responses
Ability to exert at least one scientifically-supported health promoting properties
Genetic stability
Amenability of the strain and stability of the desired characteristics during processing, storage and delivery
Viability at high populations
Desirable organoleptic and technological properties when included in industrial processes

4.1.1 Regulatory considerations

Significant progress in legislation for the safety evaluation of probiotics, has been made in USA, Canada, and Europe (FAO/WHO, 2002; EFSA, 2005a; HC, 2006); however, no unique standard is available. In the USA, microorganisms considered safe for human consumption are awarded GRAS status (Generally Regarded As Safe) by the Food and Drug Administration. In Europe, the European Food Safety Authority has introduced the concept of Qualified Presumption of Safety (QPS) similar in purpose to the GRAS approach. The QPS concept provides a generic assessment system for use within EFSA that in principle can be applied to all requests received for the safety assessments of microorganisms deliberately introduced into the food chain (EFSA, 2005b). According to recent evaluation (Wassenaar and Klein, 2008), QPS system appears more flexible because

it takes into account additional criteria to evaluate the safety of bacterial additives such as a history of safe use in the food industry and the acquisition of antibiotic resistance or virulence determinants. EFSA has published a list of microorganism, which possess a known historical safety, proposed for QPS status (EFSA, 2007). This list does not include *Enterococcus* species, even if *E. faecium* shows a long history of apparent safe use in food or feed. The main reason is due to the possibility of carrying transmissible resistance to antibiotics by *Enterococcus* spp. (EFSA, 2007).

A list of the probiotic species for studies or application in animal feed is showed in Table 4.2; these data derived from extensive literature and internet search of commercial products. In broiler nutrition, a variety of microbial species have been used as probiotics, including species of *Bacillus*, *Bifidobacterium*, *Enterococcus*, *E. coli*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, a variety of yeast species such as *Saccharomyces cerevisiae*, and undefined mixed cultures (Endo and Nakano, 1999; Mahajan et al., 2000; Casula and Cutting, 2002; Patterson and Burkholder, 2003; Huang et al., 2004; Kabir et al., 2004; Karaoglu et al., 2004; Aksu et al., 2005; Ahmad, 2006; Mountzouris et al., 2007). These probiotic species showed a beneficial effect on broiler performance (Tortuero, 1973; Owings et al., 1990; Jin et al., 1998; Zulkifli et al., 2000; Kalavathy et al., 2003; Kabir et al., 2004; Gil De Los Santos et al., 2005), modulation of intestinal microflora and pathogen inhibition (Rada and Rychly, 1995; Line et al., 1998; Pascual et al., 1999), and immunomodulation (Zulkifli et al., 2000; Dalloul et al., 2003; Kabir et al., 2004; Koenen et al., 2004). Generally, *Lactobacillus* and *Bifidobacterium* species have been used most extensively in humans, whereas species of *Bacillus*, *Enterococcus*, and *Saccharomyces* yeast have been the most common organisms used in livestock (Simon et al., 2001). However, there has been a recent increase in research on feeding *Lactobacillus* to livestock (Gusils et al., 1999; Pascual et al., 1999; Jin et al., 2000; Tellez et al., 2001). No study has been carried out on sporeforming lactic acid-producing bacteria such as *Bacillus coagulans* as probiotics in chickens. Many factors make *B. coagulans* a good candidate for probiotic use; it produces organic acids, possesses the capacity to sporulate, and is easily cultured in bulk (Hyronimus et al., 2000). In addition, in the spore form, it is more resistant to heat, which facilitates the pelleting process used in the mass production of probiotic chicken feeds.

An important point is the viability and consequently derived concentrations of viable bacteria of probiotic preparations at the moment of the administration to the animals. It is fundamental to study proper formulations which will allow the maximum viability of the bacteria species utilized.

Table 4.2. List of probiotics applied or studied for application in animal feed

Genus	**Species[(a)]**
Bacteria	
Bifidobacterium	*B. animalis (B. animalis subsp. animalis)* *B. lactis (B. lactis subsp. lactis)* *B. longum (B. longum subsp. longum)* *B. pseudolongum (B. pseudolongum subsp. pseudolongum)*

	B. thermophilum
Enterococcus	*E. faecium* *E. faecalis*
Lactobacillus	*L. acidophilus* *L. amylovorus* *L. brevis* *L. casei (L. casei subsp. casei)* *L. crispatus* *L. farmicinis* *L. fermentum* *L. murinus* *L. plantarum (L. plantarum subsp. plantarum)* *L. reuteri* *L. rhamnosus* *L. salivarius* *L. sobrius (L. amylovorus)*
Lactococcus	*L. lactis subsp. cremoris* *L. lactis subsp. lactis*
Leuconostoc	*L. citreum* *L. lactis* *L. mesenteroides*
Pediococcus	*P. acidilactici* *P. pentosaceus subsp. pentosaceus*
Propionibacterium	*P. freudenreichii*
Streptococcus	*S. cremoris* *S. faecalis* *S. faecium* *S. infantarius* *S. salivarius subsp. salivarius* *S. salivarius subsp. thermophilum*
Bacillus	*B. cereus var. toyoi* *B. licheniformis* *B. subtilis*
Yeast	
Saccharomyces	*S. cerevisiae* *S. boulardii (S. cerevisiae)* *S. pastorianus (synonym of Saccharomyces carlsbergensis)*
Kluyveromyces	*K. fragilis*
Fungi	
Aspergyllus	*A. orizae* *A. niger*

(a) In bracket valid taxonomic name.

4.1.2 Efficacy of probiotics and mode of action

Probiotic foods have been consumed for centuries, either as natural components of foods. A food can be said functional if it contains a component (which may or may not be a nutrient) that affects one or a limited number of functions in the body in a targeted way so as to have positive effects on health (Bellisle et al., 1998) or if it has a physiologic or psychologic effect beyond the traditional nutritional effect (Clydesdale, 1997). Amongst the most promising targets for functional foods are the gastrointestinal functions, including those that control transit time, bowel habits, and mucosal motility as well as those that modulate epithelial cell proliferation. Promising targets are also gastrointestinal functions that are associated with a balance colonic microflora, that are associated with control of nutrient bioavailability (ions in particular), that modify gastrointestinal immune activity, or that are mediated by the endocrine activity of the gastrointestinal system. Finally, some systemic functions such as lipid homeostasis that are indirectly influenced by nutrient digestion or fermentation represent promising targets (Roberfroid, 1996; Clydesdale, 1997). Havenaar and Spanhaak (1994) has reported that probiotics stimulate the immunity of the chickens in two ways (i) flora from probiotic migrate throughout the gut wall and multiply to a limited extent or (ii) antigen released by the dead organisms are absorbed and thus stimulate the immune system. At present it is believed that there is some relationship between the ability of strain to translocate and the ability to be immunogenic. The improvement in the immune system may be by three different ways:

(a) enhanced macrophage activity and enhanced ability to phagocytose microorganism or carbon particles;

(b) increased production of antibodies usually of immunoglobulin (Ig) IgG and IgM classes and interferon (a nonspecific antiviral agent);

(c) increased local antibodies at mucosal surfaces such as the gut wall (usually IgA).

The use of probiotics in animal feeding could be enhanced by a preliminary *in vitro* screening: antimicrobial activity, survival in the GIT, adhesive studies and antibiotic susceptibility are among the main probiotic properties that should be analysed to assess functionality and safety. The advanced molecular methods, *e.g*. microarrays, will improve the detection of these multiple characteristics allowing also the analysis, at genomic level, of phenotypic and genetic properties useful for industrial production.

Different strains of probiotic bacteria may exert different effects based on specific capabilities and enzymatic activities, even within one species (Bernet et al., 1993; Ouwehand et al., 1999). Different microorganisms express habitat preferences that may differ in various host species (Freter, 1992). Lactobacilli are among the indigenous flora colonizing the chicken's crop, stomach of mice and rats, and the lower ileum in man. Bacteria such high-transit-rate sites must adhere firmly to mucosal epithelium (Savage, 1972; Fuller, 1973; Beachey, 1980). Most of the bacterial colonies adhere to the intestinal wall and so does the probiotic. This is reason that the colonies are not swept away due to the peristalsis's along the intestinal wall. This effect prevents the pathogenic bacterial colonization along the intestinal wall and therefore, prevents disease development (Fuller, 2000). Numerous studies have shown that probiotics inhibit pathogens and disturbance of the intestinal microbiota with the antibiotics can increase susceptibility to infection but addition of probiotics increase the resistance to infection (Stavric and Kornegay, 1995;

Rolfe, 2000). Proposed mechanisms of pathogen inhibition by the intestinal microbiota include competition for nutrient, production of toxic conditions and compounds (volatile fatty acids, low pH and bacteroicins), competition for binding sites on the intestinal epithelium and stimulation of the immune system (Fuller, 1989; Gibson and Fuller, 2000; Rolfe, 2000). The mechanism of action of probiotics is not yet known and is open for research, although there are several hypotheses. There is increasing evidence to suggest that probiotics act by stimulating the host's immune systems. The only accepted example of effective protection against infections provided by living micro-organism is the "Nurmi concept", whereby one-day-old chicks acquire an enhanced protection against *Salmonella* infections when they are administered the complex intestinal flora of older chicks. The effects of probiotics on the growth, feed conversion or production of farm animals are, even in specific situations, not consistent enough to consider their use out of economic considerations (Veldman, 1992). In a very short period of time, many studies have been conducted to validate the concept of probiotics as a viable modality in the poultry production. Some known beneficial effects of probiotics include reduction in the severity and duration of rotavirus diarrhea (Oberhelman et al., 1999), reduction in the risk of traveler's diarrhea (Ribeiro and Vanderhoof, 1998), reduction in the risk of relapsing after the occurrence of *Clostridium difficile* - associated diarrhea (Pochapin et al., 1998), reduction in the risk of antibiotic-associated diarrhea in children (Vanderhoof et al., 1999), immune enhancement (Prowrie, 2001), stimulating the growth (Kumprechtova et al., 2000; Zulkifli et al., 2000; Lan et al., 2003) feed conversion ratio (Ergun et al., 2000; Panda et al., 2000; Silva et al., 2000) digestion and absorption (Fuller, 1997), competing for adhesion receptors (Savage, 1972; Fuller, 1973; Beachey, 1981). Although the number of organisms studied is small, the list is growing and it is likely that many more probiotic organisms with a variety of different benefits will be discovered. Additional organisms may eventually be developed through genetic engineering (Vanderhoof, 2001).

Probiotic activity could be related to genera, species, or strains. An approach in probiotic application could be the use of mixtures of strains belonging to different genera or species (Timmerman et al., 2004). The available body of literature offers a variety of conflicting results concerning the efficacy of probiotics for increasing growth performance in broilers; inconsistent results have been also reported from other authors (Estrada et al., 2001; O'Dea et al., 2006) showing a confusing state of the art. In fact, Stavric and Kornegay (1995) summarized several experiments in which different types of probiotics were supplied in feed or drinking water. They concluded that results were generally inconsistent. Since the report of Stavric and Kornegay (1995), the effects of probiotics administered in feed or water in poultry have been studied by many groups. Reported effects of these beneficial bacteria on chicken, hen and turkey performance varied from: little to no effect (Waldroup et al., 2003; Karaoglu and Durdag, 2005; Stanczuk et al., 2005; Donalson et al., 2008) through mixed effects (Anjum et al., 2005; Biggs et al., 2007; Bozkurt et al., 2009; Kim et al., 2011) to stimulating effects (Kabir et al., 2004; Awad et al, 2008; Mátéová at al., 2008; Willis and Reid, 2008). In addition, Timmerman et al. (2006) underlined the importance of way, dose, timing and duration of the administration as main factors affecting the efficacy of the probiotic preparation. Another determinant may be the age of the animals; during early life, colonization patterns are instable and newborn animals are then susceptible to

environmental pathogens. Initial colonization is of great importance to the host because the bacteria can modulate expression of genes in epithelial cells thus creating a favourable habitat for themselves (Siggers et al., 2007). However, it was difficult to directly assess different studies using probiotics because the efficacy of a probiotic application depended on many factors (Patterson and Burkholder, 2003) such as species composition and viability, administration level, application method, frequency of application, overall diet, bird age, overall farm hygiene, and environmental stress factors. Probiotic administration via the feed, compared to administration in the drinking water, resulted in a higher increase of average daily gain.

Eggs production has been also investigated in relation to probiotic application; Davis and Anderson (2002) reported that a mixed cultures of *Lactobacillus acidophilus*, *L. casei*, *Bifidobacterium thermophilus* and *Enterococcus faecium*, improved egg size and lowered feed cost in laying hens. Moreover, probiotics increase egg production and quality (Kurtoglu et al., 2004; Panda et al., 2008).

4.1.3. Lactic acid bacteria

Lactic acid bacteria (LAB) represent a greatly diverse group of bacteria, some of which belong to natural microbiota of the gastrointestinal tract. LAB constitute a diverse group of Gram-positive bacteria, characterized by some common morphological, metabolic and physiological traits. They are anaerobic bacteria, non-sporulating, acid tolerant and produce mainly lactic acid as an end product of carbohydrate fermentation. Moreover, several LAB strains have been demonstrated to exert a positive effect on a human or animal health, and thus being used as probiotics. Pro-health actions of LAB strains are based on their various biological activities – physical, physiological, biochemical and metabolic. Among these, a rapid and strong acidification of the environment and production of antimicrobials, like bacteriocins, seem to be very efficient in probiotic actions of LAB. It is important to identify those LAB strains which show the widest carbohydrate-digestive range, since the potential to digest sugars present in feed is important for both- lowering the inter gastrointestinal pH and protecting the avian organism against pathogenic microbes. In fact, their preservative effect is mainly due to the production of lactic acid and other organic acids which results in lowering of pH (Daeschel, 1989). Preservation is enhanced by the production of other antimicrobial compounds, including hydrogen peroxide, CO_2, diacetyl, acetaldehyde, and bacteriocins (Klaenhammer 1988; 1993; Stiles and Hastings, 1991).

Physiologically, based on the LAB strain, they are able to release to the gastrointestinal track of the host where they reside, different substances (e.g. antimicrobials) that interact with the GALT (Gut-Associated Lymphoid Tissue). This way they can stimulate cytokine production by lymphocytes, which leads to regulation of the innate and adaptive immune responses. Therefore, to assess the functional aspect of the immunomodulation of the LAB strains analyzed, the impact on the lymphocytes which are the crucial immune cells can be analyzed *in vitro*.

In addition, to the antimicrobial effects specific LAB also possess health promoting properties. Evidence from *in vitro* systems, animal models, and human clinical studies suggest that LAB function as immunomodulators and can enhance both specific and

nonspecific immune responses (Ouwehand et al. 1999; O'Flaherty and Klaenhammer, 2010) justifying their use as health promoting supplements or probiotics both for humans and animals.

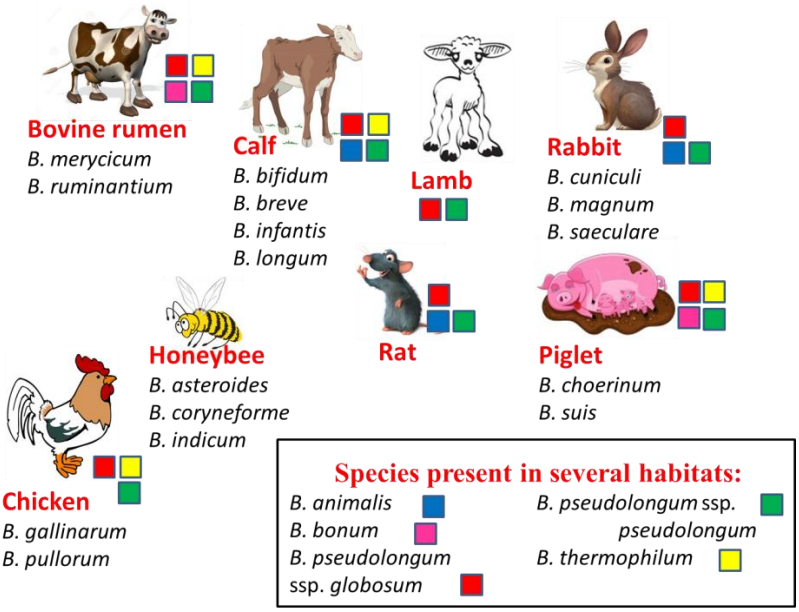
4.1.3.1 Lactococci probiotic application

Lactococci have a long history of use in milk fermentations, from small-scale traditional operations on the farm or in the family home to ever-increasing industrial scale processes. The consequent need for more robust, efficient and fine-tuned manufacturing practice has led to a sharp increase in fundamental and applied research on the bacterial species involved, *Lactococcus lactis*. *Lactococcus lactis* is able to reach intestinal cells and lives in for a lot of days and, for this, some strains, such *as Lactococcus lactis subsp lactis* and *Lactococcus lactis subsp. cremoris*, are considerable as probiotic. Many strains of *Lactococcus lactis* are resistant to some antibiotics such as tetraciclin because they contain a tetraciclin resistant gene. This observation proves that Lactococci are able to pick up multiple antibiotic resistance elements in an antibiotic challenged habitat from other member of the microbial gene exchange community that share genetic pieces. One of the most promising controllable expression systems that has been developed is based on the autoregulatory properties of nisin biosynthesis by *Lactococcus lactis* (de Ruyter et al., 1996; Kuipers et al., 1997). Nisin, a very active bacteriocin against others *Lactococcus* species and sometimes against harmful bacteria like *Enterobacteriaceae* and *Salmonella*, is a posttranslationally modified antimicrobial peptide (34 amino acid residues) that is widely used in the food industry as a natural preservative, designated as E234 (Klaenhammer, 1993) and used to protect high moisture food commodities against the pathogenic *Listeria monocytogenes* or *Clostridium botulinum*, but also against spoilage by clostridia and other Gram positive bacteria. (Delves-Broughton et al., 1996)

4.1.3.2 Bifidobacteria in animals and probiotic application

Studies on the intestinal microbiota, carried out mostly on domestic animals, have revealed a complex microbiota: *Bacteroides*, eubacteria, anaerobic lactobacilli, anaerobic Gram-positive cocci, spirillaceae and often bifidobacteria. Almost all chickens, dogs, pigs, rats and hamsters presented bifidobacteria, although in a smaller quantity than lactobacilli. Mice, rabbits and horses rarely displayed bifidobacteria, and cats and minks never had them. Many factors influence the composition of bifidobacteria microbiota in animals: the age, the species and the diet of the host. Some species apparently are host-specific: *B. magnum* and *B. cuniculi* have only been found in rabbit faecal samples, *B. pullorum* and *B. gallinarum* only in the intestine of chickens and *B. suis* only in piglet faeces (Matteuzzi et al., 1971; Figure 4.1).

Figure 4.1. *Bifidobacterium* species found in animals



In the intestinal tracts of animals and humans bifidobacteria are considered one of the key genus. Their presence in high number is associated to good health status of the host. There is a general believe that Bifidobacterium are helpful in maintaining appropriate balance of the microbiota in the GIT reducing the risk of pathogen infection. Several species are host specific (Biavati and Mattarelli, 2006). Bifidobacteria possess very promising probiotics properties; they are frequently used in food and pharmaceutical preparations and their application in animal feeding is increasing.

4.1.3.3 Lactobacillus probiotic action and application

Lactobacillus spp. are among the most frequent and better characterized microorganisms used as a probiotic. Important considerations in the choice of a probiotic include safety, functional aspects and technological aspects (Donohue et al., 1998). Many of the species are significant constituents of the normal gut microbiota of humans and animals, and their occurrence and number are host dependent. Several species of the genus are intentionally introduced in the food chains, being involved in a range of food and feed fermentations, and applied as probiotics in humans and animals (Hammes and Hertel, 2007). However, an increasing number of reports stated that these microorganisms might occasionally be involved in human diseases, where *L. casei* and *L. rhamnosus* are the most common (Vesterlund et al., 2007). No report can be found on safety concerns related to lactobacilli in animals.

4.1.4 Competitive exclusion

Competitive exclusion (CE), also indicated as the "Nurmi concept", has its origin on the finding that the newly hatched chicken could be protected against Salmonella colonization of the gut by dosing it with a suspension of gut content prepared from healthy adult chickens (Nurmi and Rantala, 1973). The introduction of CE bacteria from the gut content should occur early in life, such that the CE bacteria are preferentially established in the

gastrointestinal system to become competitive or antagonistic to opportunistic pathogens. Because of the use of undefined preparations from the cecal or fecal material could result in the transmission of pathogens, regulatory restrictions for probiotic microorganisms (SCAN, 2000) made this kind of products difficult to be authorized. However, CE products with defined and identified microorganism have been developed and applied in animal breeding (Schneitz, 2005).

4.2 Prebiotics

Prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the intestinal tract, thus improving the host's microbial balance. (Gibson and Roberfroid, 1995). For a dietary substrate to be classed as a prebiotic, at least three criteria are required: (1) the substrate must not be hydrolyzed or absorbed in the stomach or small intestine, (2) it must be selective for beneficial commensal bacteria in the large intestine such as the bifidobacteria, (3) fermentation of the substrate should induce beneficial luminal/systemic effects within the host (Scantlebury-Manning and Gibson, 2004). The effects of dietary fiber on upper and lower gastrointestinal tract are shown in Table 4.3. Most identified prebiotics are carbohydrates and oligosaccharides (OS) normally occurring in human and animal diet, with different molecular structures; dietary carbohydrates such as fibers, are candidate prebiotics, but most promising are non-digestible oligosaccharides (NDOs). NDOs which meet the critical point of the definition are fructo-oligosaccharides (FOS, oligofructose, inulin), galacto-oligosaccharides (GOS) or transgalacto-oligosaccharides (TOS), and lactulose; however a large number of other NDOs, to which less rigorous studies have been so far applied are glucooligosaccharides, glycooligosaccharides, lactitol, isomaltooligosaccharides, chitooligosaccharides (which have demonstrated to possess prebiotic activity in human) (Campbell et al., 1997; Francis Suh and Matthew, 2000; Zentek et al., 2002; Wu et al., 2005; Shoaf et al., 2006), maltooligosaccharides, xylo-oligosaccharides, stachyose, raffinose, and sucrose thermal oligosaccharides have also been investigated (Patterson and Burkholder, 2003). Although mannanoligosaccharides (MOS) have been used in the same manner as the prebiotics listed above, they do not selectively enrich for beneficial bacterial populations. Investigation on the mode of action of mannanoligosaccharide pointed out that these compounds are able to bind to mannose-specific lectin of Gram-negative pathogens that express Type-1 fimbrieae such as *Salmonella* and *E. coli* resulting in their excretion from the intestine (Baurhoo et al., 2007; Thomas et al., 2004).

Table 4.3. Intestinal functions assigned to prebiotics

Dietary fibers and gastrointestinal functions	
Effect on upper GI tract	Resistance to digestion
	Retarded gastric emptying
	Increased oro-caecal transit time
	Reduced glucose absorption and low glycaemic index
	Hyperplasia of the small intestinal epithelium
	Stimulation of secretion of intestinal hormonal peptides
	Acting as food for colonic microbiota

	Acting as substrate for colonic fermentation Production of fermentation end products (mainlt SCFAs) Stimulation of saccharolytic fermentation
Effect on upper GI tract	Acidification of the colonic content Hyperplasia of the colonic epithelium Stimulation of secretion of colonic hormonal peptides Bulking effect on stool production Regularization of stool production (frequency and consistence) Acceleration of caeco-anal transit

Dietary modulation of the human gut flora has been carried out for many years. In humans, prebiotic addition to the diet has brought positive aspects to the gut microbial balance. The use of prebiotics in animal production, as possible alternative to antimicrobial growth promoters, has given conflicting results, while their use in the modulation of the gut microbial equilibrium is worthwhile. They contribute to the establishment of a healthier microbiota where bifidobacteria and/or lactobacilli become predominant and exert possible health-promoting effects at the expense of more harmful species.

It has been shown that prebiotics stimulate the growth of endogenous microbial population groups such as bifidobacteria and lactobacilli.

Several reports have shown that supplementing a diet with OS improved the chicken growth (Kim et al., 2011), while other reports (Solis de los Santos et al., 2007) did not find any growth effect of this treatment. The reasons for the different results are not clear. However, it can be suggested that this effect may be due to the disadvantageous interaction between various fodder additions such as oligosaccharides, antibiotics, growth hormones, coccidiostatics, different chemical structure and composition of the OS used (Patterson and Burkholder, 2003), as well as the time and amount of feed and/or water intake consumption. Consequently, consumption of prebiotics is very different in the first h/d after the hatch of chickens. In this period, the contamination by the detrimental bacteria is possible. In general, available energy is a limiting factor for the growth of colonic microflora. Hence, enrichment of a diet with NDOs results in an increase of metabolic activity and the development of beneficial bifidobacteria in the colon (Mitsuoka et al. 1987; Gibson et al. 1994; Gibson, 1998; Tomomatsu 1994; Van Loo et al. 1999; Gulewicz et al. 2002). As it is known from the literature, OS as prebiotics contribute to health in many ways: (i) they influence the proliferation of bifidobacteria and reduce the numbers of detrimental microorganisms; (ii) they reduce toxic metabolites and detrimental enzymes; (iii) they prevent pathogenic and autogenous diarrhea; they prevent constipation by stimulating intestinal peristalsis and by increasing faecal moisture with osmotic pressure; (iv) they alleviate the detoxifying load of the liver; (v) they reduce the serum cholesterol level; (vi) they reduce blood pressure; (vii) they show anticancer activity; (viii) they influence the production of vitamins B1, B2, B6, B12, nicotic and folic acids (Corrier et al. 1990; Bailey et al. 1991; Waldroup et al. 1993; Choi et al. 1994; Tomomatsu, 1994; Ammerman et al. 1988).

4.2.1 FOS, fructooligosaccharides

Fructooligosaccharides are natural food ingredients commonly found in varying percentages in dietary foods. They are present in > 36.000 plant species. They are present as storage carbohydrate, together with inulin, in a number of vegetables and plants including wheat, onion, bananas, garlic and chicory. Fructooligosaccharide products include inulin, oligofructose, and short-chain fructooligosaccharide (SCFOS). FOS has been shown to be indigestible by human enzymes in the small intestine, but is extensively fermented in the large bowel (Mitsuoka et al., 1987) to short chain fatty acids (SCFA), which can be absorbed and metabolized by the host. To further investigate the role of selected oligosaccharides on concentrations of cecal SCFA, total large bowel wet weight and wall weight, and concentrations of intestinal microbiota, and to discuss the mechanisms of oligosaccharides' action to the host, it was tested the effect on the large bowel by gavage of selected oligosaccharides in mice model. SCFA and lactate of the cecum in mice were increased by the intake of selected prebiotic oligosaccharides, especially FOS and GOS. The cecal total weight and wall weight were also increased, which may be a result of SCFA change. In addition, these selected oligosaccharides stimulating the bifidobacteria and lactobacilli in the cecum may be useful in promoting gastrointestinal health (McBain and Macfarlane, 2001; Smiricky-Tjardes et al., 2003). Thus, providing these oligosaccharides as ingredients in nutritional formulas could benefit the health of the gastrointestinal tract. In addition, FOS also may help control or reduce the growth of harmful bacteria such as *Clostridium perfringens*, which is especially important to the poultry industry because this bacterium is a primary cause of necrotic enteritis that has been estimated to cost the worldwide poultry industry $2 billion each year (Dahiya et al., 2006).

In all the nutritional trials so far reported that have tested for the effect of FOS on human microbiota, the increase in the number of bifidobacteria has been reported and it has been observed that:

• the number of bifidobacteria becomes significant and reaches its maximum probably in less than a week;

• remains as long as the intake of the probiotic continues;

• progressively (within 1-2 weeks) disappears when the intake stops.

It has been demonstrated also that the intake of FOS reduces significantly the count of Bacteroides, Fusobacteria and Clostridia. The increase in bifidobacterial flora is accompanied with other beneficial effects such as: modulation of intestinal functions, increase of stool weight, decrease of faecal pH (probably linked to the suppression of the production of putrefactive substances in the colon), modulation of cholesterol levels and modulation of mineral metabolism (Roberfroid, 2000).

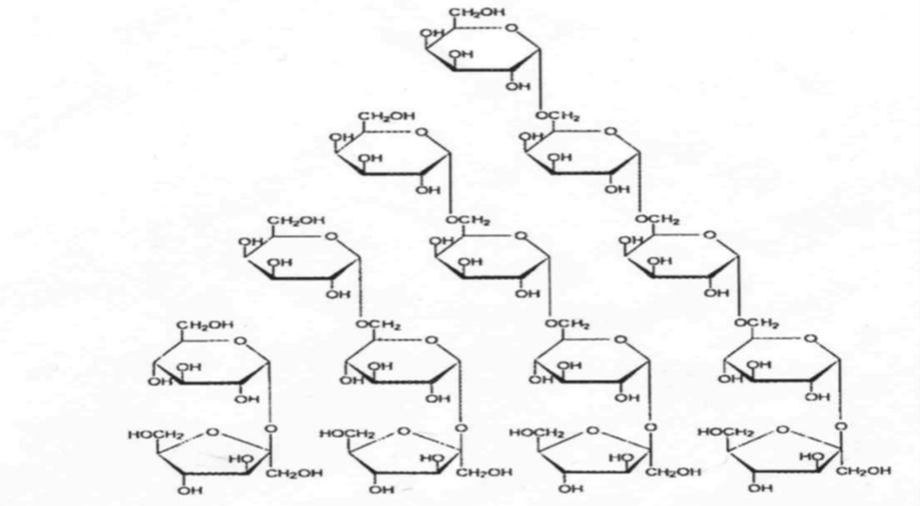
4.2.2 RFOs, raffinose family of oligosaccharides

Up to now, fructooligosaccharides have been the best known and most commonly applied oligosaccharides (Hidaka et al., 1986; Jong Won, 1996). In this aspect, compounds such as α-galactosides, also called raffinose family oligosaccharides (RFOs) are known to a lesser extent. These saccharides occur mainly in plants belonging to the Fabaceae family, where they perform a very important physiological function (Larsson et al., 1993; Horbowicz and

Obendorf, 1994; Górecki et al., 1997). RFOs accumulate in seeds, stems, leaves, roots, and tubers of plants (Avigad and Dey, 1997). The highest RFOs concentration is found in seeds, where RFOs play important roles during seed germination, acquisition of desiccation tolerance and preservation of seed longevity. RFOs are soluble carbohydrates which contain linear galactosyl residues attached to the glucose moiety of sucrose via a α-(1→6) glycosidic linkage (Avigad and Dey, 1997; Figure 4.2). These non-reducing sugars can be regarded as derivatives of sucrose with a varying number of galactosyl residues attached (Dey, 1980). The most common forms of RFOs include raffinose, stachyose, verbascose and ajugose which are tri-, tetra-, penta-, and hexa-saccharides, respectively (Figure 4.2). Dicotyledonous plants primarily accumulate stachyose and verbascose, whereas raffinose is the major RFOs in monocotyledonous plants (Peterbauer and Richter, 2001). From the nutritional point of view, RFOs have been considered thus far as antinutritional factors because they are not hydrolyzed by mucosal enzymes in the small intestine of monogastric animals and are fermented in the lower gut with liberation of gas causing arduous flatulence (Cristofaro et al., 1974; Saini and Gladstones, 1986; Prince et al., 1988). The opinion about the total function of RFOs in nutrition has recently changed due to the elaboration of a simple and rapid method of their isolation and purification from legume extracts, including lupin one (Gulewicz et al., 2000; Martinez-Villaluenga et al., 2004). It allows for wide studies of these sugars in terms of their prebiotic activity. In fact, in recent years, these compounds have been an object of growing interest of nutritionists as prebiotics due to the fact that they modify the composition of the colon bacterial microflora.

The chicken embryo is an excellent model for the investigation of biological activity of natural plant products and their effect on embryonic development.

Figure 4.2. Structures of sucrose and major RFOs (Avigad and Dey, 1997)



sucrose raffinose stachyose verbascose

4.3 Synbiotics

Probiotics and prebiotics act as growth promoters feed savers, nutritional bio-regulators, immune stimulators and help in improving performance and health (Falaki et al., 2011).

In simplest definition, symbiotic is a combination of probiotics and prebiotics (Collins and Gibson, 1999). This combination can improve the viability of probiotic microorganisms, since they are able to use prebiotics as a substra for fermentation (Bengmark, 2001). Synbiotics may be defined as a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract (Gibson and Roberfroid, 1995). The acquisition of data on the efficacy of synbiotic products as feed additives in livestock and poultry needs further investigation. However, results on *in vivo* trials are promising, either in young animals or adults: the coupling of a probiotic and prebiotic could also yield a synergistic effect in the reduction of food-borne pathogenic bacterial populations in food animals prior to slaughter (Bomba et al., 2002).

Chapter 5. Application of Probiotics, Prebiotics and Synbiotics

The adaptation to the post hatching period and the increased stressors, deriving from practices used in modern broiler production, *e.g.* feed changes or imbalances, transportation, processing at the hatchery and high stocking densities (Pinchasov and Noy, 1993), may weaken immune functions and thus predispose broilers to colonization of the gastrointestinal tract by bacterial pathogens, posing a threat to birds health and food safety. Among pathogens, *Salmonella* spp. has been the most studied because of its ability to infect chickens and hens increasing the risk of contamination through the food chain (Humphrey, 2006).

In the last years, application studies have been extended to other bacteria such as *Campylobacter jejuni* and *Clostridium perfringens*, which could be both considered an emerging and increasing threat for poultry and human health (Humphrey et al., 2007; Van Immerseel et al., 2004). Probiotics could be a possible strategy to control pathogens shedding and thus maintain a healthy indigenous gut microbiota.

The application of probiotics in poultry is strictly associated with the concept of CE. Since the first applications on new hatched chicks, several experiments with undefined and defined probiotic cultures have been developed and successfully applied to control and reduce *Salmonella* colonization. Moreover, it has been shown experimentally that the CE treatment also protect chicks against *C. jejuni*, *Listeria monocytogenes*, pathogenic *E. coli*, *Yersinia enterocolitica* and *C. perfringens* (Nisbet, 2002; Schneitz, 2005).

A variety of well-characterized probiotic strains have been selected to evaluate the modulation of the avian gut microbiota and the protection against a variety of pathogens; however there has been a recent increase in the investigation of the effect of feeding *Lactobacillus* spp. to broilers, focusing on strains previously selected *in vitro* for adhesion properties and antimicrobial activity (Patterson and Burkholder, 2003).

Higgins et al. (2008) showed that *Lactobacillus*-based probiotic cultures reduced significantly *Salmonella enteritidis* recovery in challenged neonatal broiler chicks. Furthermore, the administration by vent application, compared to traditional application by drinking water, resulted in significant reduction of *S. enteritidis* one hour following oral challenge. In a previous trial, the same probiotic cultures affected the concentration of *S. enteritidis*, both in cecal tonsils and in caeca content, whereas no relevant results were obtained towards *S. thipymurium* (Higgins et al., 2007).

No differences in cecal and colonic counts were observed testing the efficacy of *L. johnsonii* F19185 in reducing the colonization and shedding of *S. enteriditis* in newly hatched chicks; nevertheless, the colonization of *E. coli* O78K80 and *Clostridium perfringens* were compromised significantly (La Ragione et al., 2004). Lactobacilli were also successful in decreasing mortality due to necrotic enteritis from 60% to 30% in a challenge trial, when they were given orally to day-old chicks (Hofacre et al., 2003).

To date, few studies evidenced a possible role of probiotics in preventing the shedding of *Campylobacter jejuni* at the level of primary production, although *in vitro* studies reported a strong antimicrobial activity of several species of *Lactobacillus* towards this pathogen (Chaveerach et al., 2004; Fooks and Gibson, 2002). Willis and Reid (2008) showed that *C. jejuni* presence was lower in broiler chickens fed with a standard diet supplemented with a

minimum presence of 10^8 cfu/gr of *L. acidophilus, L. casei, Bifidobacterium thermophilus,* and *E. faecium*.

With regard to probiotic microorganisms, other than *Lactobacillus* spp., Vila et al. (2009) reported a reduction of *S. enteritidis* colonization and invasion by feeding continuously spores of the probiotic strain *B. cereus* var. *toyoi,* both in broiler chickens and white leghorn chickens.

In a study conducted by La Ragione and Woodward (2003), 1-day-old and 20-day-old specific pathogen free chicks were dosed with a suspension of *B. subtilis* spores prior to challenge with *S. enteritidis* and *C. perfringens*; the treatment suppressed completely the persistence and colonisation of both pathogens. Studies testing the use and efficacy of *Bifidobacterium* spp., following pathogen challenge, have not yet been described. Mainly, authors have been focused on the beneficial impact on the gut microbiota and growth performance (Estrada et al., 2001; Jung et al., 2008).

The use of bifidobacteria in poultry feeding is less common with respect to lactobacilli administration. Along with the control of foodborne pathogens in the avian gut, selected probiotic cultures, mainly *Lactobacillus* spp., may also potentially increase performance parameters; among poultry farmers, objectives such as increasing growth rate, improving feed conversion and meat quality are undoubtedly of primary importance.

Kalavathy et al. (2003) found that a supplementation of twelve *Lactobacillus* strains in broiler diets improved the body weight gain, feed conversion rate and was effective in reducing abdominal fat deposition.

Mountzouris et al. (2007) investigated the efficacy of selected probiotic bacteria, isolated from the gut of healthy chickens (*Lactobacillus reuteri*, *L. salivarius*, *Enterococcus faecium*, *Bifidobacterium animalis* and *Pediococcus acidi lactici*) and on body weight, feed intake and feed conversion ratio of broiler chickens; overall the probiotic formula added to water and feed displayed a growth-promoting effect that was comparable to avilamycin treatment. In addition, the probiotic cultures modulated the composition and the enzymatic activities of the cecal microbiota, resulting in a significant probiotic effect.

To date, probiotics are one of major food supplements for poultry industry. According to concerns about cholesterol, there are a lot of attempts to produce foods with low cholesterol. It has been reported that *L. acidophilus* can absorb cholesterol from in vitro system and this phenomenon can decrease the cholesterol level of medium (Gilliland and Speck, 1977; Gilliland et al., 1985). There are reports that probiotics can reduce the cholesterol level of blood in broiler chickens (Mohan et al., 1996; Khani et al, 2008). Panda et al. (2003) reported that probiotics cause the reduction of serum and yolk cholesterol and also the increase of egg production.

The cholesterol level of serum significantly decreased in groups supplemented with probiotics in compared to control group. *L. acidophilus* is capable to deconjucate glyco cholic and taurocholic acids under anaerobic condition (Gilliland and Speck, 1977). Deconjucation of gallbladder acids in small intestine can affects control of serum cholesterol, while deconjucated acids are not capable to solve and absorb fatty acids as conjucated acids. As a consequence,they prevent from absorption of cholesterol. Also free gallbladder acids attach to bacteria and fibres and this can increase the excretion of them.

Probiotic prescription is a good alternative for antibiotics for several reasons: suitable function, non-existence of residue in poultry productions, environmental protection and also prohibition of antibiotics usage in Europe union (Dilworth and Day, 1978; Tortuero and Fernandez, 1995).

The prebiotic approach has not a long history of use in broiler chickens (Yang et al., 2009). However, application studies have been increasing in the last years to assess their effect on gut health, performance, and reduction of pathogen shedding. Xu et al. (2003) found a dose-dependent effect of FOS on average daily gain; whereas Juskiewicz et al. (2006) reported no impact on the performance or productivity of turkeys after feeding for eight weeks with different amounts of FOS. By feeding chycory fructans to broilers, Yusrizal and Chen (2003a) showed an improvement in weight gain, feed conversion, carcass weight and serum cholesterol decrease; additionally, the supplementation of fructans resulted in increase of lactobacilli counts in the gastrointestinal tract and *Campylobacter* and *Salmonella* decrease (Yusrizal and Chen, 2003b). Klessen et al. (2003) described decreased *C. perfringens* number and a reduction in bacterial endotoxin levels by adding 0,5% of fructan-rich Jerusalem artichokes syrup in broilers drinking water. No weight gain was observed in turkeys fed with two different concentration of inulin and mannanoligosaccharides (Stanczuk et al., 2005), whereas Sims et al. (2004) reported an improvement on live weight after feeding turkeys a standard diet supplemented with MOS. Yeast cell wall containing MOS reduced intestinal *Salmonella* concentrations by 26% in broiler chicks compared with chicks fed with an unsupplemented diet (Spring et al., 2000, Gaggìa et al., 2010). Thitaram et al. (2005), with different amounts of isomaltooligosaccharide, showed a significant 2-log reduction in the level of inoculated *S. enterica* serovar *typhimurium* present in the caeca of young broiler chickens. Feed consumption, feed conversion and feed efficiency were not significant compared to the control; however, the IMO containing diets significantly increased the number of the intestinal bifidobacteria. Feeding young chicks with five different oligosaccharides (inulin, oligofructose, mannanoligosaccharide, shortchain fructooligosaccharide, and trans-galactooligosaccharide) did not registered any significant responses in weight gain for any of the oligosaccharides; moreover the study outlined that high dosage of prebiotics can have negative effects on the gut system and retard the growth rate of birds (Biggs et al., 2007).
Likewise, a recent study reported no effects in body weight, feed intake and feed conversion ratio in broiler chickens fed with a standard diet and GOS at two different concentrations; however the study clearly showed a significant increase in the intestinal bifidobacteria population (Jung et al., 2008). Mainly, prebiotics seem to enhance selectively lactobacilli and bifidobacteria populations and reduce colonization by pathogenic bacteria (Biggs and Parsons, 2008; Baurhoo et al., 2009). Results on animal performance, either with a probiotic or a prebiotic treatment, are often contradictory and mostly affected by the microorganisms or compound chosen, the dietary supplementation level, and duration of use. In many cases, the environmental and the stress status of the animals are not reported or considered as the experimental setting are often too far from the farm conditions.

Recent development and applications of synbiotic products have been focused on the assessment of beneficial effects in poultry health and production; however, there is still scarce information available to date. Mohnl et al. (2007) found that a synbiotic product had a comparable potential to improve broiler performance as avilamycin treatment. A *Lactobacillus* spp.-based probiotic product, in combination with dietary lactose, was successfully assessed, improving body weight and feed conversion in *Salmonella*-challenged turkeys (Vicente et al., 2007). Li et al. (2008), adding FOS and *B. subtilis* to the diet, observed that average daily gain and feed conversion ratio were improved; diarrhoea and mortality rate were reduced compared to aureomycin treatment. A considerable increase in the bifidobacteria, lactobacilli and total anaerobes populations has been shown by using a diet containing a combination of a GOS and *Bifidobacterium lactis*; in contrast, no effect on body weight, feed intake and feed conversion has been observed (Jung et al., 2008). Awad et al. (2009) investigated the effect of a dietary treatment with a synbiotic product (a combination of *E. faecium*, a prebiotic derived from chicory, and an immune modulating substances derived from sea algae) on broiler chickens. The body weight, average daily weight gain, carcass yield percentage, and feed conversion rate were significantly increased compared with the control, whereas no increase in organs weight was found with exception for the small intestine; a significant increase in the villus height in both duodenum and ileum was also observed. Overall, all the authors agreed that a symbiotic product displayed a greater effect than individual preparations (Awad et al., 2009; Jung et al., 2008; Revolledo et al., 2009; Vandeplas et al., 2009). This coupling could represent an important and synergic strategy to improve gut health of chickens from the first days of life and control pathogen release in the environment decreasing the risk of foodborne infections in humans. Thus, future research and applications in field trials are necessary to look for new combination with the aim to produce standard safe composition at a high functional level (Gaggìa et al., 2010).

Chapter 6. Aiding embryo nutrition in ovo

6.1 Hatchery holding period

It is common practice in the poultry industry to hold poults without feed and water for many hours after hatch. Poults may remain for up to 36 hours after hatching before they are pulled from the hatching cabinet, and then it may take an additional 72 hours before they are be serviced and transported to brooder farms where they finally have access to feed and water. Poult servicing includes sexing, toe trimming, snood removal, beak trimming, and injection of antibiotics (Donaldson and Christensen, 1991; Donaldson et al., 1991). After enduring this stress of servicing poults are often held in transportation boxes stacked in a dim room for up to 24 hours so they can recover and endure the stress of transport and placement. Donaldson et al. (1991) confirmed that poults recuperate liver glycogen concentration to levels prior to servicing when held for 24 hours, however this glycogen status recover occurs at the expense of catabolizing their own protein reserves (Donaldson, 1995; Keirs et al., 2002) since they have no access to feed or water during this time. Several studies were performed to evaluate the impact of this early fasting period on poult development, comparing hatchlings that were held for 24 hours with those given *ad libitum* access to feed and water immediately after they were removed from the hatcher. Careghi et al. (2005) observed that broiler chicks fed immediately after hatch showed higher weight gain later in life as compared to the held chicks, and that late hatchers benefit more from early access to feed. Uni et al. (1998) demonstrated that early fasting clearly delays gut maturation, affecting the development of mucosal morphology and intestinal enzyme activity. Fed poults and chicks also have more goblet cells per villus, and more proliferating enterocytes, in contrast to more apoptotic cells in fasted birds (Uni et al., 1998; Potturi et al., 2005; Smirnov et al., 2006). According to Potturi et al. (2005), poults fed immediately after hatch had 5.0μm longer and 6.8μm wider intestinal villi, and 5.6μm deeper villi crypts than fasted-held poults. These fasted poults also had more aerobic bacteria in their small intestine (Potturi et al., 2005). Feed restriction early in life also programmed birds for obesity later in life (Zhan et al., 2007) by permanently altering energy related enzyme production and function. Velleman and Mozdziak (2005) found reduced muscle growth among chicks that experienced a 72 hour of fasting after hatch. Because of the literature sited above, there is great interest in ways to aid poult nutrition before placement, as Careghi et al. (2005) suggested to provide an energy source in the hatch basked and during transport. There are several other management practices and conditions that can accentuate the adverse effects of a long post-hatch holding period, including egg storage period, egg size, and hatch window. There are many factors that may delay the initiation of feeding, and it is a challenge to manage all these factors to reduce their impact on hatchability, viability and performance. New technologies are welcome and necessary to address these problems.

While changes in the way hatcheries handle poults have not been implemented during the last 3 decades, a lot of studies have been focusing on early feeding. One hypothesis is that having the first feed formulated to improve gut development could compensate for the previous long period of fasting. For example, feeding yeast extract to turkey poults has

been shown to accelerate gut maturation in comparison to poults fed regular diets (Solis de los Santos et al., 2007). Even just the physical presence of solids with no nutritive value in the intestine is enough to stimulate maturation and growth, but with no lasting effects (Noy and Sklan, 1998a). Early feeding stimulates gastrointestinal motility and use of yolk sac nutrients necessary for growth (Noy and Sklan, 1998a, 1998b, 1999a, 2001). Offering glucose in drinking water was the earliest initiative to improve chick energy status, but the practice was later found to be detrimental because glucose suppresses gluconeogenesis, which is the main source of energy to the early hatchling (Donaldson, 1995). One other option is to offer feed in the transport boxes, which led to the design of products like Oasis (Novus International, Inc., MO), which is made of protein, carbohydrate, fat and fiber in a green granular form. The product attracts chicks and poults to eat during transport or when dressed on top of feed trays in the brooder house. Although gavage of nutrients is not practical under commercial conditions, it was found to be effective as Oasis in increasing body weight and breast meat yield (Noy and Sklan, 1999b). Even at market age early fed birds were 8-10% heavier and had 7-9% bigger breasts than birds held without feed (Noy and Sklan, 1998a, 1999b). Another option tested was subcutaneous injection of nutrients at servicing. Gluconeogenic substrates like amino acids and vitamins were injected subcutaneously in just hatched broiler chicks and resulted in 10% gain in body weight in 24 hours and nearly double their weights in 96 hours due to reduced tissue catabolism (Keirs et al., 2002; Peebles et al., 2006). Another study testing the injection of glucose and alanine concluded that under proper brooding conditions and timely feed provision, growth was not improved (Donaldson, 1995; Keirs et al., 2002; Peebles et al., 2006). John et al. (1987) tested the effect of immerging eggs in a glucose-antibiotic solution, and saw no differences in glycogen reserves, but an increase on lactate, indicating increased glycolysis. From all options tested, early feeding seems to be the most advantageous, while gavage, injections and egg dipping showed to be unpractical or have inconsistent results.

6.2 *In ovo* feeding (IOF) concept

To date, approximately 95% of broilers are vaccinated by *in ovo* injection. *In ovo* injection technology not only provides a method for vaccination, but also a practical means by which to safely introduce external nutrients into developing embryos. Vaccine manufacturers today are desiring to develop new products that can be injected *in ovo* to enhance vaccine efficacy and to improve broiler embryogenesis and posthatch performance. Nutrients and other metabolic compounds for *in ovo* injection, such as amino acids, carbohydrates, vitamins, stimulants, and hormones, are under investigation (Gore and Qureshi, 1997; Johnston et al., 1997; Henry and Burke, 1999; Kocamis et al., 1999, 2000; Ohta et al., 1999; Jochemsen and Jeurissen, 2002; Tako et al., 2004; Uni et al., 2005; Foye et al., 2006; Kadam et al., 2008; Zhai et al., 2008; Keralapurath et al., 2010a,b). During late embryogenesis, solutions injected into the amnionic fluid are subsequently swallowed, digested, and absorbed by the embryo before pipping (Uni et al., 2005). *In ovo* feeding of supplemental nutrients may help late-term embryos to overcome the constraints of limited egg nutrients (Foye et al., 2006). Rapid growth coupled with a high energy requirement, especially during late embryogenesis, may make *in ovo* feeding of supplemental carbohydrates beneficial to broiler embryos. In fact, it was hypothesized that

the administration of various types of carbohydrates to the amnion may improve the energy level of the broiler embryo and reduce internal energy consumption (proteins and lipids) during pipping, thereby increasing subsequent hatchability and chick body weight. It has previously been shown that the *in ovo* injection of a mixture of carbohydrates dissolved in saline (sucrose (a monosaccharide), maltose (a disaccharide), and dextrin (a polysaccharide) with or without β-hydroxy-β-methylbutyrate (HMB, a leucine metabolite) on day 17 or 17.5 of incubation improved embryonic intestinal development and subsequently increased total chick body weight at hatch (Tako et al., 2004; Uni and Ferket, 2004; Uni et al., 2005; Smirnov et al., 2006).

The greatest opportunity to facilitate improvements in hatchability and hatchling viability was, for a long time, to change incubation conditions. In the early days of poultry industry, Smith (1937) advised farmers to invest in turkey hen nutrition because, after the egg was laid, it was impossible to increase any of the food essentials for development and growth of the embryo until it hatches. In the beginning of the 80's, *in ovo* technology has become an alternative to the vaccination of broiler chicks against disease (Sharma and Burmester, 1982). In fact, *in ovo* vaccination against Marek's disease was proven to be effective against early exposure to the virus (Sharma and Burmester, 1982). These same authors developed a successful method of *in ovo* injection. Experimental injection of small amounts of drugs, vaccines and nutrients in the egg during incubation was tested along the years. The injection of nutrients in the egg during incubation is done by using special needle that is introduced through the shell and membranes until the tip reaches the target (the amnion), delivering nutrients without harming the embryo (Figure 6.1)

Figure 6.1. Schematic representation on *in ovo* injection

A summary of early research papers mentioning *in ovo* administration is presented on Table 6.1.

Table 6.1. Summary of research papers mentioning *in ovo* administration of substances

Article	*In ovo* injected substance	Target
Balaban and Hill, 1971	L-thyroxine, thiourea	embryo
Al-Murrani, 1982	amino acids	yolk
Decuypere et al., 1982	Iopanoic acid	allanotic circulation
Sharma and Burmester, 1982, 1984	vaccines	amnion/embryo
Sharma, 1984	vaccines	amnion/embryo

Sharma, 1985	vaccines	amnion/embryo
Wakenell and Sharma, 1986	vaccines	amnion/embryo
Iqbal et al., 1987	methimazole	allanotic circulation
Hargis et al., 1989	growth hormone	albumen
Ahmad and Sharma, 1992	vaccines	amnion/embryo
Sharma et al., 1994	vaccines	amnion/embryo
Johnston et al., 1997	vaccines	amnion/embryo
Edens et al., 1997	lactobacillus	air cell, amnion, embryo
Kocamis et al., 1999	growth hormone	albumen (pre-incubation)
Henry and Burke, 1999	testosterone, antiandrogen	albumen (pre-incubation)
Coles et al., 1999	peptide yy	air cell
McReynolds et al., 2000	antibiotics	amnion
Williams and Brake, 2000	fungicides, mold inhibitors	air cell
Williams et al., 2000	contaminated air antibodies against adipocyte	air cell
Wu et al., 2000	membrane	allantoic circulation
Kocamis et al., 2000	insulin-like growth factor-i	albumen
Ohta and Kidd, 2001	amino acids	yolk
Otha et al., 2001	amino acids	yolk
Jochemsen and Jeurissen, 2002	detectable particles and vaccines	amnion
Weber et al., 2004	Eimeria	air cell
Tako et al., 2004	carbohydrates, HMB	amnion
Uni et al., 2005	carbohydrate, HMB	amnion
Tako et al., 2005	zinc-methionine egg white, HMB	amnion
Foye, 2005	carbohydrate, arginine	amnion
Moore, 2005	egg white, HMB	amnion
Smirnov et al., 2006	carbohydrates	amnion
Foye et al., 2006	egg white, HMB, carbohydrates	amnion
Kim et al., 2006	antibodies against myostatin	albumen or yolk
Matsushita et al., 2006	pesticides	albumen (pre-incubation)
Pedrosa et al., 2006	glucose	amnion
Kim et al., 2007	antibodies against myostatin	yolk

The *in ovo* studies presented on Table 6.1 show a variety of substances being injected in different compartments of the egg. The first studies of Balaban and Hill (1971), Al-Murrani (1982), Sharma and Burmester (1982), Sharma and Burmester (1984) determined that injecting solutions in the air cell or on the chorioallantoic membrane depressed hatchability. Balaban and Hill (1971) proved that hatchability is dependent on thyroid hormones, which was later confirmed by Decuypere et al. (1982). Al-Murrani (1982) was the first to attempt improving embryo body weight by adding amino acids to the yolk sac of chicken embryos at 7 days of incubation. He concluded that embryos used the extra protein to grow heavier only when they reached late embryonic growth, and that supplemented chicks were heavier all the way to market age. His idea was not to make *in ovo* supplementation commercially viable, but to prove that laying hens needed additional protein in their diets.

Most of the studies done during the following two decades were focused on *in ovo* vaccination (Johnston et al., 1997). Research was done to test the effect of growth hormone

(Hargis et al., 1989; Kocamis et al., 1999), testosterone (Henry and Burke, 1999), peptide YY (Coles et al., 1999), antibiotics (McReynolds et al., 2000), insulin-like growth factor-I (Kocamis, et al., 2000), pathogens (Williams et al., 2000; Weber, et al., 2004), and fungicides and toxic compounds (Williams et al., 2000; Matsushita et al., 2006). Otha et al. (2001) resumed Al-Murrani's work, with the goal to improve hatchability and chick weight (Ohta and Kidd, 2001; Ohta et al., 2001); to that end, they injected a mixed amino acid solution similar to the amino acid profile of egg white into different embryonic compartments on day 7 of incubation and then, they concluded that the yolk sac or the extra-embryonic cavity was the best target for amino acid injection at 7 days of incubation to positively affect hatchability. However, injecting this amino acid solution into the amnion at 7 days of incubation resulted in embryonic death within 24 hours. Yolk and extra-embryonic coelom supplemented chicks hatched heavier than the controls (Ohta and Kidd, 2001), and had higher amino acids concentration in their tissues (Ohta et al., 2001).

In ovo vaccination has become a widely adopted practice by the poultry industry (Johnston et al., 1997), and after the technology was patented (Sharma and Burmester, 1984). In fact, Uni and Ferket (2003) patented (Uni and Ferket 2003, US patent, 6.592.878) the concept of administrating a nutritive solution into the amniotic fluid so as to "feed" supplemental nutrients to the embryo which consumes the amniotic fluid prior to hatch. According to Uni and Ferket (2004), if early access to feed is critical for early development post-hatch, then feeding the embryo before hatch by *in ovo* administration would be expected to enhance hatchability, and development of the digestive tract, and increase body weight and nutritional status of the hatchling. Smith (2006b) mentioned *in ovo* techniques as one of the biggest contributions of poultry research, along with feather sexing, nutritional strategies to avoid leg problems, blood screening to eliminate pathogen carriers, and genome sequencing. The advantage of *in ovo* feeding over early feeding is the possibility of helping the struggling embryos to hatch. The IOF may not replace the benefits of early feeding, but it should potentialize its effects if both practices were combined, and at least minimize the adverse effects of post-hatch holding if the poults do not have access to early feeding. Many studies have been conducted concerning the development of techniques for *in ovo* feeding and, to that end, researchers concluded that *in ovo* feeding must be applied while the embryo consumes the amniotic fluid, being around embryonic day (ED) 17 until ED 18 for chicken embryos, as the amniotic fluid is orally consumed by the embryos towards hatch and, therefore, nutrients are delivered to the embryonic intestine. Stimulation of gastrointestinal development *in ovo* is initiated with intestinal presentation of amniotic contents while feed intake after hatching will cause a further stimulation. In addition, both IOF and early feeding after hatch improve body weight until at least 35 days post-hatch (Uni and Ferket, 2004; Uni et al., 2005). Therefore, the question arises if there is a carry-over effect of IOF on the already beneficial effects of early feeding after hatch. An argument for this carry-over effect is based on muscle growth post-hatch. Satellite cell mitotic activity is the highest in the perinatal period and decreases with birds age (Velleman, 2007). Delay in feed access decreases satellite cell mitotic activity when compared to their fed counterparts (Halevy et al., 2000; Mozdziak et al., 2002; Moore et al., 2005). Therefore, *in ovo* feeding may induce proliferation of myoblasts during embryonic development and could be responsible for a higher number of satellite cells in

early post-hatch development. *In ovo* feeding, gender and incubation profile affected embryo development in terms of enteric development and maturation, (Uni and Ferket, 2004; Tako et al., 2005; Smirnov et al., 2006; Uni et al., 2006), improves hatchability (Ferket et al., 2005), and energy status (Uni et al., 2005). and post-hatch performance increasing body weight and relative pectoral muscle size at hatch (Ferket et al., 2005; Uni et al., 2005).

This improved nutritional status of *in ovo* fed birds is expected to yield several advantages: more efficient feed nutrient utilization; reduced post-hatch mortality and morbidity; improved immune response to enteric antigens; deduced incidence of developmental skeletal disorders; increased muscle development and breast meat yield. These benefits will ultimately reduce the production cost per kg of consumable poultry meat. At the time of hatch, *in ovo* fed birds had gastro-intestinal tracts that were functionally at a similar stage of development as a conventional 2-day old chick offered feed immediately after hatch.

Therefore, the *in ovo* technology may revolutionize early nutrition and even incubation practices. The challenge is to overcome the IOF formulation and delivery constraints in order to be commercially applied on large scale application and even to achieve a better return on investment.

6.3 The application of pre/probiotic *in ovo* injected

The pre/probiotics could be a reasonable alternative to antibiotics, however their exact mode of action is still not defined. There are several hypotheses which are discussed below. Initially, it was suggested that administration of probiotic bacteria directly to neonates reduced infection by pathogens because of the "competitive exclusion" between the bacteria. Currently, it is understood that the commensal bacteria can prevent infection by pathogens through maintenance of the integrity of the gastrointestinal tract and regulation of the immune system. The suggested mode of probiotics action, in poultry includes: (i) maintaining normal intestinal microflora by competitive exclusion and antagonism (ii) altering metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production (iii) improving feed intake and digestion; (iv) stimulating the immune system (reviewed in Kabir, 2009). Understanding the complete mechanism, effectiveness, and potential effect of bioactives like pre-/pro-/ and synbiotics have been hampered till now by the unavailability and insensitivity of methods used, especially:

a) mode of bioactive administration;

b) microbiota identification;

c) microbiome-host interactions.

A major problem with the use of bioactives is their efficient administration under fully controlled conditions. In order to be effective, they have to be administered to animal as early in life as possible. Above that uncontrolled variables (i.e. water quality) should be minimized. To eliminate some of these factors that could influence the responses to bioactives, the *in ovo* injection technology of these bioactives, directly into a chicken embryo, has been defined (Gulewicz and Bednarczyk, Polish patent Nb. 197726). By *in ovo* injection, pre-/pro-/synbiotics are administered as early in life as possible, and

uncontrolled environmental factors are minimized and/or eliminated. The chicken is one of the primary models for embryology and development because its embryonic development occurs *in ovo*. Avian eggs offer a mechanism for studying embryonic development and pathology in detail, because the chicken embryo is readily accessible from the period of gastrulation through neurulation and organogenesis until hatching. In addition, the period of embryonic development of chicken comprises 21 days of incubation only, and as a result of it reproduction a large number of eggs, embryos or progeny are possible. It is a common opinion that embryonic development of the chicken happens in sterile environment (Amit-Romach et al., 2004) and the first contact with microflora occurs only after hatching. Although Deeming (2005) found that microorganisms may be internalized from yolk at 18 day of embryonic development. Pedrosa (2009) discovered that the embryo's intestinal tract is far from sterile and this pioneer microbial community demonstrates signs of evolution in the last 4-5 days before hatch. Inoculation of pre- or probiotics to the embryo can stimulate/modulate very early the developing immune mechanisms of the innate and adaptive immunity, and this fact may be more important than competitive exclusion the digestive tract, especially cecum. Studies comparing the biological activity of various oligosaccharides using the chicken embryo model require the preliminary determination of many factors as time of injection and range of preparation dose. According to Johnston et al. (1997), different factors influence effective delivery of *in ovo* injected substances to embryos. In case of prebiotics, the additional source of variation that influenced the response to *in ovo* administration is the time from its injection to hatching, necessary to promote the growth of bifidobacteria. On the other hand, after twelve days of incubation, the completely developed and highly vascularized allantochorion serves as the more efficient transport route from air cell to the blood (Romanoff, 1960). However, studies concerning the way that oligosaccharide preparations applied during embryogenesis influence post-embryonic development of organisms (for example on meat trials of broiler), the effects of oligosaccharides on the intestinal profile of other bacteria including harmful and toxic ones, or oligosaccharides as substitutes of antibiotics injected during embryo development are presently being undertaken (Villaluenga et al., 2004).

It has been demonstrated that a single *in ovo* prebiotics injection into 12 day old chicken embryo leads to an increased number of bifidobacteria at the moment of hatch, and it also assures the long-term maintenance of a high level of intestine bifidobacteria (Villaluenga et al., 2004; Pilarski et al., 2005). The same *in ovo* prebiotic injection approach realized on a large scale (1.9 milion broilers) in the field condition suggests that the application of prebiotics to the chicken diet can be successfully replaced by injecting these compounds *in ovo* in very low doses (Bednarczyk et al., 2009, 2011a). This method of pre/(and possibly) probiotic injection has an additional advantage. Such an individual injection with fully defined dose and content of bioactives provided directly to the chicken embryo realizes two goals: firstly an immune system is fully developed and can profit from these bioactives, and secondly a chicken embryo is not contaminated and might be populated with the beneficial bacteria.

Prebiotic administration *in ovo* could have, also, a positive effect on chicken hatchability even if literature reports few works about this investigation and not so clear results. In fact,

Bednarczyk et al. (2011b) found that the hatchability percentage was significantly higher for chicken treated with antibiotics in drinking water in comparison to the eggs injected *in ovo* with RFOs prebiotics. The exact reason for the decline in hatchability of the injected embryos is unknown. In fact, different factors influence the hatchability of *in ovo* injected embryos, as site of injection, features and doses of injected substances and technology of incubation. The use of a caecal culture containing highly proteolytic organisms resulted in depressed hatchability, when the material was introduced into the air cell. Introducing the preparation into the amnion killed all the embryos (Edens et al., 1997). However, *Lactobacillus reuteri* could be administered *in ovo* without loss of hatchability (Edens et al., 1997). Their unpublished data, concerning 1.9 million of treated embryos, indicated that the different hatchability effect of *in ovo* injection depended on the incubation technology used, and was +1.7%, -3.2%, and -1.5%, compared to the control group (Bednarczyk et al., 2011b).

6.4 *In ovo* constraints

Enting et al. (2007) tested breeder diets with different nutrient densities and concluded that available practical diets produced eggs with reduced egg white compared to a control diet. The authors concluded that the amount of egg white in these eggs at the time of lay was insufficient to sustain proper embryonic development. This is one situation where IOF can be beneficial to the embryos. However, several technical questions need to be addressed for IOF to succeed under commercial conditions: 1) what is the best time to inject (embryo age or stage of development?); 2) how much volume can or need to be injected?; 3) what is the best osmolality of the solution so it will not harm amnion/embryo osmotic balance?; 4) what nutrients and how much can be *in ovo* fed without increasing metabolic load or causing toxicity to the embryo?. Therefore, questions related to the physical constraints of the egg and metabolic constraints of the embryo must be answered.

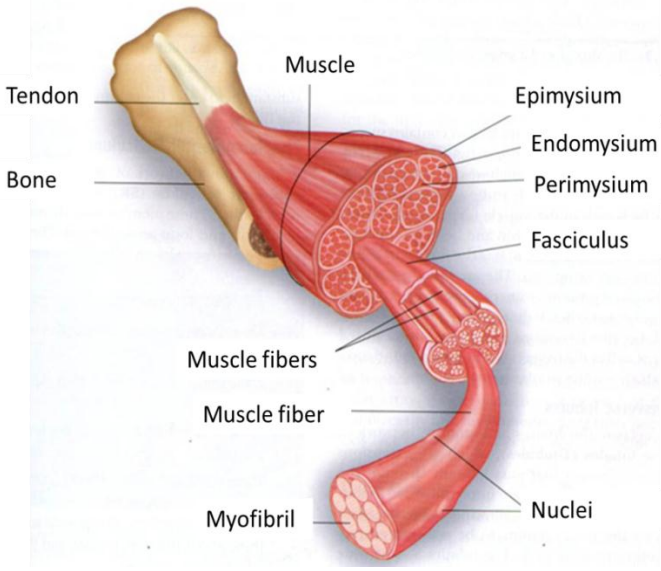
The idea of supplementing carbohydrates to chicks and poults into the amnion before hatch may seem simple, but it is technologically difficult. As already mentioned, just giving glucose in the drinking water to chicks at placement ended up being detrimental because it suppressed gluconeogenic enzymatic activity (Donaldson, 1995). So the kind of nutrient that can be *in ovo* fed and how it will affect embryonic metabolism must be carefully studied. A popular trade magazine asked the questions: “Can nutritionists find ways to put more vitality into day-olds?”, and “Will it be via breeder feed or by direct application into the egg?” (Horrox, 2006). Since it is difficult to influence egg composition via hen nutrition, *in ovo* feeding offers an intriguing solution.

Chapter 7: Structural and biochemical aspects of poultry muscle

7.1 Muscle structure

The meat sold in the market is based on skeletal muscle. A muscle is usually enclosed by a thick sheath of connective tissue, the epimysium (Figure 7.1a), and divided into bundles of fibres by a connective tissue network, the perimysium. The individual muscle fibres are surrounded by a plasma membrane itself bound by a thin connective tissue network, the endomysium. This consists of a base membrane surrounded by a reticular layer, in which collagen fibrils are set in a matrix.

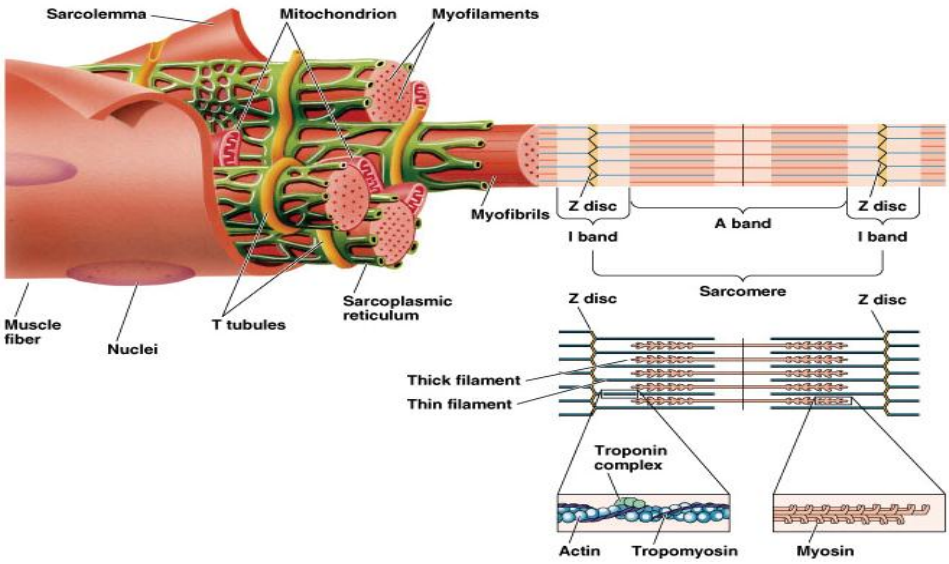
Figure 7.1.a Diagram of the structure of muscle and associated connective tissues



From http://www.ivy-rose.co.uk (*viewed April 25th , 2013*)

The skeletal muscle is composed of numerous muscle fibers. Each muscle fiber is a multinucleated, cross-striated cylindrical cell and shows very regular crossways striations along their length (between 1 and 300 mm). Fiber consists of a cell membrane called sarcolemma, which encloses the cytoplasm (sarcoplasm). There are two types of substances embedded in the sarcoplasm: (i) several nuclei arranged at the periphery beneath the sarcolemma; (ii) a number of evenly distributed longitudinal threads called myofibrils. Each myofibril shows alternate light and dark bands. Dark bands are anisotropic and thus are known as A-bands. The light bands are isotropic and thus are known as I-bands. The bands of adjacent fibrils are aligned transversely, so that the muscle fiber appears cross striated. In the middles of the A band (dark band) there is a light H band. In the middle of the H band there is a dark M line. In the middle of the I band (light band) there is a dark Z disk also known as Krause's membrane. The segment of myofibril between successive two Z-line is called sarcomere, which represent the structural unit of myofibrils (Figure 7.1b). The striations of the myofibrils are caused by a highly organized array of two kinds of filaments: the thick filaments and the thin filaments (Warris, 2000).

Figure 7.1b The fibrous microstructure of meat



From http://comenius.susqu.edu/bi/320/L5%20Muscle.ppt *(viewed April 25th , 2013)*

The sliding of myofilaments relative to each other occurs in muscle from living animals with the contraction initiated by the nervous system. This contraction occurs by the actin and myosin filaments sliding with respect to each other. This activity is initiated by release of calcium from the sarcoplasmic reticulum. Then activated and continued by ATP. Usually as a consequence of death, the muscles are in rigor which means in the presence of calcium and absence of ATP. The depletion of muscle energy stocks leads to rigor mortis and a change in status i.e. the muscles becomes meat (Warris, 2000).

7.1.1 Protein accretion

Increasing growth rate also changes the protein dynamics. Accretion of muscle is a dynamic process between opposing anabolism and catabolism and changes in either or both sides of the equilibrium will result in changes in size of the muscle. Administration of b-agonists results in muscle hypertrophy in rats, lambs, cattle, and chickens by changing the proteolytic activity. In the study of Reeds et al. (1986), administration of the b-agonist, clenbuterol, to rats suggested that the hypertrophy was entirely due to a reduced protein catabolism. Degradation of muscular proteins therefore constitutes an important regulatory mechanism for muscle growth (Goll et al., 1992). There are three proteolytic systems in muscle: the cathepsins (lysosomal), the calpains (calcium-dependent) and the proteasome (adenosine triphosphate (ATP)/ubiquitin dependent). All of these enzymes have been sequenced and specific inhibitors (cystatins and calpastatin, respectively) have been purified for the first two systems. *In vivo*, the proteasome appears to be responsible for the majority of protein turnover (Goldberg et al., 1997), calpains appear to be responsible for the degradation of the cytoskeleton, whereas lysosomal activity increases after damage or disease. The cathepsins and calpains have been studied extensively, but little information is available on proteasome in relation to muscle growth. Studies of the effect of growth rate on the proteolytic capacity of breast muscle (Table 7.1) show that slow growing birds have

a higher ratio of enzyme to inhibitor. In the slow-growing birds, the enzyme is in excess, whereas in fast-growing birds, the inhibitor is in excess. White Leghorns showed the largest proteolytic capacity of the calpain system and a high activity of cathepsin H and cystatins.

Table 7.1. Proteolytic capacities in relation to growth rate[1]

Strain	Feed conversion ratio	μ-Calpain	Cathepsin B and L	Cathepsin H
White Leghorns	2.527	7.08	3.40	1.44
Ross	1.758	0.37	1.53	1.28
Very high growth rate	1.701	0.57	1.29	1.09

[1]*Values are the potential proteolytic activities for calpain and cathepsins taken as the ratio of the amount of enzyme to that of their specific inhibitor for Pectoralis muscles from male chickens from White Leghorn (650 g at 6 wk), Ross (2.4 kg at 6 wk), and one selected for very high growth rate (2.5 kg at 6 wk). Data taken from Schreurs et al. (1995). Enzyme activities are in micromoles of substrate per minute per gram of muscle.*

These studies suggest that the increased growth and muscle mass in modern lines could be largely governed by reduced protein catabolism. Cathepsins, and particularly calpains, have been implicated in postmortem proteolysis and weakening of the muscle fibers leading to tenderization. With the reduced proteolytic potential in faster growing lines, there is less activity and, therefore, reduced tenderization. (Dransfield and Sosnicki, 1999)

7.1.2 Postmortem events

After death of the animal, anaerobic metabolism reduces the pH from about 7.2 in muscle to 5.8 in meat and stiffness develops (rigor mortis). The rate of rigor mortis development can be affected at all stages of production (Froning et al., 1978), both pre- and postslaughter, and variations in its rate in turn affect the sensory and functional properties of raw meat and of further processed products (Richardson, 1995). Heat stress is one of the prominent ante mortem environmental factors that cause a rapid early postmortem glycolysis (McKee and Sams, 1997). Glycolytic fibers have a more rapid rigor mortis development. Thus in beef, rigor would normally take about a day, whereas in pork, rigor is complete in several hours and in chicken breast muscle takes about 1h. Growth performance can influence the rate and extent of rigor development in the meat. For example, the rate of pH fall was the same in breast muscle of selected lines for high growth rate but a protein-inefficient line (White Leghorn) had higher ultimate pH values (Schreurs et al., 1995). At high ultimate pH, water-holding properties of myosin will remain high. Also, in fast-growing turkeys, the rate of pH decline in pectoral muscle was about 0.04 units/min, about twice the rate than that of a slow growing line (Santé et al., 1995). The rate of pH decline varies among chicken genetic lines and between individual birds, typically pH values at 15 min. after slaughter vary from 6.2 to 6.6 (Gardzielewska et al., 1995). Arbor Acres broilers showed the fastest decline with 6% of breast muscles having a pH 5.7. In commercial production, with the variety of environmental factors, pH at 15 min

postmortem may typically vary from 6.2 to 6.8 in breast muscle from 10-wk-old turkey hens and vary more widely in male and female chicken muscles. Rapid pH decline at high temperature will inactivate the calpain system and reduce postmortem tenderization (Dransfield, 1994), leading to toughening (Table 7.2). With a faster pH decline, myosin will be more susceptible to denaturation. Rapid denaturation of myosin increases the likelihood of reduced water holding capacity, pale color, similar to pale, soft, and exudative (PSE) condition in pork. *In vitro* studies show that lowering the pH by 1 unit increases the rate of denaturation 12 times (Offer, 1991).

Table 7.2. Interaction of rate of rigor mortis development and temperature in determining the quality of poultry meat

	Rapid chill	Slow chill
Rapid rigor		High drip, pale meat, tough, rapid aging
Slow rigor	High drip, tough, slow aging	

Temperature is also critical, with an increase in temperature of 10 C (in the region of 30 C) increasing denaturation 20-fold. Thus, the potential detrimental PSE-like effect of fast-growing lines could be partially offset by increasing the rate of carcass cooling (Table 7.2). However, rapid cooling will toughen slow glycolysing muscles (Wakefield et al., 1989). Ideally, the most tender meat will be produced by reaching about 10°C when the pH is 6.2. So, for example, those carcasses with rapid rigor should be chilled quickly to reduce protein denaturation and those with slower rigor mortis development chilled more slowly reducing their toughening (Table 7.2) (Dransfield and Sosnicki, 1999).

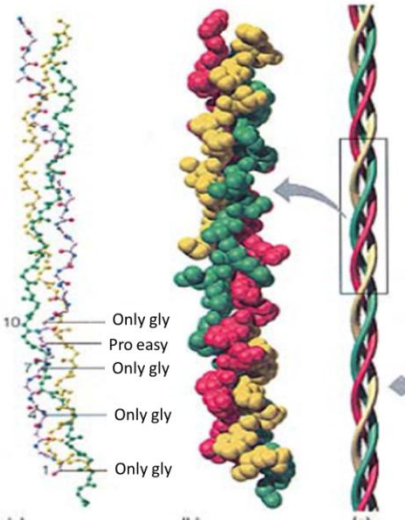
7.2 Connective tissue

Unlike other tissue types that are formed mainly by cells, the major constituent of connective tissue is its extracellular matrix (ECM), composed of collagen, elastic fibers, glycoproteins and proteoglycans.

7.2.1 Collagen

The collagens are a large family of molecules. Each collagen molecule is composed of three polypeptide chains which form a unique triple helical structure (Figure 7.2). The chains are composed of repeating unit of –Gly-Xaa-Yaa, where Xaa and Yaa can be any amino acid but are frequently the imino acids proline and hydroxyproline (Kadler et al., 1996). The collagen molecule contains about 33 % of the amino acid glycine, 12 % proline and 11 % hydroxy-proline. The different chains are designated α1, α2, α3. The chains of a collagen molecule can be similar or different, depending on the type of collagen. The chain is stabilized by H-bonds. For the three chains to wind into a triple helix they must have the smallest amino acid, glycine at every third residue along the chain (Kadler et al., 1996).

Figure 7.2. Collagen molecule



From https://chempolymerproject.wikispaces.com/Collagen-D-apml (*viewed April 29th, 2013*)

7.2.2 Collagen types and organization

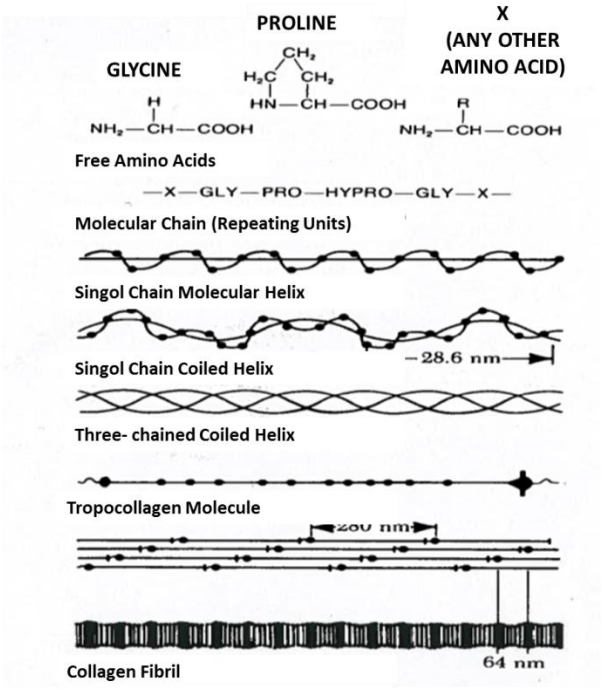
Connective tissue envelopes muscles, muscle bundles and muscle fibers. The endomysium is the fine connective tissue layer separating individual muscle fibres. The vast majority of its thickness is made up of a near- random feltwork of fine, wavy collagen fibres. This collagen feltwork can easily reorientate with changing the muscle length (Purslow and Trotter, 1994). The connective tissue layer that separates each muscle into muscle fiber bundles, or fascicles is the perimysium. There are large (primary) fascicles and smaller (secondary) fascicles. Primary and secondary perimysiums are arranged in a crossed-ply arrangement of two sets of wavy collagen fibers (Rowe, 1981). Reorientation of this collagen network allows the perimysium to easily follow elongation or shortening of the muscle fascicles. The epimysium is the connective tissue sheath delineating and separating individual muscle. In many muscles collagen fibers in the epimysium are arranged into a crossed-ply arrangement of two sets of wavy collagen fibers or in muscles where the epimysium clearly participates in transferring load to adjacent structures (e.g bovine semitendinosus), the collagen fibers are more closepacked and longitudinally arranged, like a tendon (Purslow, 2002).

Collagen can be divided into three major groups; fibrous collagen (types I, II and III), nonfibrous collagen (type IV/basement collagen) and microfibrillar collagen (types VI, VII, V IX, X), VIII and XI) (Xiong, 1994). The microfibrillar collagen molecules form a loosely packed filamentous structure with anti-parallel alignment of individual molecules. Type IV collagen molecules are the only members of the non-fibrous category found in the muscle. They form a “chicken wire” structure, which can be found in basement membranes (Weston et al., 2002).

The fibrous collagen self- assembles to form a characteristic band pattern. The formation of collagen fibril is shown in Figure 7.3. The fibrous collagen is created by monomers of tropocollagen molecules. Tropocollagen is a long thin molecule with a molecular weight of 300000 Da and a length of 280 nm (Weston et al., 2002).

Each tropocollagen molecule overlaps its lateral counterpart by slightly less than one-fourth of its length and is aligned in a quarter – stagger fashion (similar to building bricks). Each unit extends about three- quarters the length of its neighbor and is bonded together at frequent intervals to prevent sliding under tension. These bonds are referred to as crosslink (Bailey, 1972).

Figure 7.3. Diagrammatic illustration of the molecular structure of collagen, tropocollagen and the amino acid sequence. It shows the collagen fibril formation (Jugde et al., 1989).



After the collagen molecules are synthesized, they are secreted from the cell and into the extracellular space and align into a quarter-stagger array. Larger fibrils are formed by crosslinking between the fibrils (Reiser et al., 1992).

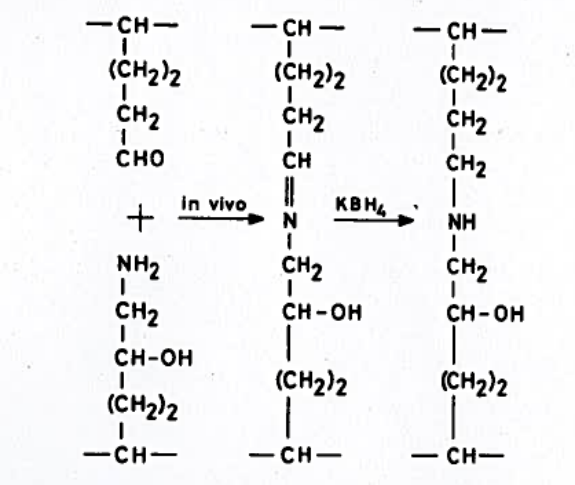
7.2.3 Biochemical property

Fibril collagens have unique biomechanical properties. The collagen fibers have a high tensile strength due to intra- and intermolecular cross-linkages. Intramolecular cross-linkages are those which are formed between the α-chains within the same molecules. Intermolecular cross-linkages are formed between α-chains in different molecules (Bailey and Light, 1989). Intermolecular cross links are important in the stabilization of the collagen fibers (Weston et al., 2002).

The collagen molecules are cross-linked internally and to other collagen molecules by different mechanism. Intra- or inter molecular disulfide bonds are confined to a few collagen types such as type III and type IV. In collagen type III molecules three cysteine side chains are present, one in each α-chain. It is possible for two of these residues to react intramolecularly to form a disulphide bridge. The third free cysteine side chain can make intermolecular disulphide bonds with other cysteins in adjacent type III molecules (Bailey and Light, 1989).

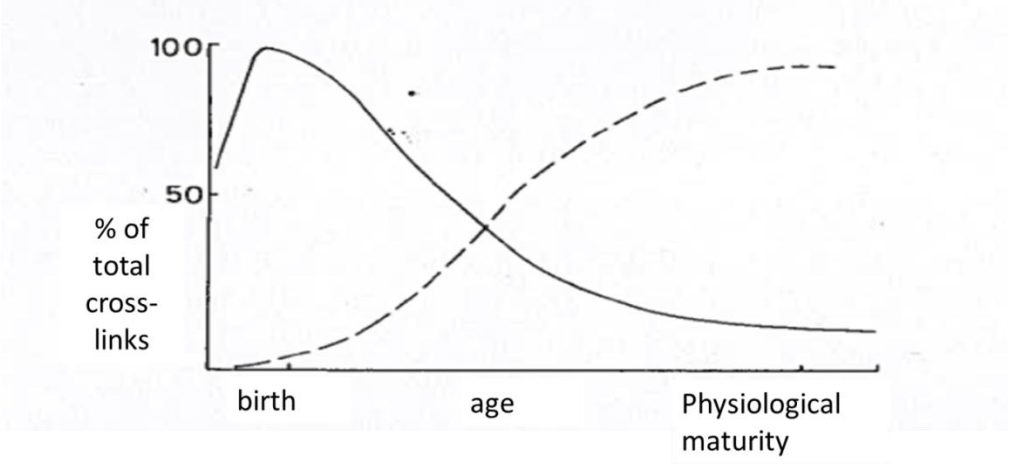
Collagen crosslinks with divalent bonds link two collagen chains in the same or different molecules. This is initiated by the enzyme lysyl oxidase which converts the amine group of lysine or hydroxylysine residues in the non-helical N- and C- terminals of each α-chains region into aldehydes. The aldeydes derived from lysine and hydroylysine are called allysine and hydroxallysine. Allysine and hydroxallysine react with the amino group of hydroxylysine in an adjacent collagen molecule, where allysine forms an aldimine bond and hydroxyallysine form a ketoamide bond. The aldimine bond contains a double bonded system formed between the amino nitrogen and the aldehyde carbon (Figure 7.4). The aldimine bond is stable under physiological condition but is disrupted by heat and low pH. The aldimine bond can therefore be chemically reduced with agents such as sodium borohydride (Figure 7.4). The ketoamide bond is stable at both low pH and high temperatures (Bailey and Light, 1989).

Figure 7.4. The formation of the aldimine bond: A double bond between the amino nitrogen and the aldehyde carbon is formed during cross-linking. The bond is reducible at low pH and when heated (Bailey and Light, 1989).



During age the collagen matrices become stronger and more rigid. One might expect that this is not because of formation of more reducible cross-links; however, it has actually been shown that the number of reducible cross-links decreases during aging. It is suggested that these cross-links were not disappearing but where further reacting with other components to be more complex and non-reducible. The non-reduible crosslinks forms trivalente and tetravalente cross-links. The divalente aldimine cross-links can for example react with histedine in a third molecule to form a trivalent cross-link which is non-reducible. The content of non-reducible and heat stable cross-links increases with age of the animals as shown in Figure 7.5 (Bailey and Light, 1989).

Figure 7.5. Changes in cross-linking in collagen during aging are shown

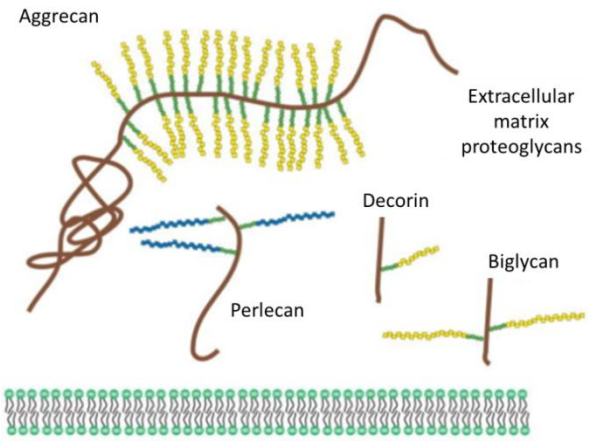


—— = reducible crosslinks, ———— = non-reducible mature crosslinks (Bailey and Light, 1989).

7.3 Proteoglycans

Proteoglycans (PGs) are complex and multifunctional molecules consisting of a protein core with variable number of covalently attached carbohydrate side chains. The polysaccharide chains are named glycosaminoglycans (Esko et al., 2009). Examples of some ECM proteoglycans are showed in Figure 7.6. The structural diversity due to disaccaride composition gives the proteoglycans unique features and functions. The number of attached Glycosaminoglycans chain varies from only one (e.g., decorin) to more than 100 chains (e.g., aggrecan) (Esko et al., 2009).

Figure 7.6. Some ECM proteoglycans. The proteoglycans consist of a protein core (brown) and one or more covalently attached glycosaminoglycan chains (blue and yellow) (Esko et al., 2009)



Proteoglycans have a variety of biological functions, they act as tissue organizers, influence cell growth and maturation of specialized tissue, play a role as biological filters and modulate growth-factor activities, regulate collagen fibrillogenesis and skin tensile strength, affect tumor cell growth and invasion, and influence corneal transparency and neurite outgrowth (reviewed in Iozzo, 1998).

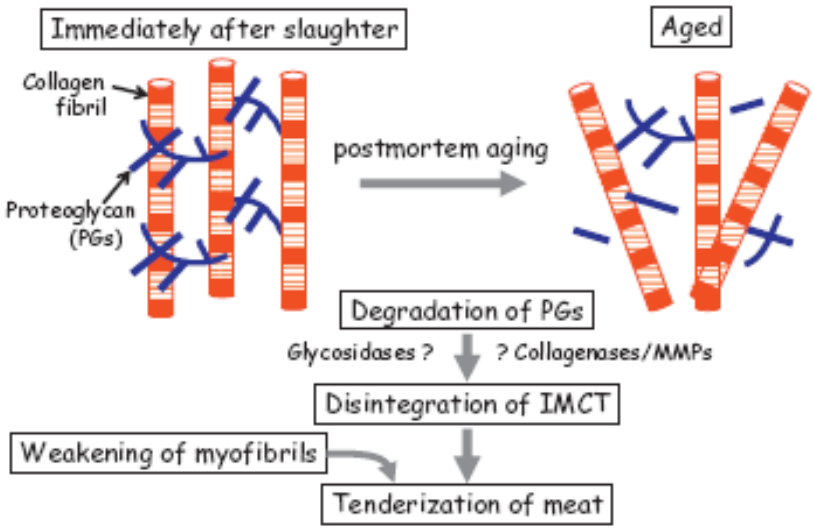
7.4 Connective tissue degradation

Muscles are active components of the body, responsible for locomotion under strict control of the nervous system and well supplied with oxygen and nutrients. After slaughter of an animal, muscle become isolated structures with no supply of oxygen and nutrients. Many of the biochemical reactions present in the living state retain. Due to residual intracellular glucose and glycogen, glycolysis continues for some time. In absence of oxygen the final product of glycolysis is lactic acid, which accumulates and induces a decrease in the pH of the muscle. The contractile apparatus, deprived of its store of ATP, goes into a state of excessive and uncontrolled contraction called rigor mortis (the stiffness of dead). The muscle does not remain in this "stiffened" state but becomes soft due to a series of enzymatic degradation (Bailey and Light, 1989). When the pH reaches the isoelectric point (~ pH 5) of actin and myosin, there will be an equal number of positively and negatively charged groups. At this point the groups tend to be attracted to each other, and there will be few groups that are available for water binding. The swelling of collagen on either side of its isoelectric point has been well investigated. It has been shown that when the swelling increases, the tensile strength of the fiber decreases, and the collagen is more label to heat (Bailey and Light 1989).

It has been suggested that collagen with large fiber diameters and highly packed bundles contribute to tough meat. The bundles and the thick collagen fibers are more difficult to damage by proteases during storage.

The large proteoglycan with aggrecan characteristic has been shown to be degraded the first 24 h after slaughter (Eggen et al., 1994) *postmortem* (Figure 7.7). The tenderness of meat is improved during post mortem ageing. The weakening mechanism in intramuscular connective tissue is not known. Some components in ECM are degraded by metalloproteinases and lysosomal enzymes *in vitro* (Bailey and Light, 1989). But it is not clear whether the extracellular matrix in muscle is degraded by these enzymes during *post mortem* aging. PGs are degraded by β- glucuronidase which is released from the lysosomes in postmortem muscle (Møller et al., 1976). The activity of free β- glucuronidase increases with the *postmortem* aging (Dutson and Lawrie, 1974).

Figure 7.7. Schematic representation of changes in intramuscular connective tissue during *post mortem* aging



In muscle immediately after slaughter PGs link collagen fibrils and stabilize the intramuscular connective tissue. During post mortem aging, PGs are degraded and the linkage between collagen fibrils is weakened. This structural change in the intramuscular connective tissue contributes to the tenderization of aged meat (Nishimura, 2010).

Chapter 8. Hystological and hystopathological aspects of poultry muscle

8.1 Muscle fiber

Skeletal muscle is a very heterogeneous tissue that is composed of a large variety of functionally diverse fiber types. One of the unique features of skeletal muscle is its numerous fiber types and their distinct functional characteristics and compositions, which contribute to a variety of functional capabilities (Pette and Staron, 2001). These fiber types can be grouped according to various parameters, including myofibrillar protein isoforms, metabolic enzyme profiles, and structural and contractile properties (Schiaffino and Reggiani, 1996; Bottinelli and Reggiani, 2000). Therefore, the morphological and biochemical characteristics of muscle fiber types are major factors that influence energy metabolism within the skeletal muscles of live animals, as well as during the postmortem conversion of muscle to meat (Ryu and Kim, 2005, 2006).

Because muscle fibers occupy 75–90% of the muscle volume, morphology of muscle fiber is a major determinant factor of muscle mass. Morphology of muscle fiber is represented by their total number of fibers (TNF), cross-sectional area of muscle fiber (CSAF), and length of muscle fiber. Muscle growth potential is closely related to TNF and CSAF (Rehfeldt et al., 2000; Ruusunen and Puolanne, 1997).

8.1.1 Classification of muscle fiber type

Fiber type composition can vary markedly in different species and muscle types, depending on function (Klont et al., 1998). Moreover, there are many factors that contribute to fiber type variation, such as sex (Ozawa et al., 2000), age (Candek-Potokar et al., 1998), breed (Ryu et al., 2008), hormones (Florini et al., 1996), and physical activity (Jurie et al., 1999). These fiber type variations differ according to their molecular, metabolic, structural, and contractile properties (Schiaffino et al., 1989). Therefore, having an understanding of such muscle fiber characteristics is important for the study of overall muscle characteristics and subsequent meat quality.

The structure and function of avian muscle fiber types are revealed in metabolic differences in the red and white fibers. Red fibers are narrow in diameter, myoglobin rich, and adapted to aerobic (oxidative) metabolism for rapid, fatigue-resistant activity, whereas white fibers are larger in diameter, adapted to anaerobic (glycolytic) metabolism, fast-fatiguing, and used for brief bursts of activity (George and Berger, 1966). With increasing growth rate, fibers become more glycolytic (fast twitch, glycolytic; type IIB fibers).

The functional, structural, and metabolic characteristics of the four major muscle fiber types differ in adult animals (Bottinelli and Reggiani, 2000). Fiber type I, or slow-twitch fibers, generate energy for ATP resynthesis predominantly by aerobic energy transfer. They possess a low myosin ATPase activity level and a glycolytic capacity that is less developed than fast-twitch fibers. Slow-twitch fibers have a wider Z-band than fast-twitch fibers, with type IIB fibers having the thinner Z-band (Sjostrom and Squire, 1977). In the intrinsic speed of contraction, the shortening velocities of fast-twitch fibers are approximately three times faster than those of slow-twitch fibers (Schiaffino and Reggiani, 1996). Types IIX and IIA fibers display shortening velocities that are similar to each other, but are slower than type IIB fibers (Schiaffino and Reggiani, 1996). Type I fibers contain

relatively large and numerous mitochondria, myoglobin, and iron-containing cytochrome of the electron transfer chain. High concentrations of mitochondrial enzymes and myoglobin also support an enhanced aerobic metabolic capacity (Nemeth and Lowry, 1984). Moreover, type I fibers contain a higher amount of lipid, some of which presumably serves as a source of aerobic metabolic fuel; they also contain lower amounts of glycogen and glucose than type IIB fibers (Peter et al., 1972; Hintz et al., 1984). Thus, type IIB fibers predominantly use glucose as fuel. Also, type IIB fibers have a more extensively developed Sarcoplasmic Reticulum (SR) and T-tubule system, both of which are consistent with their more rapid contraction speed; however, they are relatively easily fatigued. Therefore, type II fibers, especially type IIB, have the ability to rapidly transfer energy for quick, forceful muscle actions. For example, the ATP splitting rate is three to four times faster in type IIB fibers than in type I fibers, and types IIA and IIX fibers are intermediate (Bottinelli et al., 1994, Stienen et al., 1996). In addition, the tension cost, which is the ratio between ATPase and tension, is several times lower in type I fibers than in types IIA, IIX, and IIB fibers (Bottinelli et al., 1994; Stienen et al., 1996). These results imply that when movements require the generation of mechanical power, type I fibers are energetically more economical than IIA, IIX, and IIB fibers (Bottinelli et al., 1994, Stienen et al., 1996).

8.1.2 Muscle fiber size

Skeletal muscle fiber is made up of multinucleate, membrane-bound cells that are typically 10 to 100 μm in diameter, and their lengths can vary from several millimeters to more than 30 cm (Bechtel, 1986). This myofibril striation pattern repeats with a periodicity of approximately 2 to 3 μm, and in vertebrate muscle, the sarcomere is a complex structure containing at least 28 different proteins (Craig and Padron, 2003). The diversity of skeletal muscle can be attributed to the heterogeneous characteristics of the individual muscle fibers and the mosaic composition of the numerous fiber types (Schiaffino and Reggiani, 1996; Bottinelli and Reggiani, 2000).

In general, the number of fibers is related to changes throughout growth, and fast-growing farm animals have more muscle fibers than slower growing strains. Within strain, the fiber number may increase with increasing average daily gain and gain:feed ratio (Stickland, 1995). In poultry, the muscle fiber cross-sectional area increases with age (Table 8.1). Geese, selected for meat yield, have larger fibers than birds selected for egg production (Klosowska et al., 1993) and fast-growing chickens have larger diameter fibers than slow-growing lines (Table 8.1). This increase is also associated with an increase in the number of giant fibers, which typically have cross-sectional areas three to five times larger than normal, although these may also result from severe contraction (hypercontracted fibers). Smaller fiber diameters may allow a higher packing density and increase toughness of the meat.

Table 8.1. Growth, fiber diameter, and fiber type in two strains of chicken[1]

Age	Live weight		Fiber area		Citrate synthase activity[2]	
	Rapid	Slow	Rapid	Slow	Rapid	Slow
(wk)	(g)		(μm^2)			
0	36	31	20	23	6.4	7.4
11	1,882	675	1,256	664	4.4	3.9
55	3,285	1,883	2,755	1,946	4.6	3.6

[1]*Values are the means of the Pectoralis muscle for rapid- and slow-growing strains of male chickens. Data from Rémignon et al. (1995).*

[2]*Micromoles of substrate per minute per gram of muscle.*

It is well known that the myofiber number in chickens is established before hatching. So, any increase in muscle weight post-hatching depends on the increase in length and diameter of the myofibers. Myofiber numbers differ between breeds regardless of nutrition treatment and sex, and remain constant during growth to market weight (Swatland, 1984). In essence, genetically programmed increases in muscle mass must be due to a larger number of myofibers, larger myofibers, or a combination of these two factors (Smith, 1963). Aberle and Stewart (1983) studied fiber characteristics of muscles from broiler and laying strain chicks between 3 and 11 weeks of age. Myofibers of broiler muscles had a greater cross-sectional diameter than those from the laying strain. Moreover, Fowler et al. (1980) found that the difference in *Semimembranosus* size between a large-bodied line of Japanese quail and the small-bodied line was primarily due to cell numbers rather than cell size. Several other experimental avian models had been used to study genetic influences on mechanisms of muscle growth, and it appeared that different muscle growth rate of different populations resulted from significant differences in myofiber numbers, differences in myofiber area and undoubtedly differences in muscle length. Prentis et al. (1984) demonstrated that the breed difference in *Iliotibialis lateralis* muscle weight resulted from different fiber diameters, while, in contrast with this, the breed difference in *Pectoralis* muscle was due to different numbers of fibers.

Muscle development in avian species occurs in 2 distinctive periods. First, during the embryonic phase, the muscle fiber number (MFN) is established when a large number of precursor cells are committed to the expression of muscle-specific genes (Christ and Brand-Saberi, 2002). Later, during the post-hatch period, the hypertrophy of the muscle takes place, mainly by accretion of protein and nuclei originating from the proliferation and fusion of satellite cells (Moss, 1968). Embryonic muscle growth is the result of a balance between proliferation and differentiation of myoblasts into myotubes, such that an increased myoblast proliferation rate is expected to increase the MFN, resulting in stimulation of muscle growth and enlargement of muscle size (Coutinho et al., 1993; Thomas et al., 2000; Christ and Brand-Saberi, 2002). Higher myoblast proliferation rate might be related to the timing in myogenesis, as delay in the appearance of the somites and the expression of myogenic regulatory factors and myosin heavy chain was observed for growth-selected quail when compared with control lines (Coutinho et al., 1993).

8.2 Neuromuscular desease

Neuromuscular diseases are generally divided into two categories: the neuropathies and the myopathies. Neuropathic disorders are those in which motor nerve cells or their processes are first affected, resulting secondarily in atrophy of muscle. In the primary myopathies the disease process is assumed to involve the muscle directly, without damaging its nerve supply. Clinical, laboratory, and electromyographic findings are often useful in distinguishing between these two classes of disorders, but the muscle biopsy may be the deciding factor.

In general, interpretation of the muscle biopsy has been based on two principles: (1) following denervation, muscle fibers belonging to the same motor unit undergo atrophy together and are located in groups. By contrast, myopathies attack individual muscle fibers at random, giving rise to scattered changes throughout the muscle. (2) In myopathic disorders architectural changes are found in medium-sized and larger muscle fibers. On the other hand, denervation leads to simple atrophy of muscle fibers. Degenerative changes are said not to occur until the denervated fibers have become very small. The following list gives the histological features considered to be typical of myopathy by the most authoritative sources available. The degree of reliance placed on each of these descriptive features varies from source to source:

1. random variation in fiber size (triangular, trapezoidal, square);
2. random variation in fiber shape (atrophic fibers, giant fibers);
3. fiber degeneration: loss of striations (hyaline change), granular change, floccular change, vacuolar change;
4. phagocytosis;
5. regeneration (basophilia, vesicular nuclei, prominent nucleoli);
6. endomysial fibrosis;
7. fiber splitting;
8. central nuclei;
9. ringed fibers (“ringbinden”).

The muscle histology may permit a clear-cut distinction between neuropathy and myopathy. However, in long-standing disorders, the muscle frequently presents a confusing picture which is difficult to interpret according to the conventional criteria. (Drachman et al., 1967).

8.2.1 Breast muscle myopathies

Rising demand for meat and meat products give a reason for intensification animal production. Long-term selection work allowed to obtain bigger, faster growing, better fodder utilizing and more muscular animals. Genetic studies contributed to improve the animal production potential; however, the effect of this actions are pathological changes in muscles (Dransfield and Sosnicki, 1999).

Constant selection for performance, especially heavier weights, has its consequences. One big setback is that selection for higher post-hatch growth is negatively correlated with embryo survival (Nestor and Noble, 1995; Christensen et al., 2000). Collin et al. (2007) pointed out that selection for breast meat yield leads to poor visceral system. This phenomenon was explained by Foye (2005), who said that selection for growth is pushing

precocial poultry to become more altricial by directing resources to growth stead of viscera maturation. One of the consequences of the reduction of visceral capacity relative to body size among modern broilers selected for rapid growth is exemplified by the increasing incidence of ascites. Ascites have been liked to limited cardiopulmonary capacity of the animal to attend body demands. One of the strategies to reduce the incidence of ascites is to slow down early growth by feed restriction; but if it is too severe, the restriction may not be compensated later in life, resulting in lower weights and yields at processing (Ozkan et al., 2006). Selection for weight gain also increases risk of obesity, lower fertility, and lower hatchability (Joseph and Moran, 2005). The value of poultry industry has increased tremendously in the last twenty years. The broiler industry has grown from $5.68 billion in 1985 to $20.9 billion in 2005. Associated with this increase in meat poultry production was annual increase in broiler eggs set in hatcheries of 98%.

Adverse effects resulting from the increased growth rate of farmed birds, are best seen and studied in adult individuals. They involve a number of disorders, ranging from skeletal deformities to reproductive disorders (Dickinson et al., 1968). Dunnington and Siegel (1996) represent the five main groups of traits that are affected by selection for high body weight: growth and development, metabolism, reproductive traits, nucleotide sequence or resistance. It has been found that individuals selected for higher body weight were characterized by reduced antibody production in response to infection. Study of Helminowska-Wenda et al. (2004), and Klosowska et al. (1993) indicate that hystopathological changes are most extensive in birds with higher growth rate and high meatiness. There is a high probability of skeletal muscle myopathy along with the intense increase in the thickness of muscle fibers in these birds. Sosnicki et al. (1991) and Hoving-Balink et al. (2000) demonstrated that many cases of myopathy in modern lines of fast growth chickens and turkeys result from inadequate blood supply to the pectoral muscle. Significant increase in the diameter of muscle fibers, which transport oxygen through the capillaries is limited and consequently it leads to hypoxia of the muscle cells and necrosis. Necrosis is the most profound and irreversible retroactive change. It arises as the effects of external and internal factors (Gallup and Dubowitz, 1973; Sosnicki and Wilson, 1991) leading to cell death (Rowińska-Marcińska et al., 1998).

Differences in the histochemical structure of the breast muscle in galliform poultry (chickens, turkeys) and geese can also affect the different responsiveness of these birds to the selection and specific environmental conditions. Chickens broiler breast muscle is characterized by a far smaller share of red fibers (ßR) with oxygen metabolism and worse blood circulation demonstrated by a smaller number of capillaries in a muscle fiber and a lower concentration of myoglobin in muscle tissue (Elminowska-Wenda, 2004). Regarding the muscle composition, the main changes caused by selection pressure include the number of muscle fibers and their microstructure. The diameter of the muscle cell, in normal conditions, depends mainly on the age and activity of the animal and the type of muscle. The formation of giant fibers is often found in the nervous stimulation. Formation of giant fibers with muscle contractions and it can be a preliminary step to the hialin degeneration (Klosowska et al., 1993).

Muscle fibertrophy is a pathological process resulting from many different factors and consisting of concentration decline of nutrients in the cells and a general reduction in the

metabolic rate with a predominance of catabolism. As a result of these changes cells, tissue and ultimately the entire organs reduce in size and the degree of the blood supply. The reason for this may be nerve damage, aging or physiological state of cachexia (Rowińska-Marcińska et al., 1998). So, muscle hypertrophy, defined as excessive growth, is considered as abnormal increase in the number of myofibrils and it is the result of the excessive activity. Transportation of oxygen and energy material to the central parts of the muscle fiber is hindered by the considerable increase of its diameter causing the formation of secondary structural changes in the fiber (Rowińska-Marcińska et al., 1998). The most common reactions include splitting and necrosis of central parts of the fibers (Gallup and Dubowitz, 1973). Splitting is a common non-specific change in the fiber structure. The emergence of the gap separating the partially hypertrophic fibers indicates the onset of this pathology. The next phase is complete separation of one or more parts of fiber. The reason of splitting the fibers is overloaded cell. Hypertrophic fibers undergo fission (Gallup and Dubowitz, 1973).

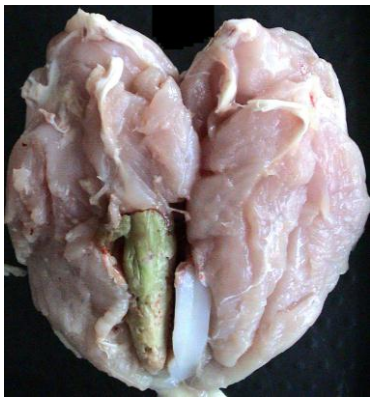
Connective tissue hypertrophy is a characteristic histopathological change of stress myopathy (Sośnicki and Wilson, 1991). Grow rank connective tissues may lead to the oxygen deficiency of fibers and to the formation degenerative changes by pressure on blood vessels capillaries in the muscle (Gallup and Dubowitz, 1973).

8.2.2 Deep pectoral desease

Deep pectoral disease (DPM) has the more important impact on final product quality issues (Figure 8.1). Deep pectoral disease, also known as Oregon disease or green muscle disease, was first described in 1968 as "degenerative myopathy" in turkeys (Dickinson et al., 1968) and it was subsequently studied at the Oregon State University (Harper et al., 1983; Siller, 1985). Even though this condition was first recognized in adult meat-type turkey and chicken breeders, it has become more and more common in meat-type growing birds (Richardson et al., 1980; Bilgili and Hess, 2002.) DPM occurs exclusively in birds that have been selected for breast muscle development (Siller, 1985). It is generally recognized that deep pectoral myopathy is an ischemic necrosis that develops in the deep pectoral muscle (*supracoracoideus* or *pectoralis minor* muscle) mainly because this muscle is surrounded by inelastic fascia and the sternum, which do not allow the muscle mass to swell in response to the physiological changes occurring when muscles are exercised, as in wing flapping (Jordan and Pattison, 1998). 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. 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. The lesion does not impair the general health of birds and is generally found during cut-up and deboning; moreover, it can be both unilateral or bilateral, affecting just one or both *pectoralis minor* muscles, respectively. No public health significance is associated to deep pectoral myopathy, but it is aesthetically undesirable. The fillet should be removed whereas the rest of the carcass is still fit for human consumption. However, the required trimming operations cause the downgrading of the products and produce an economic loss for the industry, especially because it affects the more valuable part of the carcass. The

incidence of carcasses affected by deep pectoral myopathy was estimated to be just below 1% (Bianchi et al., 2006). The incidence of DPM increases with market weight in broilers, with more cases reported in higher-yielding strains and in males. Increased bird activity (flock nervousness, flightiness, struggle, and wing flapping) induced by factors such as feed or water outages, lighting programs and intensity, human activity, and excessive noises in and around chicken houses should be looked at as a trigger for the development of DPM in broilers (Bilgili et al., 2000).

Figure 8.1. Deep pectoral myopathy (Bilgili et al., 2000)



Based on data obtained by Bianchi et al. (2006), DPM prevalence can be different when diverse breeds are considered, suggesting that genetics may play an important role in the determination of this condition. As a consequence, genetic selection against DPM has been undertaken by broiler breeding companies. Moreover, recent developments in whole-genome selection using dense DNA-markers should provide effective and powerful tools to reduce DPM importance in the future (Petracci and Cavani, 2012).

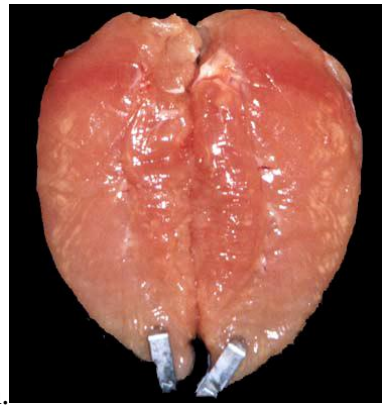
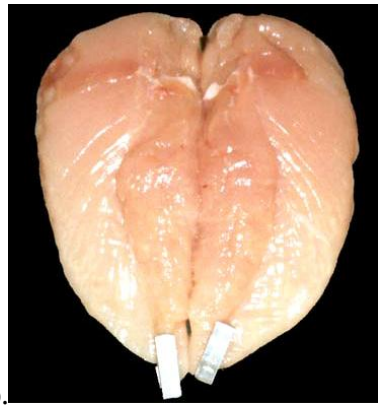
8.2.3 PSE-like breast meat

Color is an important quality attribute that influences consumer acceptance of many food products, including poultry meat. Consumers will often reject products in which the color varies from the expected normal appearance. Birren (1963) pointed out that color is everywhere and that psychological responses to color, as they relate to appetite, are considered important to processors and consumers. Consequently, color is often used to determine economic value of food (Qiao et al., 2001).

Color is important for both the consumer's initial selection of a raw meat product in the marketplace and for the consumer's final evaluation and ultimate acceptance of the cooked product upon consumption. Froning (1995) reviewed the many live bird production and processing factors associated with poultry meat color. One of the most frequent challenges to the meat industry associated with the intensive selection for increased muscling is the development of pale, soft and exudative meat. The term PSE was originally a descriptor for a pork product, characterized by light color, flaccid texture, poor water-holding capacity and substantially reduced cooking yield. With the advent of technologies to identify and eliminate this major cause of extreme cases of PSE, a great reduction in the incidence and severity of PSE has been realized, even if products with poor water holding capacity still exist (Barbut et al., 2008). The suggestion that a pale, soft, and exudative (PSE-like)

condition exists in poultry was mentioned some decades ago (Figure 8.2). Barbut (2009) reported that the occurrence of PSE meat in broiler chickens ranged from 0 to 28% in seven different flocks. A grocery store survey of 1,000 boneless, skinless, broiler breast fillet packages showed that approximately 7% of the multiple-fillet packages had one or more fillets that were significantly different in color, either lighter or darker, than the other fillets in the same package (Fletcher, 1999). However, to date, there is no evidence to support or refute a genetic mutation in chicken and turkeys as related to PSE development (Strasburg and Chiang, 2009). The metabolism of the breast muscle and conditions at slaughter could contribute to PSE-type meat because there are large glycogen stores within the breast muscle with a high propensity to produce lactic acid (entirely glycolytic) and therefore the potential for rapid drop in pH and/or low ultimate pH post mortem exists. In addition, there is the potential for high muscle temperatures due to flapping, struggle, stress, and high metabolic rate in the lead-up to slaughter and the large breast muscle mass (particularly in turkeys) being difficult to chill post mortem (Barbut et al., 2008).

Figure 8.2. Pale, soft and exudative (PSE)-like broiler breast meat

a.  b. 

a. Normal; b. PSE-like.

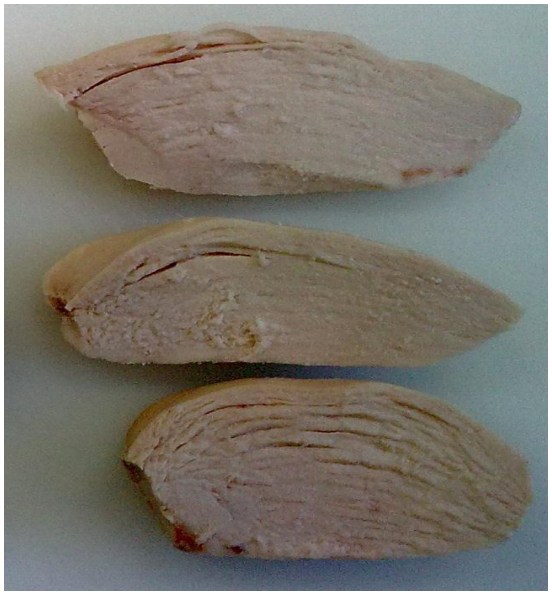
Several studies have been conducted to establish directly or indirectly the main causes of PSE-like condition in poultry (Petracci et al., 2009; Owens et al., 2009). These studies can be divided into two categories: those evaluating the role exerted by genetic selection and those concerning the effect of environmental factors. As for genetics, it has been shown that selection for body weight or muscle development has induced histological and biochemical modifications of the muscle tissue, which can be related with PSE-like condition (Barbut et al., 2008). Among environmental factors to induce PSE-like meat occurrence, heat stress during the end of the growing phase or preslaughter period seems to play the major role (Petracci et al., 2010). Faster growing or heavier birds have been shown to be more susceptible to heat stress indicated by great metabolic heat production, increased body temperature, and mortality. Sandercock et al. (2001, 2006) found that rapidly growing lines of birds may exhibit a reduced thermoregulatory capacity compared with their genetic predecessors and may thus be more susceptible to heat stress during the preslaughter period and to consequent problems including muscle damage, acid-base disturbances, and reduced meat quality (Petracci and Cavani, 2012).

Although color variations and their related problems occur in the poultry industry, they tend to be sporadic, inconsistent in severity, and are often not well described (Qiao et al., 2001).

8.2.4 Intramuscular connective tissue defects

A newer emerging quality issue in poultry is the poor cohesiveness of meat due to immaturity of intramuscular connective tissue (IMC) in relation to the very early slaughter age of modern chicken and turkey strains. The strength of IMC is based on collagen fibrils and there are cross-bridges between the collagen molecule units. These cross-bridges determine the physical strength and heat stability of IMC. The number and stability of cross-bridges increase with age determining a reduced tenderness. Modern poultry is not tough, but the problem is increasingly the opposite. The collagen content of lean meat is 0.2–0.4%. In fast-growing birds the collagen is immature resulting in low heat stability. Consequently, poultry meat is tender, but may turn fragile, even mushy (Poulanne and Voutila, 2009). Voutila et al. (2009) indicated that currently there are two emerging types of defect in commercial poultry meat: (1) cooked chicken breast meat is generally fragmented (soft) (Figure 8.3); and (2) raw turkey breast meat is so loose in structure (disintegrated) that it is possible to pull the muscle fiber bundles away with the fingers. (Swatland, 1990). The mushy structure of cooked chicken breast meat can be perceived so that the need to chew before swallowing the piece of meat is minimal (Voutila, 2009).

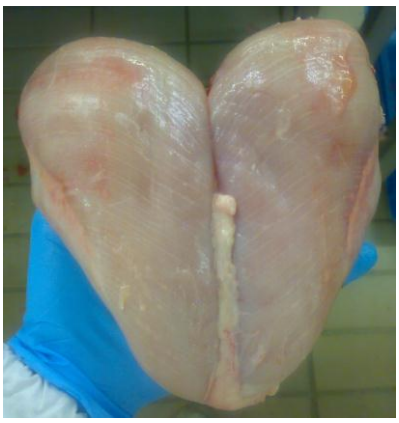
Figure 8.3. Broiler breast meat with poor cohesiveness



As a consequence of this IMC defect, new quality issue was recently observed regarding the appearance of breast muscle. McKee et al. (2010) indicated that one of emerging meat quality problems is the appearance of white striping or striations in poultry breast fillets following the directions of muscle fibers (Figure 8.4). While the phenomenon has not be linked to any particular eating attributes of cooked poultry, it does affect the appearance of raw meat and would possibly lead to consumers not selecting the product due to its

appearance. Histological observations indicated an increase in degenerative and atrophic fibers in breast fillets affected by white striping.

Figure 8.4. Broiler breast meat with “white striping” defect



As previously discussed for PSE-like issue, proteomics can be also very helpful to identity potential protein markers for understand defects such as poor texture and white striping in poultry related with intramuscular connective tissue development. For example, there are an increasing number of publications trying to reduce toughness problems in beef meat (Hollung et al., 2007; Eggen and Hocquette, 2003). However, until now, no clear relationship between collagen content and fiber type composition has been reported in livestock species (Lefaucheur, 2010). Unfortunately, to date, proteomic studies related this topic in poultry are limited because of less practical importance in respect to meat tenderness issues in beef and pork meat.

It seems that main current problems related to meat quality in poultry are related to selection of the birds for growth rate and breast yield, even if involved underlying genetic mechanisms are not fully understood. This continues to create new challenges for the food scientists who must work with meat from birds selected primarily for quantitative traits. Today, with the advent of “omics” science there are more possibilities to further investigate these issues. Overall proteomics, even if is at an early stage, may allow the identification of markers for muscle growth and meat quality properties and understanding the molecular mechanisms that influence texture and water-holding capacity of meat (Petracci and Cavani, 2012).

Chapter 9. Qualitative and nutritional properties of poultry meat

9.1 Meat quality concept

The concept of meat quality has received a great deal of attention from food manufacturers, small traders, as well as public institutions and health centers. Food quality is considered to be the most difficult to define the concept of the food industry, which has become particularly acute problem in recent years (Brunso et al., 2004). There is no standard definition of meat quality that meets all the quality components of the meat production; however the quality of meat production is determined by sustainability and safety of food chain (Akkerman et al., 2010). In fact, it is very difficult to develop common quality standards for the meat market as meat quality concept is changing significantly over time (Frisby et al., 2005; Bogosavljević-Bošković, 2007). The supply of meat which is wholesome, safe, nutritious, and of high quality to the consumer will ensure continued consumption of meat. In affluent countries, consumers are increasingly demanding meat products which are of high quality 100% of the time. In order for livestock industries to consistently produce high quality meat, there must be an understanding of the factors that cause quality to vary, and implementation of management systems to minimize quality variation. Meat quality is defined by those traits the consumer perceives as desirable which includes both visual and sensory traits and credence traits of safety, health and more intangible traits such as 'clean' and 'green' or welfare status of the production system (Becker, 2000). Important visual traits include; color and texture of the meat, fat color, amount and distribution of fat as well as the absence of excess water (purge) in the tray (Glitsch, 2000). Once cooked, consumer satisfaction is largely determined by how tender the meat is as well as its flavor/odor and juiciness (Glitsch, 2000).

So, meat quality is a generic term used to describe properties and perceptions of meat. It includes attributes such as carcass composition and conformation, the eating quality of the meat, health issues associated with meat such as *Escherichia coli* 0157 contamination and bovine spongiform encaphalopathy, and production-related issues including animal welfare and environmental impact. These factors combine to give an overall assessment of meat quality by the ultimate arbiter, the consumer. The critical point of appraisal of meat quality occurs when the consumer eats the product, and it is this outcome, together with views of color, healthiness and price, that determines the decision to repurchase (Boleman et al., 1997). The main source of consumer complaint and the primary cause of failure to repurchase is the variability in eating quality, especially tenderness (Tarrant, 1998; Bindon and Jones, 2001). Despite the efforts to control and optimise the peri-slaughter environment (Meat and Livestock Commission, 1991; Tatum et al., 1999; Moloney et al., 2001), which has a particular impact on tenderness (Ferguson et al., 2001), there is still unacceptable variation in eating quality, suggesting that determinants of meat eating quality are multifactorial and complex. This situation is not surprising since muscle is intrinsically a highly organized and complex structure, so that the properties of meat are likely to be determined at different levels ranging from the molecular to the mechanical.

9.2 The major factors affecting meat quality

9.2.1 Appearance and technological characteristics

Generally, consumers decide to purchase meat based on its appearance. The colour of the meat greatly affects its saleability. Moreover, its water-holding capacity (WHC) is also important to the consumer. It can be said that appearance and technological characteristics are connected. The importance of WHC can be classified into three sections; firstly, poor WHC can be connected to the appearance of the meat. WHC is obvious to the consumer when examining the Styrofoam packaging in the retail stores. Poor WHC results in the drip remaining in the package - resulting in a negative appearance of the meat. Secondly, the drip loss is connected to the weight of the meat.

In processed meats, poor WHC may reduce water retention and therefore yield of product is reduced. Finally, the juiciness of the meat after cooking is also affected by the WHC. Poor WHC meat may be dry or taste may be negatively affected. Beside colour and WHC, there is also a relationship between appearance and intramuscular fat (IMF) or marbling. This is also an important factor for determining appearance of the meat. High marbling is a requirement for some consumers such as in Japan, whereas low marbling is required by some other countries as in France (Monin, 1999; Warris, 2000).

9.2.2 Palatability

Palatability or eating quality of meat can be defined by three characteristics. Those are tenderness, juiciness, and flavour or odour. In most countries, people want their meat tender, but that is not the case for many African countries, where they prefer their meat chewy. Juiciness of the meat is mainly related to the WHC of the meat or low IMF level. Flavour and odour are closely related. Generally, flavour is linked to water-soluble materials, and odour is related to fat-soluble volatile elements. If the meat smells unpleasant, it is mostly related to the quality of the meat. It can be an indicator of the spoilage. But it is not always the case. For instance, some unpleasant smell can be caused by the boar's taint of male pigs (Warris, 2000).

9.2.3 Wholesomeness

According to the Canadian Food Inspection Agency, "Wholesomeness" is defined as free from decomposition, bacteria of public health significance or substances toxic or aesthetically offensive to man. Wholesomeness has two components. First, meat should be safe to eat. This means the meat must be free from parasites, microbiological pathogens and hazardous chemicals (Heitzman, 1996).

Second, people want meat to be beneficial to their health in contributing minerals, vitamins, high value protein, and possibly essential fatty acids, such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids to their diet (McCance and Widdowson, 1997).

9.3 Composition and nutritional aspects of poultry meat

Over the past few years, meat production and market have undergone several negative events that have impaired the image of this essential food product from the consumer's standpoint (Toldrá and Reig, 2011). Generally, the image of meat and meat products is

relatively negative due to their content in fat and saturated fatty acids, most prevalent diseases of Western societies like cardiovascular diseases and diabetes mellitus (Micha et al., 2010) and cancer (Cross et al., 2010; Ferguson, 2010; Santarelli et al., 2010). In fact, epidemiological data suggests a relationship between meat consumption or dietary heme and risk of colon cancer (Cross et al., 2010; Bastide et al., 2010). Reduction in meat consumption has been accentuated by a series of scandals and animal health problems which have hit livestock production, such as BSE, dioxins in meat and avian influenza. Within this context, the poultry meat has maintained its identity and a higher value compared to other species for several reasons. Indeed, worldwide poultry meat production and consumption have increased rapidly and, in many parts of the world, per capita consumption of poultry meat will continue to grow (Cavani et al., 2009). Poultry meat represents more than 30% of global meat production, the most common sources of poultry meat are chickens and turckey (87% and 7% of total poultry production, respectively). Relatively low and competitive prices compared to other meats, the absence of cultural or religious obstacles, and dietary and nutritional properties are the main factors explaining poultry meat's attractiveness (Valceschini, 2006). The quality of meat in general and hence poultry meat is an extremely complex notion that can be assessed from different points of view. From the standpoint of consumer interests and the slaughter industry, broilers should have not only high slaughter yields and desirable carcass conformation scores but also good aesthetic, sensory and nutritional characteristics. Nutritionally speaking, the main quality features of poultry meat are chemical composition and the ratio of muscles to fat in carcass. The structure of meat consists of a carcass muscle, fat, bone and connective tissue, as well as cartilage and ligaments. The less bone and cartilage and more muscle and adipose tissue in the carcass determine the higher categorical and nutritional value of meat. Content of muscle tissue of the carcass varies between 40-70%. In the case of broiler crosses muscle tissue proportion in the pectoral muscle is from 94 to 98% and in the leg from 92 to 97%. The most valuable is the ratio of carcass flesh and bones 4-4.5:1. The muscle is approximately 75% water (although different cuts may have more or less water) and 20-25% protein with the remaining 5% representing a combination of fat (1.2-2.5%), carbohydrate and minerals (Maiorano et al., 2012). The nutrient composition of chicken is a response to the diet it consumes particularly in the early stages of life. As a broiler chicken grows the composition of its carcass changes (Jones, 1986) and fat deposits increase. The intramuscular fat also palys an important role in the quality (flavor and juiciness) of chicken meat (Chizzolini et al., 1999). Regarding the fat composition, based on a sinlge value for a 100g lean fillet serve, lean chicken breast has relatively higher proportions of monounsaturated fatty acids (>20% compared to beef and tuna). and polyunsaturated fatty acids (>30% compared to beef, lamb and pork) (Rule et al., 2002; Probst, 2009). Fat is very important from a sensory aspect since it is a source of many aromatic substances affecting the meat taste. Intramuscular fat increases energy value, improves the taste, but too much body fat inhibits gastric acid secretion and complicates protein digestibility (Jukna et al., 2007). Consumers prefer lean meat with reduced content of fat. However, overmuch low intramuscular fat content in worse taste qualities of meat (Valsta et al., 2005; Jukna et. al., 2010). Intramuscular fat is the most variable part of the meat. Its coefficient of variation is several times higher than other meat characteristics.

Meat is an important source of protein, vitamin B1, niacin, B2, B6 and B12 and vitamin A. It is also a major source for phosphorus, iron, copper, zinc, and selenium. When all animal products are included whole in protein supply for humans, red meat and poultry make up one sixth of all protein consumption.

The main component of meat is muscle tissue. Proteins are the most valuable part of the muscle, which accounts for about 80 percent muscle tissue materials. Proteins determine the nutritional value of meat, they influence changes in the technological processes and physical - chemical parameters of meat. In fact, proteins are the major component of dry matter of meat, and their content in muscles is variable between 18 and 22% and depends on the function of a particular tissue. According to Simeonovová (1999) breast muscles contain approximately 22% proteins, while in thigh muscles, which contain more fat, approximately 17.20% of proteins was found.

Poultry meat well fit the current consumer demand for a low-fat meat (3-8%), low sodium (0.09%) and cholesterol levels (Suchý et al., 2002) with a low energetic value (519–741 kJ/100 g). It should also mentioned that the changes in consumer's lifestyle in developed countries have led to a meat market more and more addressed towards easy-handled and processed products ("convenience food"). This trend has been exploited since long time by the poultry industry, which made strong investments in the processing area, by increasing the availability of poultry in a large variety of processed ready meals (Cavani et al., 2009). Poultry meat may also be considered as "functional foods", which provide bioactive substances with favorable effects on human health, like conjugated linoleic acid (CLA), vitamins and minerals, and a balanced n-6 to n-3 polyunsaturated fatty acids (PUFA) ratio (Barroeta, 2006; Givens, 2009). There is considerable evidence to suggest that long-chain n-3 fatty acids are important to certain tissues such as the brain and retina and play a role in the maintenance of human health by protecting against metabolic diseases (Mori et al., 2000; Kris-Etherton et al., 2002; Delarue et al., 2004; Perez-Matute et al., 2007).

9.3.1 Factors affecting poultry meat quality

The quality and composition of poultry meat are influenced by numerous factors such as genotype of birds (Genchev et al., 2005; Alkan et al., 2010), divergent selection (Maiorano et al., 2009, 2011), feeding (Gardzielewska et al., 2005), sex (Genchev et al., 2008), age (Tserveni-Gousi and Yannakopoulos, 1986), and stress (González et al., 2007). Genotype, sex and age stand out among biological factors (Lewis et al., 1997; Bokkers and Koene, 2003; Hellmeister et al., 2003). Meat quality is also affected by environmental factors, including broiler nutrition (diet composition, feed consumption, feed additives), broiler rearing system, feeding management and stressful preslaughter conditions favoring meat defects (Holm and Fletcher, 1997; Owens and Sams, 2000).

No study has been conducted on the interaction between genotype and pre-slaughter stress in poultry. Significant effects of preslaughter conditions on meat characteristics and some interactions with the genotype were observed only in the study made by Debut et al. (2003). However, preslaughter conditions had an effect of lower magnitude than genotype and were limited to thigh characteristics. These results suggest that thigh meat is more sensitive to environmental factors than breast meat. The difference in sensitivity have been previously suggested by the genetic study of Le Bihan-Duval et al. (2003) on turkey meat

quality, in which environmental factors appeared most dominant for some thigh characteristics (pH and color) that showed extremely low heritable features. Surprisingly, breast meat characteristics were unmodified by heat stress in the present study, which was also reported by Petracci et al. (2001), suggesting that the influence of acute heat stress on meat quality could vary according to the conditions of application (duration or intensity) but also according to the genotypes and muscle used. Moreover, Debut et al. (2003) assert that in chickens as in pigs the postmortem pH decline strongly affects the quality of the meat, particularly by the strong effect of ultimate pH on processing yield.

The main indicator of the quality of poultry meat is the category of carcass which is determined by its nutritional status, taking into account the degree of fat and muscle tissue (Groom, 1990). The main edible components of chicken are:

- breasts (white meat and relatively dry);
- legs: the "drumstick" which is the lower part of the leg and the meat is dark; the "thigh", upper part of the leg and the meat is dark;
- wing: the "drumette", shaped like a small drumstick; "wingette";
- giblets: neck, liver, heart and gizzard.

So, as above reported, several are the factors that affect the overall quality of poultry meat but other 3 extremely important traits influence its quality: appearance, meat tenderness and flavor. (Fletcher, 2002). Appearance (e.g., color of skin and meat) is critical for both consumers' initial selection of the product and final product satisfaction. Poultry is unique because it is sold with and without its skin. In addition, it is the only species known to have muscles that are dramatic extremes in color, white and dark meat (Qiao et al., 2002). Breast meat is expected to have a pale pink color when it is raw, while thigh and leg meat are expected to be dark red when raw. There are times when poultry meat does not have the expected color and this has created some special problems for the poultry industry. In addition, raw meat color lightness, has a close relationship with various physical and chemical properties and functionalities of both raw and cooked poultry breast meat (reviewed in Zhuang and Savage, 2010). Poultry meat color is affected by factors such as age of animal, sex, strain (exercise), diet, intramuscular fat, meat moisture content, pre-slaughter conditions and processing variables (reviewed in Petracci et al., 2004). Color of meat depends upon the presence of the muscle pigments myoglobin and hemoglobin. Myoglobin is a protein responsible for the majority of the red color. It doesn't circulate in the blood but is fixed in the tissue cells and is purplishin color. When it is mixed with oxygen, it becomes oxymyoglobin and produces a bright red color. In addition, the color of meat and poultry can change as it is being stored at retail and at home. When safely stored in the refrigerator or freezer, color changes are normal for fresh meat and poultry.

Meat tenderness is an important meat quality trait and the biological, structural and physiological mechanisms underlying meat tenderness have been extensively investigated (Dransfield and Jones, 1980; Koohmaraie, 1988; Tornberg, 1996; Harper, 1999). Intramuscular fat also indirectly influences meat tenderness (Tornberg, 1996; Harper, 1999; Nishimura et al., 1999; Hocquette et al., 2010) as does the rate and extent of post-mortem energy metabolism (Thompson et al., 2006). Essentially, meat tenderness is determined by the amount and solubility of connective tissue (collagen morphology), sarcomere shortening during rigor development (sarcomere length, pH), and post-mortem

proteolysis of myofibrillar (fiber resisitance) and myofibrillar-associated proteins (Koohmaraie and Geesink, 2006). Each of these factors are generally non-linear in their effect on meat tenderness, with continuing debate over the importance of each factor, depending on the muscle, the species, the age of the animal and the data available. Whether or not poultry meat is tender depends upon the rate and extent of the chemical and physical changes occurring in the muscle as it becomes meat. Raw chicken meat is generally very soft and, when cooked, it can even be cohesive. In addition, the genetic progress has put more stress on the growing bird and has resulted in histological and biochemical modification of the muscle tissue by impairing some meat quality traits (reviewed in Maiorano et al., 2012).

Also the rate of pH decline plays an important role on post-mortem meat tenderization and all stages of production; it varies among chicken genetic lines and between individual birds (Gardzielewska et al., 1995). After death of the animal anaerobic metabolism reduces the pH from about 7.2 in muscle to 5.8 in meat and stiffness develops (rigor mortis) (reviewed in Dransfield and Sosnicki, 1999). A fast pH decline, when the carcass has a high temperature, inactivates the calpain system and reduce the process of postmortem tenderization and the meat will be tough. In addition, with a rapid pH decline the myosin will be more susceptible to denaturation, as consequence the meat is characterized as pale and with low functional properties (lower water-holding capacity soft and exudative) normally refers to the PSE-like condition found in poultry. (Van Laack et al., 2000).

Flavor is an important quality attribute which relates to the organoleptic characteristics of meat; it is another quality attribute that consumers use to determine the acceptability of poultry meat. Although perception of flavor is a complex phenomenon, odor is the most important single factor contributing to the overall characteristics of flavor. A large number of compounds have been identified in the volatile fraction of red meats and poultry. An overview of the chemical constituents present in the volatiles of beef, pork, mutton and chicken is presented according to species and arranged by chemical class (hydrocarbons, alcohols, acids, aldehydes, ketones, sulfides, hetorocyclic compounds) (Shahidi et al., 1986).

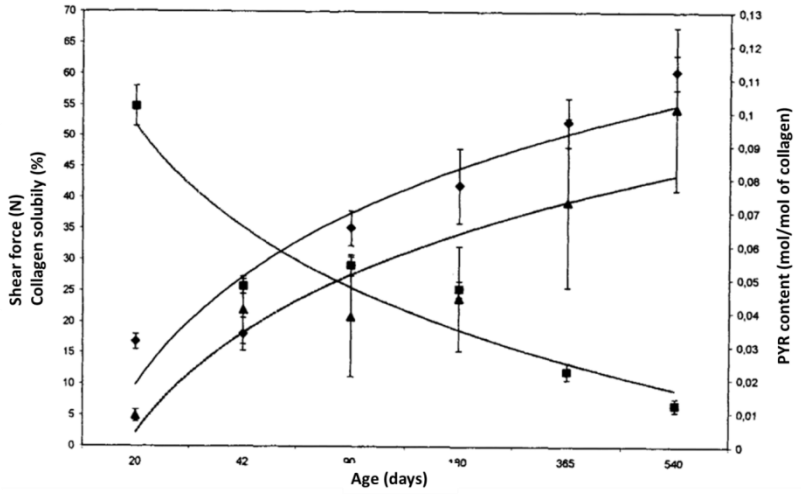
9.3.1.1 Effect of intramuscular connective tissue on meat toughness

Connective tissue, where collagen is the dominating component plays a role in meat quality. Collagen form strong fibrous structures linking muscle elements together, therefore it is hypothesized that the total amount of this component determines the meat texture. In fact, the morphology, composition and amount of intramuscular connective tissue (IMCT) vary tremendously between muscles, species, breeds and with animal age. It intrinsically acknowledged the large influence of IMCT on meat toughness in age-old preferences in choosing cuts of meat to purchase. Although only a small component (in terms of mass fraction), the disproportionately large influence of IMCT on meat toughness has resulted in studies trying to relate its amount, or various aspects of its composition and distribution in muscle tissue, to variations in meat texture for more than three-quarters of a century (Lehmann, 1907; Hammond, 1932; Carpenter et al., 1963; Marsh, 1977; Light et al., 1985; Brooks and Savell, 2004). Modern production practices for animal production clearly aim to minimize the variations in texture due to animal maturity, so that young

animals of a narrow age range at slaughter of the same breed show variation in the texture of a meat from a given muscle that is only minimally related to characteristics such as collagen content and solubility. Given the large influence of IMCT on meat texture, even small manipulations of its expression and turnover may have considerable potential for reducing unwanted variations in meat tenderness. (Purslow, 2005)

Meat texture sensation is dictated by the presence of several factors including the amount of intramuscular fat, water holding capacity and actomyosin complex (Avery and Bailey, 1995; Bosselmann et al., 1995). However, it is the quality of collagen, the major component of the intramuscular connective tissue, which gives the toughness to mea of different domestic animals including birds (Maiorano et al., 2011, 2012). It has been established that collagen covalent cross-linking mediated by lysyl oxidase enzyme changes with advancing age (Bailey and Shimokomaki, 1971; Robins el al., 1973). In fact, the immature collagen crosslink, hydroxylysinoketonorleucine (HLKNL) decreases its concentration with aging of the animal toughening the meat (Shimokomaki et al., 1972). The main mature crosslink derived from two reducible HLKNL is pyridinoline (PYR), which stabilizes the collagen molecule (Fujimoto, 1977; Eyre and Oguchi, 1980; McCormick, 1999). PYR has been shown to be related to meat texture (Nakano et al., 1991; Bosselman et al., 1995) and to bridge different types of collagen (Shimokomaki et al., 1990) thus stabilizing further the extracellular macromolecular organization (Shimokomaki et al., 1990). Furthermore, the collagen molecule stabilization is measured by its increasing insolubility (Robins et al., 1973; Young et al., 1994). Although, chicken is commercially slaughtered at the age that collagen normally does not constitute a texture problem; in addition, knowledge on collagen crosslinks in poultry intramuscular connective tissue is scarce. (Corò et al., 2003). PYR biosynthesis is considerable 40 to 50 days after hatch. The presence of PYR within the collagen fibers would create environmental conditions to slow down the formation of *de novo* collagen synthesis. There is an inverse relationship between collagen solubility and amount of PYR. These results supported the thesis that collagen crosslinking is an important factor for the mechanism for regulating the rate of *in vivo* catabolism (Krane, 1987). Up to 100 days after hatch, there was a noticeable increase in collagen synthesis. As PYR was synthesized into collagen fibrils it imparted resistance to degradation by collagenase as measured by the rate of diminution of collagen solubility. While the rate of collagen synthesis became proportionally constant with aging of poultry, PYR was continually formed (Figure 9.1) bringing about the slowdown of collagen turnover. Since there was no relative increase in collagen content after 180 days posthatch, the increase in texture seemed to be related to collagen crosslinking (Figure 9.1) and this fact was corroborated by diminution of collagen solubility (Figure 9.1). Furthermore, it seemed that the amount of collagen crosslinks was related to growth rate and nutrition status depending on the poultry genetic line specificity (McCormick, 1994).

Figure 9.1. Variation in breast meat (*Pectoralis Major* m.) shear value (-●-), pyridinoline (pyr) content (- - ▲ - -) and collagen solubility (---■---) with increasing hen age



These data are averages with standard deviation bars from four separate experiments in triplicate for each experiment.

In fact, comparing the results obtained by Corò et al. (2003) with those reported by Velleman et al. (1996), there is at least five times more collagen in breast White Leghorn than Ross and PYR quantitative pattern was again ten times more concentrated in White Leghorn of comparable age samples. It is known that White Leghorn chicken breast presented the concentration of over 1.0 mol PYR/mol collagen by 1 year of age, the highest value reported so far in muscle (Velleman et al., 1996). This condition can be explained by the fact that White Leghorn hen is designed to be an egg producer and is not submitted to a high energy diet showing a relatively slow-growing process. Conversely, the meat producer Ross is submitted to a high plane energy diet and consequently is a fast growing bird with protein accretion. In fact, Schreurs et al. (1995) demonstrated that White Leghorn had higher proteolytic activities for calpain and cathepsins in comparison to Ross. The reduced catabolism in fast growing birds would dictate the pattern of having less collagen crosslinks formed thus lower shear values in breast meat. Therefore, the relationship between labile to insoluble collagen, for the fast growing hen is favored although there was not necessarily a diminution of insoluble collagen (Etherington, 1987). Corò et al. (2003) results are in agreement with other reports for other animals (McCormick, 1989), the rapid growth and increased collagen synthesis resulted in more tender meat because less collagen crosslinking takes place.

PART 2: AIM OF DISSERTATION

In poultry farming, the gut microbiota has received increased attention in the past decade. Researches on poultry microbiota mainly focus on minimizing food-borne illness in humans, improving animal nutrition and reducing the use of antibiotics as growth-promoters.

Antibiotics have often been used in animal breeding as growth promoters to improve feed efficiency and to control the so-called "production related" bacterial infections e.g. infections associated with early weaning, high animal densities, poor sanitary conditions and frequent transportations. However, concerns about development of antimicrobial resistance and transfer of antibiotic resistance genes from animal to human microbiota, led to withdrawal approval for antibiotics as growth promoters in the European Union since January 1th, 2006. In the post-antibiotics era, probiotics and prebiotics could represent a strategy to improve intestinal health and growth performance of broiler chickens. Adding and/or stimulation of the beneficial bacteria in the digestive tract of poultry is not a new concept. Studies carried on these bioactives, administered in feed or water, show conflicting results due to the different environmental conditions (experimental and field conditions) and the way of use. In order to be effective, they have to be administered to animal as early in life as possible and uncontrolled variables (i.e. water quality) have to be minimized. To eliminate some of these factors that could influence the responses to bioactives, the *in ovo* injection technology of pre-/pro-biotics and their combination (synbiotics) has been used, an emergent and original technique directly into a chicken embryo during embryogenesis (Gulewicz and Bednarczyk, Polish patent Nb. 19772).

Therefore, the aim of this research was to evaluate the effects of prebiotic used alone or in combination with strictly selected and characterized probiotic bacteria (synbiotics; Bardowski and Kozak, 1981; Boguslawska et al., 2009) *in ovo* administrated performance, meat quality and hystopathological changes in breast muscle of broiler chickens.

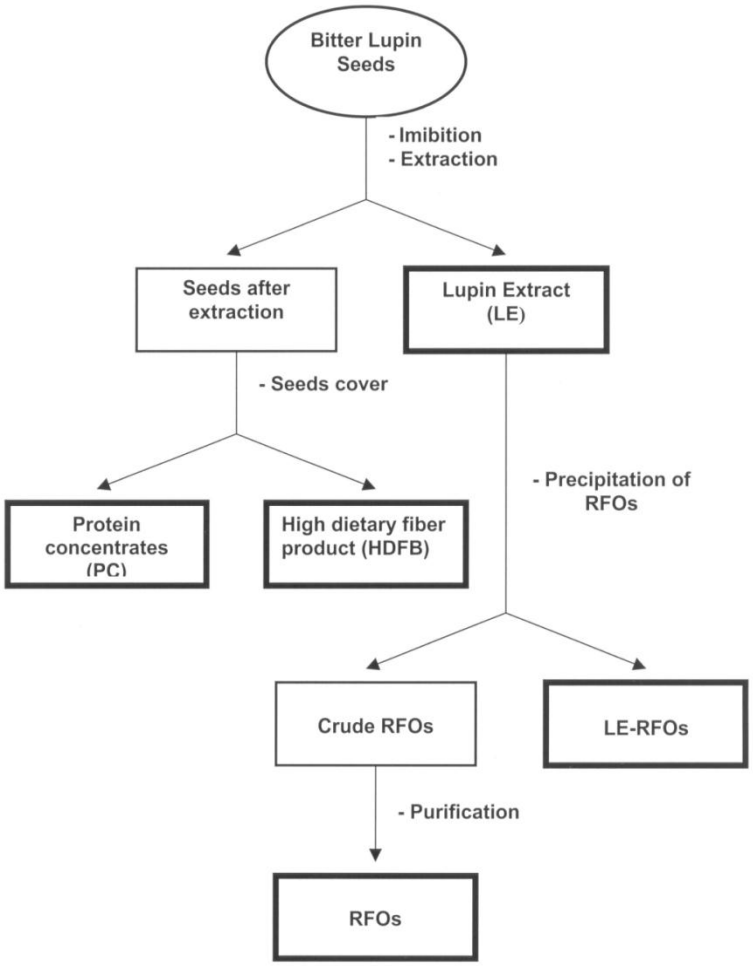
PART 3: MATERIALS AND METHODS

Chapter 10. Debittering process of alkaloid-rich lupin seeds

10.1 RFOs isolation and purification from seeds of lupin

Seeds of various species of lupins have been used as food for over 3000 years around the Mediterranean (Gladstones, 1998) and for as much as 6000 years in the Andean highlands (Uauy et al., 1995; Bhardwaj and Hamama, 2012). People used to soak the seeds in running water to remove most of the bitter and toxic alkaloids and then cook or roast the seeds tomake them edible (Hill, 1977). Unfortunately, this mode of debittering led to removal of the valuable biological compounds that were significant from an economic point of view. This important problem was resolved with a method described by Gulewicz (1988, 1991a, 1991b) and Gulewicz et al. (2000). A general scheme of this process is presented in Figure 10.1. The main products of this process are protein concentrate (PC), high dietary fiber product (HDFP), lupin extract (LE), and raffinose family oligosaccharides.

Figure 10.1. General scheme of bitter lupin seeds processing



10.1.1 Imbibition of seeds.

First of all, lupin seeds (*Lupinus luteus* L. *cv.* Lord) are imbibed in a determined volume of distilled water (required for full imbibition). The quantity of water used in this step depends on seed species and ranges from 80 to 120 mL/100 g of legume seeds. The imbibition of seeds was carried out at +4°C for 10-12 h. During this process the vessel with seeds should be shaken from time to time.

10.1.2 Extraction of RFOs.

The imbibed seeds were then extracted with 200 mL of 50% ethanol (v/v) per 100 g of seeds at 40°C overnight. The water that was not absorbed by seeds was used for preparation of proper ethanol concentration for extraction. After extraction the supernatant was decanted. The seeds were reextracted with fresh alcohol under the same conditions. The supernatants from two cycles of extraction were boiled under reflux for 10 min and combined together. In the case of lentil seeds extraction the supernatants containing any precipitate should be centrifuged before the next step.

10.1.3 RFOs precipitation.

The clear supernatant was concentrated on a rotary vacuum evaporator at 50 °C to the volume of 25 mL, placed in glass separator and dropped into 100% ethanol with continuous stirring. The ratio of water extract volume to volume of 100% ethanol should be 1:10. The crude RFOs precipitate was separated from supernatant by centrifugation at 3000 rpm for 15 min. The RFO precipitate was then placed into a vacuum desiccator in order to remove of any ethanol residue.

10.1.4 Purification of RFOs on diatomaceous earth and charcoal.

The crude RFOs extract was dissolved in distilled water (25 mL) and placed onto diatomaceous earth and charcoal (1:1 w/w) located in a sintered glass funnel (pore size G4, 7 cm x 5 cm i.d.) and connected to a vacuum. The funnel was then washed with 200 mL of distilled water. The RFOs were eluted with 70% ethanol (500 mL). The presence of RFOs in the eluate was checked by reaction with naphthoresorcinol. Afterward, RFO alcohol solutions were concentrated to dryness on a rotary vacuum at 50 °C.

10.1.5 Cation-exchange chromatography.

The purified RFOs (about 3 g) were dissolved in 10 mL of distilled water and then applied into a Dowex 50WX8 column (12 x 1.5 cm i.d.) and washed with distilled water (50 mL) until oligosaccharides were not identified in the eluate. The presence of RFOs was monitored on TLC by reaction with naphthoresorcinol. The acidic solution of RFOs (pH 1.5) was adjusted to pH 7.0 using 4% freshly prepared $Ca(OH)_2$. The solution was then boiled for 2 min and centrifuged. Supernatant containing a high purity of RFOs was than evaporated to dryness on a rotary vacuum evaporator at 50°C.

10.1.6 Qualitative evaluation of RFO composition using Thin-Layer Chromatography (TLC).

Qualitative evaluation of chemical composition of RFOs at each particular stage of purification was done by TLC according to Stahl (1969), and Dey (1990). For the separation of RFOs, 2-propanol-acetic acid-water (5:2:3 v/v) as mobile phase was used. The RFOs were visualized by naphthorezorcinol. For qualitative and quantitative separation of RFOs, silica gel 60 F_{254} TLC plates were used.

10.1.7 Determination of total soluble sugars and α-galactoside content at particular stages of the procedure.

To 0.4 mL of an aqueous solution from each particular stage of purification, containing 2.0-15.0 µg of soluble sugars, 10µL of 80% phenol in water (w/w) and 1 mL of concentrated H_2SO_4 were added. After mixing, the solution was kept at room temperature for 10 min and then cooled in a bath of cold water for 20 min. The same procedure was also performed for the standards. Resulting absorbance obtained using a DU-62 spectrophotometer (Beckman) at 485 nm was referred to the standard curve obtained for glucose (Fry, 1994).

10.1.8 HPLC analyses of RFOs.

The analysis of separation and quantification of RFOs from legume extracts was carried out by high performance liquid chromatography using a refraction index detector (HPLC-RI) (Frias et al., 1994). The analysis was performed on a HPLC chromatograph (Waters Associates, Milford, CT) equipped with a Waters model 510 pump, a Rheodyne model 7000 sample injector, a reflection type differential refractometr detector model R410 (Waters). The chromatographic system was controlled by an computer with a Maxima HPLC system controller software (Waters). A pre-column (0.32 cm i.d. x 4.0 cm) packed with C18 Porasil B and a m-Bondapak/carbohydrate column (0.39 i.d. x 30 cm) (Waters) were employed. Acetonitrile-distilled water (75:25 v/v, HPLC grade) was used as the mobile phase at the flow rate of 2.0 mL/min. Solvents were filtered through a Millipore FH (0.45 µm) membrane and degassed under helium. Injection volumes were 100 µL.

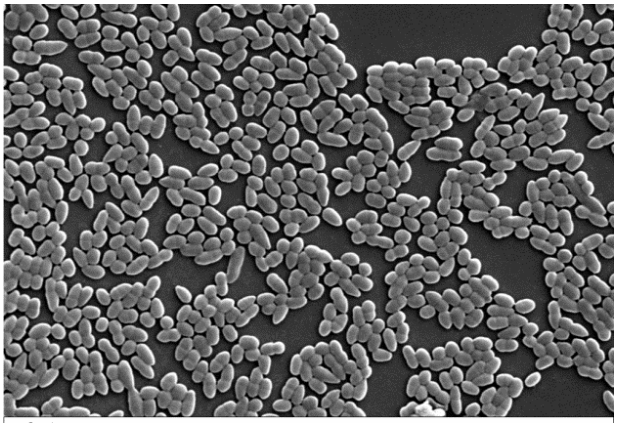
Chapter 11. Preparation of the synbiotic for the in ovo injection

11.1. Bacterial strains and growth conditions.

The strains used were *Lactococcus lactis ssp. cremoris* tetracycline-resistant strains (IBB SC1), and *Lactococcus lactis ssp. lactis* bacteriocin-typing strain (IBB SL1) and they were provided by Institute of Biochemistry and Biophysics in Warsaw, Poland (Collection IBB PAN).

The strains (Figure 11.1) were grown on GM17 agar medium.

Figure 11.1. *Lactococcus lactis* viewed by microscope



The typical composition of this medium is listed below:

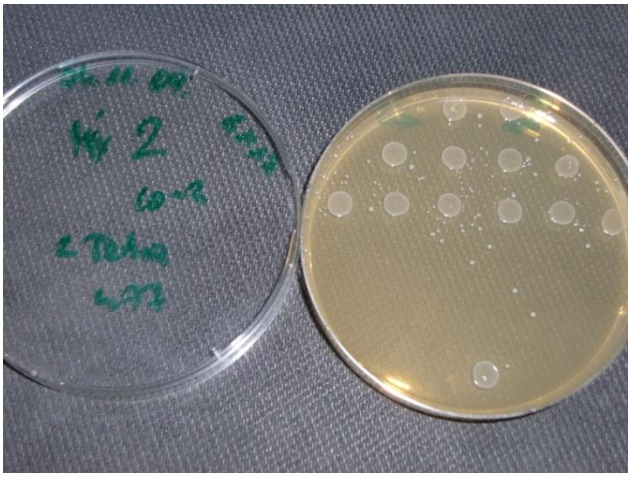
-5.0 g Pancreatic Digest of Casein;
-5.0 g Soy Peptone;
-5.0 g Beef Extract;
-2.5 g Yeast Extract;
-0.5 g Ascorbic Acid;
-0.25 g Magnesium Sulfate;
-10.0 g Disodium-β-glycerophosphate;
-11.0 g Agar .

The medium preparation:

1. Heat with frequent agitation and boil for 1 minute to completely dissolve;
2. Autoclave at 121°C for 15 minutes. Cool to 50°C;
3. Add 50 ml filter sterilized 10% glucose solution obtaining the GM17 agar medium.

Lactococcus lactis ssp. lactis grown on GM17 agar medium for about 18h at 25-28°C under aerobic conditions. *Lactococcus lactis ssp. cremoris* grown on GM17 agar medium supplemented with tetracycline, (the selection agent for growing) at 10mg/ml concentration for about 18h at 25-28°C under aerobic conditions (Figure 11.2).

Figure 11.2. *Lactococcus lactis ssp. cremoris* grown on GM17 plus tetracycline



The pure cultures of this two strains were centrifuge at 7000xg for 1min. The bacteria pellet was washed twice and resuspended in a prebiotic solution in order to obtain a final concentration of 10^5cfu/ml. Then, 190ml of prebiotic solution was mixed with 10ml of bacterial suspension (10^5cfu/ml) to obtain a final concentration of 10^3 bacteria/embryo contained in 0.2ml of synbiotic solution for injection.

11.2 Commercial synbiotic strains.

The commercial symbiotic used was Duolac (producer Biofaktor) which contains in 100g:
-*Lactobacillus acidophilus:* 10^9 cfu;
-*Streptococcus faecium:* 10^9 cfu;
-1g of lactose.
1 g of Duolac was dissolved in 100 ml of distilled water (10^7 cfu of *Lactobacillus* + 10^7 cfu of *Streptococcus*/100ml) to obtain the of 10^5 cfu of *Lactobacillus* + 10^5 cfu of *Streptococcus*/ml). A serial dilution 10x were made:
-10 ml Duolac + 90 ml distilled water: 10^4 cfu/ml for both strains;
-50 ml Duolac 10^4 cfu /ml + 150 ml distilled water: a concentration of 2.5 x 10^3cfu/ml for both strains in order to reach a concentration of 10^3 cfu bacteria/embryo contained in 0.2ml of commercial synbiotic solution for injection.

Chapter 12. Study design

12.1 Birds

Broiler chicken (Ross 308) hatching eggs (480) were incubated in a commercial hatchery Drobex (Solec Kujawski, Poland), a Petersime incubator (vision version, Petersime NV, Zulte, Belgium) (Figure 12.1)

Figure 12.1. Broiler chicken (Ross 308) hatching eggs and Drobex Agro commercial hatchery

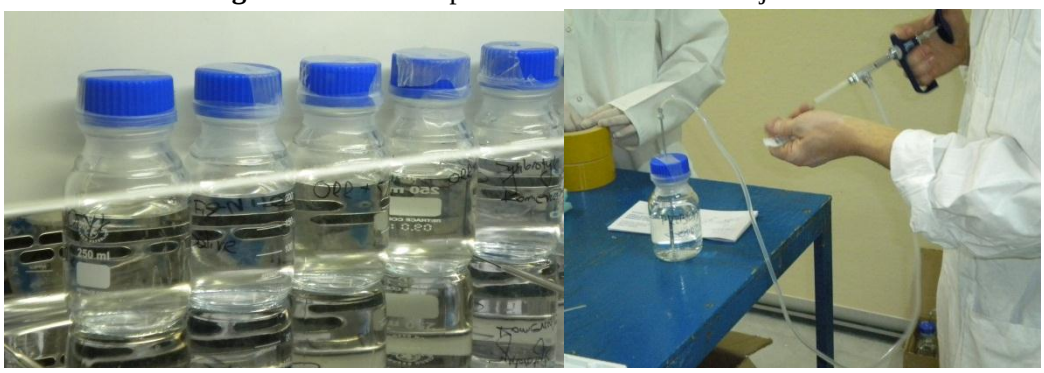
On d 12 of incubation, the eggs were randomly divided into five experimental groups treated with different bioactives, administered *in ovo*. Prior to the injection, the eggs were candled and those unfertilized or with dead embryos were discarded (Figure 12.2).

Figure 12.2 Egg candling

An aqueous solution at the equal volume of 0.2 ml was injected manually into the air chamber by using self-refilling syringes (Socorex, Ecublens, Switzerland, Figure 12.3):
The C group (control) was injected with physiological saline.
The T1 group was injected with a solution containing 1.9 mg of raffinose family oligosaccharides/egg (RFOs).
For the T2 and T3 groups, the injection solutions consisted of homemade synbiotics: 1.9 mg of RFO enriched with different probiotic bacteria, specifically, 1,000 cfu of

Lactococcus lactis ssp. lactis SL1 (group T2) and 1,000 cfu of *Lactococcus lactis ssp. cremoris* IBB SC1 (group T3). (Patent P-339113).
The T4 group was injected with a commercially available synbiotic Duolac (Biofaktor, Skierniewice, Poland), that contained combined 500 cfu of *Lactobacillus acidophilus* and 500 cfu of *Streptococcus faecium* with addition of lactose (0.001 mg/embryo).

Figure 12.3. Five experimental solutions for injection



After injection, each hole was sealed with adhesive tape and the egg incubation was continued until hatching (Figure 12.4).

Figure 12.4. *In ovo* injection

Among the hatched chickens (Figure 12.5), sixty males were randomly chosen (12 birds for each group) and reared according to the animal welfare recommendations of European Union directive 86/609/EEC in an experimental poultry house that provided good husbandry conditions (e.g., stocking density, litter, ventilation).

Figure 12.5. Hatched chickens

The birds were grown to 42 d of age in collective cages (n = 3 birds in each 4 cages: replications for experimental groups). The cages (30 x 40 x 35 cm; length x width x height) had wire floors and solid metal walls, a feeder space of 12.2 cm/bird, and 1 water nipple. The lighting program was 23L:1D (Figure 12.6).

Figure 12.6. Birds in collective cages

The conditions of management of broiler chickens were the same in all groups. The broilers were fed *ad libitum* commercial diets (Table 12.1) according to their age, and water was provided ad libitum. Amounts of feed offered to each cage were recorded, and uneaten feed in each cage was weighed daily (from 1 to 42 d). Cumulative feed intake and feed conversion ratio were calculated on a cage basis.

Table 12.1. Composition and nutritive value of the diets

Item (% unless noted)	Period	
	1 to 14 d	15 to 42 d
Component		
Wheat	35.0	40.0
Maize	17.05	22.85
Soybean meal 45%	37.0	27.8
Soybean oil	5.7	4.6
Calcium monophosphate	1.3	1.3
Limestone	0.6	0.5
$NaHCO_3$	0.1	-
Lysine 20%	0.9	0.8
Methionine 20%	1.0	0.8
NaCL	0.35	0.35
Vitamin-mineral premix[1]	1.0	1.0
Calculated nutritional value		
ME (kcal/kg)	3,150	3,100
N x 6,25	23.0	20.0
Ca	1.1	1.2
P available	0.47	0.60
Na	0.23	0.23
NaCL	0.50	1.0

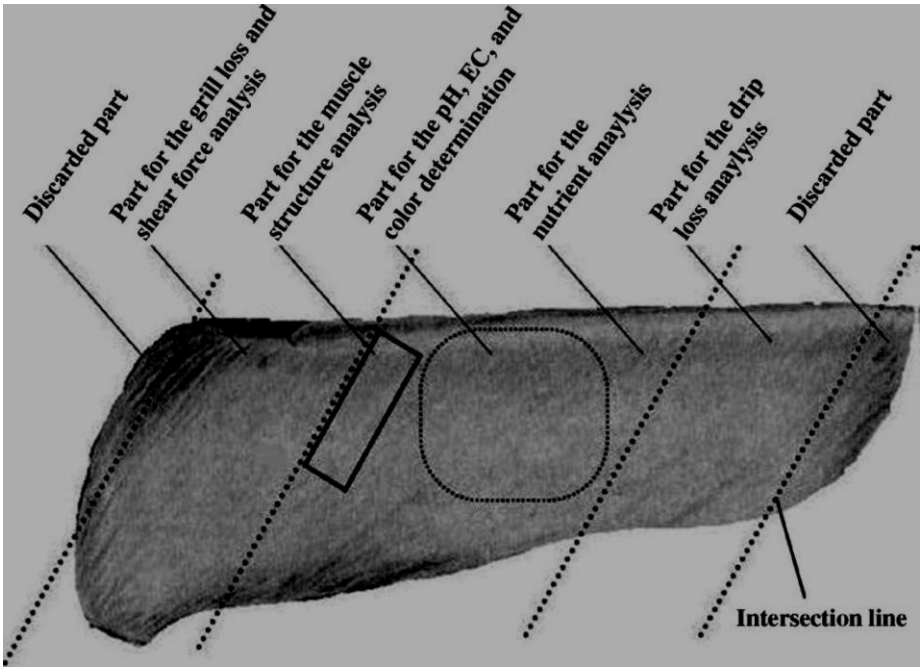
[1]*Provided the following per kilogram of diet: vitamin A (retinyl acetate), 13,000 IU; vitamin D3 (cholecalciferol), 4,000 IU; vitamin E (DL-α-tocopheryl acetate), 80 IU; vitamin K (menadione sodium bisulfite), 3 mg; riboflavin, 6.0 mg; pantothenic acid, 6.0 mg; niacin, 20 mg; pyridoxine, 2mg; folic acid, 0.5 mg; biotin, 0.10 mg; thiamine, 2.5 mg; vitamin B12, 20 μg; Mn, 120 mg; Zn, 90 mg; Fe, 30 mg; Cu, 10 mg; I, 1.5 mg; Se, 0.2 mg; and ethoxyquin, 100 mg.*

12.2 Slaughter surveys and analyses

At 42 d of age, broilers (12 for each group), identified by numbered permanent wing bands, were weighed individually (after a fasting period of 12 h) and transported within 1.0 h (including careful catching and loading) to a commercial poultry slaughterhouse. After careful unloading and hanging in randomized order, the birds were electrically stunned and slaughtered (according to the local Animal Research Ethics Committee at University of Technology and Life Sciences in Bydgoszcz), hot carcass weight was recorded, and carcass yield percentage was calculated. At slaughter, pectoral muscle and abdominal fat were removed from all carcasses (n = 60) and theirs percentages were calculated based on hot carcass weight. In addition, pectoral muscle pH was recorded at 45 min (pH_{45}), 12 h (pH_{12}), and 24 h (pH_{24}) *postmortem* using a portable HI 9625 pH meter (Hanna Instruments, Padova, Italy, Figure 12.7). Samples of the right pectoral muscle of 40

animals, 8 birds from each experimental group, were taken and frozen in liquid nitrogen (-196ºC) for histological and histopathological analyses. The left pectoral muscle was vacuum packaged and stored frozen (- 40°C) until intramuscular collagen and cholesterol analyses.

Figure 12.7. Positions for the determination of pH, muscle structure and meat quality characteristics of *Pectoralis superficialis* muscle



The image shows the ventral view of the PS muscle (From Werner et al., 2008)

12.3 Histological and histopathological analyses

The muscle samples were cut into 10 μm cross-sections in a Leica cryostat. The samples were stained with Hematoxylin and Eosin according to the method of Dubowitz and Brooke (1973) to measure the diameters and the number of muscle fibers and to determine the extent of histopathological changes in the pectoral muscle. Using the InterVideo WINDVR program (Kworld Computer Co. Ltd., New Taipei, Taiwan), 12 images of microscopic pictures were taken per sample, using magnification 12.5 x 10. Afterwards, all muscle fibers were counted and their diameters were measured at the surface of a 1 mm^2 cross-section of the pectoral muscle of the chickens using the computerized image analysis system MultiScanBase v. 14.02 (Computer Scanning System Ltd., Warszawa, Poland). Percentage of histopathological changes in muscle structure was evaluated in an area of 2mm^2 cross-section. Changes in fiber size and shape, and degenerative changes of fibers were analyzed according to Dubowitz and Brooke (1973).

12.4 Measurement of muscle cholesterol

The muscle cholesterol content was determined using the method by Maraschiello et al., (1996). 100mg of breast muscle with 2ml of methanolic KOH (0.5N) was heated in water bath at 80°C for 60min. All analyses were carried out in duplicate. After cooling, cholesterol was extracted and centrifuged (at 3000rpm for 10min) twice with 2ml of NaCl and 3ml of ether/hexane (1:1) (first extraction) and with 3ml of ether/hexane (1:1) (second

extraction). Then, the supernatants were evaporated to dryness in water bath at 30°C using Rotavapor, redissolved in 1ml of acetonitrile/isopropanol (1:1) and injected into HPLC. A Kontron HPLC (Kontron Instruments, Milan, Italy) model 535, equipped with a C18 reverse-phase column (250 x 4.6 mm x 5 μm; Hamilton Company, Switzerland), was used. The mobile phase was acetonitrile/2-propanol (55: 45 vol/vol) at a flow rate of 1.2 mL/min. The detection wavelength was 210 nm, and retention time was 12.89 min.

12.5 Collagen

Approximately 100 g of muscle (wet weight) was thawed at room temperature, trimmed of fat and epimysium, lyophilized for 48 h, weighed, 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. All analyses were carried out in duplicate. The IMC concentration was calculated, assuming that collagen weighed 7.25 times the measured hydroxyproline weight (Eastoe and Leach, 1958) and expressed as μg hydroxyproline per milligram of lyophilized tissue. Hydroxylysylpyridinoline (HLP) concentration, the principal nonreducible crosslink of muscle collagen (McCormick, 1999), was concentrated and separated from the bulk of the other amino acids by selective elution from a CF1 cellulose column (Skinner, 1982), using the HPLC procedure developed by Eyre et al. (1984). The HPLC was equipped with a Kontron 450 MT2 (Kontron Instruments, Milan, Italy) data system and with an Altex (Beckman Instruments, Fullerton, CA) Ultrasphere-ODS (C-18; small pore; 4.6 × 250 mm) column. Pyridoxamine.2HCl (Sigma Chemical, St. Louis, MO) was added as an internal standard to the eluent containing HLP and analyzed by HPLC. Identification of HLP in tissue hydrolyzates was made by comparison with a purified HLP standard and the known relationship between the elution time of HLP and pyridoxamine (Eyre et al., 1984). Purified HLP standard was routinely prepared from bovine cartilage hydrolyzates using the technique described by Eyre et al. (1984). The concentration of HLP residues in samples was calculated based on the concentration of collagen in each hydrolyzate and assuming that the molecular weight of collagen was 300,000 and the molar fluorescence yield of pyridoxamine was 3.1 times that of HLP (Eyre et al., 1984). The HLP was expressed as moles of HLP per mole of collagen and also as microgram of HLP per milligram of lyophilized tissue.

12.6 Statistical analyses

To verify significant differences in relation to the treatments, the data were evaluated by using one-way ANOVA and means were separated by Scheffe's battery of pairwise tests (SPSS Inc., 2010).

PART 4: RESULTS AND DISCUSSION

Chapter 13. Effect of in ovo prebiotic and synbiotics administration on slaughter performance of broiler chickens

13.1 Slaughter traits, Feed Conversion Ratio and pH of broiler chickens.

The effect of prebiotic and synbiotics administrated *in ovo* on slaughter traits, feed conversion ratio, and pH of broiler chickens is showed in Table 13.1. Compared with the control (C) group, the treatment groups (T1, T2, T3 and T4) had slightly higher final body weight (BW), more evident in the prebiotic (T1) group (+ 4.54%), even if these differences were not statistically significant. Chickens from T1 group showed also a slightly higher carcass weight compared with others groups ($P > 0.05$). Carcass yield percentage was higher (+ 2.9%) in C group than in commercial synbiotic (T4) group ($P < 0.05$) while the proportion of carcass yield of T1, T2 and T3 broilers was intermediate between C and T4 groups ($P > 0.05$). The poultry feed efficiency, traditionally measured as FCR (the ratio of feed intake to weight gain), was found to be similar (Table 10.1) among the groups C, T1 and T2 ($P > 0.05$); however, FCR was higher in the homemade synbiotic (T3) and commercial synbiotic (T4) groups compared with other groups ($P < 0.05$). In the study conducted under commercial conditions on 1,381,212 broilers (M. Bednarczyk, personal communication), the *in ovo* injection of prebiotic (RFOs) improved growth performance. The registered broilers survivability, final BW, and FCR were 4.7%, 2,304g, 1.87, and 4.2%, 2,325g, and 1.86, in controlled and injected groups (RFOs), respectively. In a recent study, Bednarczyk et al. (2011b) observed under field conditions that the prebiotic effect of RFOs (administered by single *in ovo* injection) and growth promotant antibiotic (in water) had a similar influence on broiler performance (survivability, growing period, and European production efficiency factor); instead, BW and FCR were significantly higher in injected group (RFOs) in comparison with the control. The lack of consistency between broilers performance registered in an experimental farm or in field conditions can be explained by the study of Timmerman et al. (2006). Based on their 4 studies in combination with 9 other studies published earlier, the authors suggested that the effect of probiotics becomes smaller when productivity rates of broilers is higher.

In the present study, the growth performance and FCR depend on the combination of prebiotic and probiotic *in ovo* injected, similar to the effect of bioactives administered in chickens feed (Falaki et al., 2011). Bozkurt et al. (2008) maintained that dietary antibiotic growth-promoter and mannano-oligosaccharides supplementation additives resulted in higher body weight of broiler chickens at the level of 2.2 and 5.1% ($P < 0.05$) respectively, at day 42. In a similar pattern, this advantage for additive programs in terms of growth rate was sustained through finisher period from 22-42 d. In addition, the results of this study also demonstrated that MOS, a non-antibiotic additive, was equivalent to AGPs (avilamycin) with respect to technical performance while giving hopeful signs replacing for AGPs. Zhou et al. (2010) evaluated, also, the effect of probiotic via the basal diet on growth performance of Guangxi Yellow chickens. It was clear that the dietary administration of probiotic (*Bacillus coagulans* ZJU0616) had beneficial effects on both

final body weight and daily weight gain of chickens. Other authors also showed that supplementation of the adherent *Lactobacillus* cultures to chickens, either as a single strain of *Lactobacillus acidophilus* or as a mixture of 12 *Lactobacillus* strains, increased significantly ($P < 0.05$) the BW of broilers after 40 d of feeding (Jin et al., 2000) and promoted the growth performance of birds (Noh, 1997; Zulkifli et al., 2000; Lan et al., 2003; Timmerman et al., 2006). On the contrary, Kumprechtova et al. (2000) investigated the effect of probiotic, *S. cerevisiae* Sc47, on chicken broiler performance and showed that the bacteria strains could not improve the live weight at 21 and 42 d of age. No positive effect on the growth performance of chickens was even found in the study conducted by Priyankarage et al. (2003) and by Midilli et al. (2008), who maintained dietary pre- and pro-biotics supplementation in broilers did not cause any significant effect on carcass weight and carcass yield. There are many reports that indicated the carcass weight increased by increasing protein amount of diet. Hosseini Mansoub (2010) maintained increase in the carcass weight could be probably because of increase in available protein and it was demonstrated that adding bacteria to diet directly enhance the protein availability (Nahanshon et al., 1993). Moreover, Falaki et al. (2011) reported that the use of mixture of commercial probiotic (combined preparation of live microorganisms including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus faecium* and *Bifidobacterium bifidum*) and commercial prebiotic of the mannan-oligosaccharides family (obtained by extraction from the outer cell wall of the yeast *Saccharomyces cerevisiae*) can improve the carcass quality by improving the uptake of nutrients (especially fatty acids and glucose), the increase in nitrogen stability and the reduction in excretion of fat in the feces and microbial urea (Willis et al., 2007). In addition, the prebiotic dietary administration reduces, also, the number of bacteria, toxins and their secondary products in the GI tract (Gunal et al., 2006).

However, it is difficult to directly assess different studies using prebiotic and probiotics because the efficacy of these additives application depended on many factors (Patterson and Burkholder, 2003). In general, these additives have proved most effective under conditions of stress, possibly the presence of unfavourable organisms, extremes in ambient temperature, diseases, crowding and poor management. In commercial broiler production one or more of these conditions are invariably present. Further, possible causes of variations in response to probiotic and/or prebiotic supplementation in broilers could be differences between strains, hybrids, age, sex, plane of nutrition, nutrient composition of the diet, microbial population of gastrointestinal tract, levels of inclusion of probiotics and prebiotics in the diet, duration of supplementation or other environmental conditions. Moreover, Patterson and Burkholder (2003) suggested that the efficacy of prebiotics and probiotics is more effective when the animal is producing well below its genetic potential.

Improvements in feed efficiency were attributed to an encouraged growth of the beneficial micro flora in the GIT induced by dietary supplementation of prebiotic and probiotic. Midilli et al. (2008) reported that in broilers of 6 weeks of age, the additives led to significant changes ($P <0.05$) in the feed conversion ratio (kg feed/kg gain) compared with the control. Improvements in feed conversion ratios of 3.19, 2.66 and 4.26%, respectively, were recorded in the probiotic, prebiotic and synbiotic treatments, compared to the control (1.82, 1.83, 1.80 *vs* 1.88, respectively, $P <0.05$). Findings reported by Zhou et al. (2010)

showed, also, that the use of the probiotic at a certain concentration (2.0×10^6cfu·g^{-1} and 5.0×10^6 cfu·g^{-1}) in the diet could significantly reduce FCR of Guangxi Yellow chickens and similar improvements in feed efficiency had been reported for poultry receiving probiotic *Lactobacillus* strains (Mohan et al., 1996; Lan et al., 2003). Sahane (2001) and Pelicia et al. (2004) suggested that these improvements might be due to a better ileal digestibility of nutrients. In fact, Endens et al. (2003) reported that probiotics improved digestion absorption and availability of nutrition accompanying with a positive effect on intestine activity and increasing digestive enzymes. Thus, in addition to an antimicrobial activity, a significantly increased intestinal amylase enzyme activity was observed by Jin et al. (1997) when adding *L. acidophulus* and a mixture of Lactobacilli to the diets. Yeo and Kim (1997) reported also that the improvement in feed efficiency of birds receiving probiotic supplemented diets could be due to decreased urease activity in the GI tract of the broiler chicks. In contrast to these findings, the work investigating the efficacy of a new multibacterial species probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in male Cobb broilers had shown inconsistent results instead (Mountzouris et al., 2007). In addition, O'Dea et al. (2006) reported no effect of any of the probiotic treatments used on FCR at any point during the production period or on the overall FCR over the entire production period as well as others authors have not found differences in FCR between probiotic-treated birds and untreated control birds (Watkins and Kratzer, 1983, 1984; Estrada et al., 2001; Huang et al., 2004). These varying results may be due to differences in the bacterial strains used in the above-mentioned studies and the origins of these strains. Because in most studies no information is provided as to whether the strain used was isolated from poultry, it is not possible to assess whether it is host-specific and would be able to attach to the GIT epithelial cells (Jin et al., 1998; Cox et al., 2001). In all of the mentioned studies, no information as to the origin of the bacterial strains used was provided by the manufacturers, so it was impossible to assess whether this may have played a role.

The abdominal fat percentage (Table 13.1), ranging from 1.2 to 2.4% (SEM = 0.20) was similar ($P > 0.05$) among the groups. Fontana et al. (1993) found similar value of the abdominal fat pad weight (2.39%) of carcass weight. Abdominal fat pad weight is an objective indicator of carcass fatness in broilers (Yalçin et al., 1999). Selection of meat-type chickens has previously focused not only on increased growth performance but also on improved carcass quality. In particular, the emphasis has been on better body composition, with higher breast meat yield and lower abdominal fat. This focus responds to the consumer desire for healthier meat, and to the evolution of the market through a rising demand for portioned and processed products (Barton, 1994). Karaoglu and Durdag (2005) maintained that the use of probiotics in broiler diets appeared to decrease subcutaneous and intermuscular fat accumulation, based on subjective visual assessment but it was unaffected by probiotic supplementation. Moreover, by observing a reduction in the fat level of birds fed by prebiotic, it is suggested that this product can interfere in the accessibility to fat for the formation of fat tissue in the birds (Falaki et al., 2011).

Deposits of fat in the abdominal area of broilers are considered as waste in the poultry industry. Not only does abdominal fat represent a loss in the market, but it also represents an added expense during the treatment of effluent produced when processing broilers.

Zhou et al. (2009) revealed that this type of waste could be reduced by reduction of the fat content in the abdominal area of broilers induced by chitooligosaccarides supplementation. Furthermore, the concentration of abdominal fat decreased (linear, $P < 0.05$) as the concentration of COS increased from 0.2% to 0.4% in the basal diet of chickens. The important inhibition characteristics of fat digestion by chitosan from observations of the ileal contents were due to the fact that it dissolved in the stomach and then changed to a gelled form, entrapping fat in the intestine. This lipids-lowering effect of chitosan was, even, documented in earlier studies (Kanauchi et al., 1995).

The pectoral muscle percentage was slightly influenced by the treatments (Table 13.1). In fact, compared with C and T2 broilers, those of T1, T3 and T4 groups had slightly higher ($P = 0.070$) pectoral muscle percentage. Similarly, Pilarski et al. (2005) found a slight but not significant influence of different doses of fructooligosaccharides or α-galactoside (RFOs) injection on the final body, carcass, breast muscle, and leg weight, and on abdominal fat ratio of Hubbard broilers. Conversely, studies conducted by several authors (Tako et al., 2004; Tako et al., 2005; Smirnov et al., 2006) have shown that the administration of 1 ml of *in ovo* feeding solution including dextrin (as a source of carbohydrates), increased hatching weights by 5% over controls and elevates relative breast muscle size of broilers (calculated as % of body weight) by 6%. *In ovo* feeding is expected to yield several advantages, among them reduced post-hatch mortality and morbidity, greater efficiency of feed-nutrient utilisation at an early age, reduced incidence of developmental skeletal disorders, and increased muscle development and breast meat yield (Noy and Uni, 2010).

Table 13.1. Effect of *in ovo* prebiotic and synbiotics administration on slaughter traits, FCR of broiler chickens

	GROUP[1]						
Item	**C**	**T1**	**T2**	**T3**	**T4**	**SEM**	**P value**
N	**12**	**12**	**12**	**12**	**12**		
Final BW (g)	2708.4	2837.1	2717.7	2799.3	2784.5	26.34	0.491
Carcass weight (g)	1993.0	2048.2	1977.1	2010.1	1965.9	19.79	0.726
Carcass yield (%)	73.6^{a}	72.2^{ab}	72.7^{ab}	71.8^{ab}	70.7^{b}	0.31	0.043
FCR[2]	1.54^{b}	1.55^{b}	1.53^{b}	1.64^{a}	1.67^{a}	0.01	0.001
Abdominal fat (%)	1.4	1.5	2.4	1.3	1.2	0.20	0.351
Pectoral muscle (%)	24.6	28.3	24.7	26.2	27.0	0.48	0.070

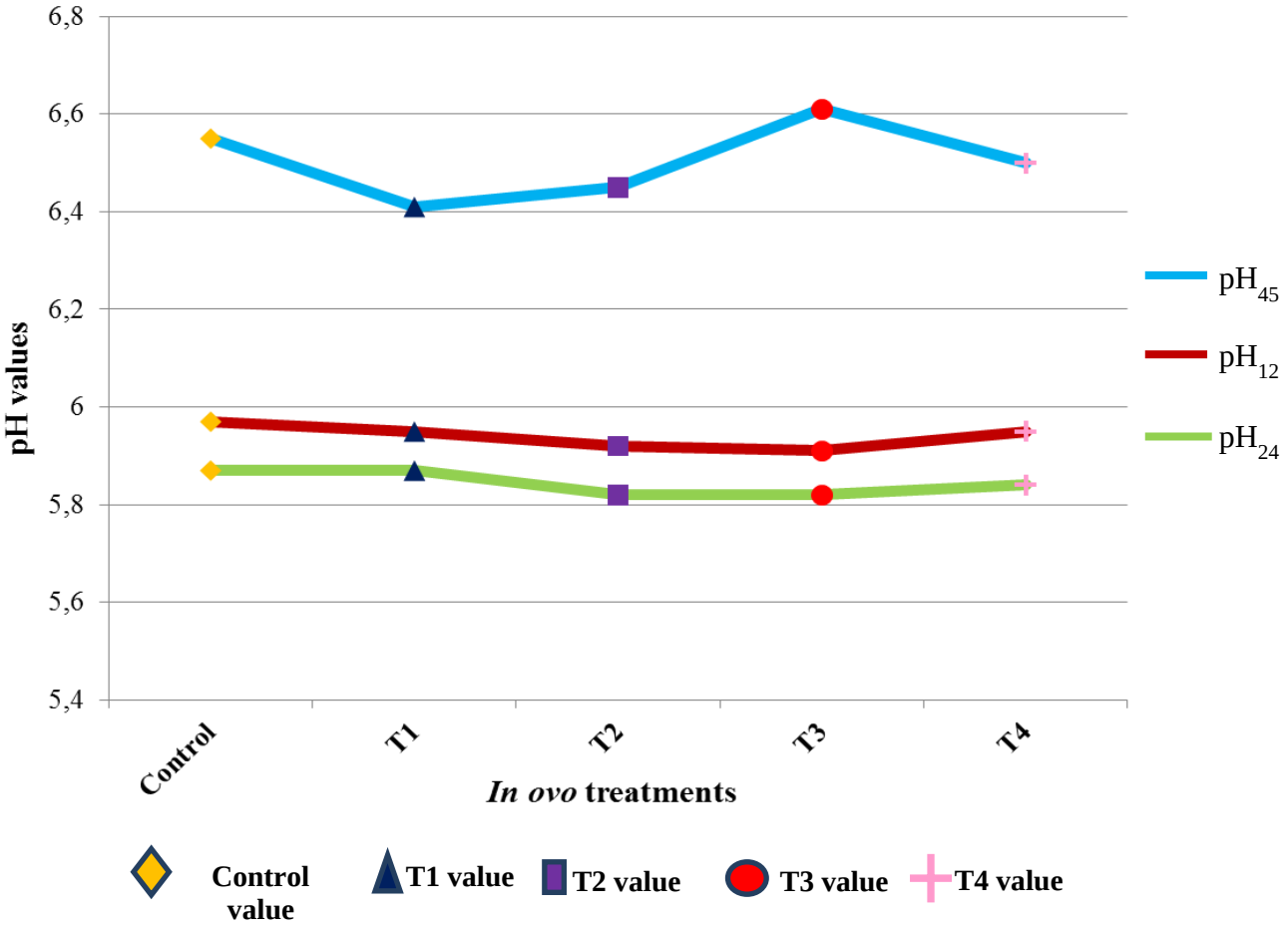
$^{a–c}$Means within a row lacking a common superscript differ ($P < 0.05$).

[1]C = control group; T1 = prebiotic group; T2 = synbiotic group (prebiotic + 1000 cfu of *Lactococcus lactis ssp. lactis* SL1); T3 = synbiotic group (prebiotic + 1000 cfu of *Lactococcus lactis ssp. cremoris* IBB SC1); T4 = commercial synbiotic group (*Lactococcus acidophilus* + *Streptococcus faecium*).

[2]FCR = feed conversion ratio (cumulative feed intake/weight gain); cage was used as the experimental unit.

The pH_{45} differed significantly (P = 0.003) among groups (Figure 13.1) while the parameters pH_{12} and pH_{24} were found to be similar (P > 0.05) among groups. Pelicano et al. (2005) registered similar breast meat pH values ranging from 5.74 to 5.82, independently by the prebiotic and synbiotic diet supplementation. The ultimate pH values observed in the present study (ranging from 5.82 to 5.87) varied within the pH range accepted for commercial meats. It is well known that the ultimate pH of the muscle is an important contributing factor to meat quality expressed as tenderness, colour, and storage life (van Laack et al., 2000).

Figure 13.1. Effect of *in ovo* prebiotic and symbiotic administration on pH decline of broiler chickens

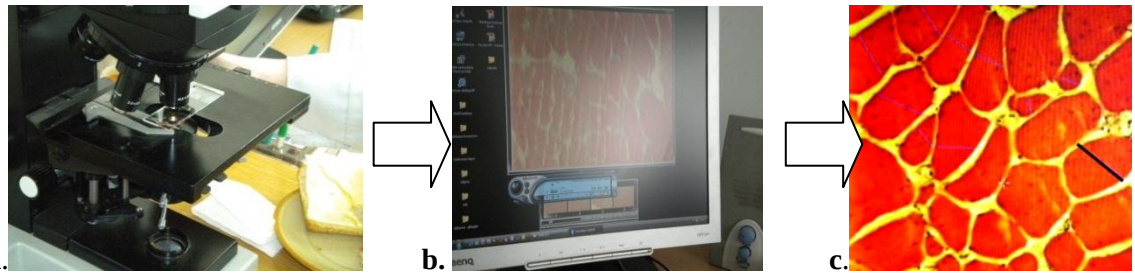


Chapter 14. Effect in ovo prebiotic and synbiotics administration on pectoral muscle qualitative traits and cholesterol content of broiler chickens

14.1 Fibre histological characteristics and intramuscular collagen properties

The effect of *in ovo* prebiotic and synbiotic administration on fibre histological characteristics and collagen properties in the pectoral muscle is presented in Table 10.2. The radial muscle fiber size was estimated by measuring the distance across the narrowest part of the fiber profile passing through the centroid (Figure 14.1), defined as the minimum fiber diameter (Dubowitz and Brooke, 1973; Cumming et al., 1994). A greater diameter of muscle fibres was found in the experimental groups (T1, T2, T3 and T4) of chickens compared with the control group (Table 14.1); however, the differences were not statistically significant, possibly due to significant variation of body weight within the experimental groups.

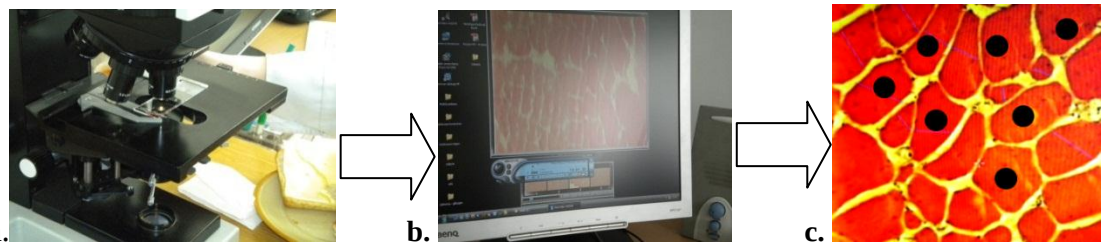
Figure 14.1. Measurement of radial muscle fibre



a. *Visualization of staining by microscope;* ***b.*** *Computerized analysis system MultiScanBase v.14.02;* ***c.*** *The black line indicates the minimum fiber diameter.*

In contrast, differences (P = 0.045) in the total number of fibres (Figure 14.2) in the 1 mm^2 of the cross-section of pectoral muscle were more evident between control and homemade synbiotic (T3) group (P < 0.05), while no significant differences were found among T1, T2 and T4 groups (Table 14.1).

Figure 14.2. Way of counting the number of fibres



a. *Visualization of staining by microscope;* ***b.*** *Computerized analysis system MultiScanBase v.14.02;* ***c.*** *The black point is used to count the number of fibres.*

The size and the number of muscle fibres are two factors that affect muscle growth potential and meat quality (Ryu and Kim, 2005; Rehfeldt and Kuhn, 2006). The TNF and the cross-sectional area of fibres are inversely correlated with each other (Lee et al., 2010), and they are positively correlated with muscle mass (Handel and Stickland, 1988; Dwyer et

al., 1993; Henckel et al., 1997; Larzul et al., 1997; Fiedler et al., 1999; Rehfeldt et al., 2000). In the present study, the control and the homemade synbiotic (T2) groups with the highest number of the muscle fibres (285.1 and 255.0 number/mm^2, respectively) showed the lowest pectoral muscle percentage (24.4 and 24.7%, respectively). On one hand, it can be assumed that the application of prebiotic or synbiotics has a positive impact on muscle weight. On the other hand, it probably leads to even greater congestion of already hypertrophic fibres. In fact, Macrae et al. (2007) have proposed that during the postnatal period, avian muscle growth occurs only by hypertrophy (increased radial growth of muscle fibres) and not by hyperplasia (increased muscle fibre number), with fibre number becoming fixed before or shortly after hatch. In studies that Lisowski et al. (2003) carried out on meat type chickens, a beneficial effect of the RFO on breast muscle weight and on carcass percentage was observed. The increased body weights of modern broilers are due to increased muscle yield, particularly of the *Pectoralis major* breast muscle, which in turn may be related to increased muscle fibre sizes. The genetic selection, in fact, has led to a gross overdevelopment of the broiler breast muscle *Pectoralis major* resulted in a larger muscle fiber number set and greater post-hatch growth potential (Rèmignon et al., 1995); this may have as a consequence an increased susceptibility to spontaneous myopathy caused by radial fiber growth outstripping the support systems and large fibers splitting because of metabolic stress (muscle damage; Mahon, 1999; Mitchell, 1999; Kranen et al., 2000; MacRae et al., 2006). Additionally, this may have induced detrimental effects on meat quality attributes (Santé et al., 1991; Le Bihan-Duval et al., 1999; Sandercock et al., 2001). Both commercial broilers and turkeys have larger diameter muscle fibres compared to their traditional or unselected counterparts at the same age (Mills, 2001). There may be a threshold fibre size, above which fibre metabolism is compromised due to the large diffusion distances for oxygen, metabolites and waste products (Mahon, 1999). The increased oxygen diffusion distances of large fibres may reduce oxidative capacity, alter mitochondrial distributions within the fibre and may result from a combination of larger fibre size and inadequate development of the supporting capillary supply. In fact, according to Elminowska-Wenda et al. (2004), accelerated growth of skeletal muscle in different species of birds is associated with reduced oxidative capacity. This is most often the case in the pectoral muscle of broiler chickens, and it confirms the high proportion of glycolytic fibres (white) that function through the anaerobic metabolism of glycogen.

The effect of prebiotic and synbiotics administration had a partial influence on IMC properties (Table 14.1). In general, the IMC concentration was notably reduced by the treatments ($P = 0.001$). In particular, the IMC concentration was higher ($P < 0.05$) in meat of control chickens than for treated groups and a lower value was found in the meat of the commercial synbiotic (T4) group. The IMC content found in the T4 group differed ($P < 0.05$) from that of chickens of the homemade synbiotic (T3) group. Muscle HLP concentration (μg/mg) and collagen maturation (mol of HLP/mol of collagen) did not differ ($P > 0.05$) among experimental groups.

Table 14.1. Effect *in ovo* prebiotic and synbiotics administration on pectoral muscle qualitative traits of broiler chickens

	GROUP[B]						
Item[A]	**C**	**T1**	**T2**	**T3**	**T4**	**SEM**	**P**
n.	***12***	***12***	***12***	***12***	***12***		**value**
Fiber diameter (μm)	45.9	50.5	51.2	51.6	50.8	0.85	0.200
Fiber density (num./mm^2)	285.1b	239.6ab	255.0ab	225.6a	249.2ab	6.91	0.045
IMC (μg/mg[C])	25.27a	19.39bc	20.49c	21.06c	18.87b	2.67	0.001
HLP (mol/mol of collagen)	0.065	0.079	0.076	0.075	0.085	0.003	0.400
HLP (μg/mg[C])	2.33	2.20	2.23	2.21	2.35	0.10	0.984
Cholesterol (mg/100g)	71.60	78.12	74.21	70.45	72.56	1.30	0.389

[a–c]Means within a row lacking a common superscript differ (P < 0.05).

[A]Fiber density and fiber diameter = 8 animals for each group; fiber diameter = 200 fibers; IMC = intramuscular collagen; HLP = hydroxylysylpyridinoline.

[B]C = control group; T1 = prebiotic group; T2 = synbiotic group (prebiotic + 1,000 cfu of *Lactococcus lactis ssp. lactis* SL1); T3 = synbiotic group (prebiotic + 1,000 cfu of *Lactococcus lactis ssp. cremoris* IBB SC1); T4 = commercial synbiotic group (*Lactococcus acidophilus* + *Streptococcus faecium*).

[C]of lyophilized muscular tissue.

Velleman et al. (1996) found a similar collagen content (25%) in the pectoral muscle of 6 weeks White Leghorn chickens but more crosslinked (almost 0.5 mol of HLP/mol of collagen). This marked difference in collagen maturity could be due mainly to the modern chicken strains and are probably a consequence of genetic selection for desirable meat characteristics such as lean breast meat in commercial broilers, which are usually slaughtered at very early slaughter age (Petracci and Cavani, 2012). In fast-growing birds, in fact, collagen looks like immature, resulting in low heat stability; consequently, poultry meat is tender, but may turn fragile, even mushy (Puolanne and Voutila, 2009). This poor cohesiveness of meat due to immaturity of intramuscular connective tissue in relation to the age represent a newer emerging quality issue in poultry (Petracci and Cavani, 2012). Modern poultry is, in fact, not tough, but the problem is increasingly the opposite. Voutila et al. (2009) indicated that currently there are two emerging types of defect in commercial poultry meat: (1) cooked chicken breast meat is generally fragmented (soft); and (2) raw broiler breast meat is so loose in structure (disintegrated) that it is possible to pull the muscle fiber bundles away with the fingers. The disintegration of cooked broiler meat has,

even, been reported by Swatland (1990). The mushy structure of cooked chicken breast meat can be perceived so that the need to chew before swallowing the piece of meat is minimal (Voutila, 2009).

The strength of IMCT is based on collagen fibrils and there are cross-bridges between the collagen molecule units and also between the collagen molecules. These cross-bridges determine the physical strength and heat stability of IMCT. The number and stability of cross-bridges increase with age determining a reduced tenderness. Additionally, the amount of collagen is related both to muscle growth and fibre diameter (Das et al., 2010) and in fast-growing chickens selected for meat production the growth of the intramuscular collagen in muscle does not keep pace with muscle fibre radial growth and the fibres outgrow the supporting connective tissue, leading to muscle damage (Swatland, 1990; Kranen et al., 2000). In agreement with Das et al. (2010), the broiler chickens of the present study with a slightly greater pectoral muscle, in which the fibre diameter was slightly higher, had a lower amount of collagen. McCormick (1999) suggested that mature crosslinks and collagen concentration have an additive effect on the toughening of meat. Maiorano et al. (2001) give a tenderness index, which is the amount in HLP crosslinks per gram of lyophilized muscular tissue in different muscles in goat meat. In agreement with the suggestions of McCormick (1999) and Maiorano et al. (2001), the results of HLP crosslinks concentration (μg/mg) of the present study indicate that meat produced from all birds could be similar in background toughness.

14.2 Cholesterol content

The effect of *in ovo* prebiotic and synbiotic administration on muscle cholesterol content is presented in Table 10.2. The breast muscle cholesterol content, ranging from 70.45 to 78.12 mg/100g (SEM = 1.30), was found to be similar among experimental groups ($P > 0.05$). These findings are in contrast with those obtained by Pilarski et al. (2005) in Hybro G broiler breeder eggs, who reported that the fructooligosaccharides (FOS) caused a decrease of breast muscle cholesterol in comparison with the control group. Moreover, the cholesterol values found in the present study are higher than those reported by Pilarski et al. (2005) in breast muscle of 42-d-old broiler chickens (ranging from 49.3 to 54.7 mg/100g). On the other hand, Salma et al. (2007) observed higher cholesterol values (93.6 mg/100g of meat) in *Pectoralis major* of 56-d-old male Chunky broilers than those obtained in the present study. The knowledge about cholesterol content in foods is important, especially in poultry meat, because consumption of this food is currently increasing based on the recommendations of healthy nutrition. There has been growing interest over recent years in the modulation of the cholesterol content and fatty acid composition in poultry products (Sacks, 2002). It is widely acknowledged that there is an urgent need to return to a balanced fatty acid diet by decreasing intake of cholesterol and saturated fatty acid (SFA) (Evans et al., 2002). In recent years, in fact, research has been focused to reduce fat, cholesterol, and SFA contents of poultry meat by dietary supplementation of garlic (Konjufca et al., 1997), copper (Pesti and Bakalli, 1996), α-tocopherol acetate (Ashgar et al., 1989), and n-3 fatty acid (Ayerza et al., 2002). Meat cholesterol concentration is usually associated with total fat content of the tissue, which is more abundant in thigh than in breast muscle. Several reports indicated that dietary

supplementation of bacteria such as *Lactobacillus* cultures (Jin et al., 1998) reduced serum cholesterol in broilers. Additionally, observations made by Salma et al. (2007) reveal that cholesterol concentration in thigh and breast muscle of the broilers has a positive correlation with the change of the cholesterol content in serum. In fact, these authors found that not only the serum cholesterol and triglyceride, but also the meat cholesterol and triglyceride concentrations were reduced by supplementation of probiotic bacteria to the broiler diet. For that, Salma et al. (2007) maintained the application of dietary bacteria into diet may be feasible to reduce cholesterol concentration and improve the ratio of UFA (unsaturated fatty acid) to SFA in broiler meat. The modulation of the fatty acid composition of poultry meat by dietary means is relatively easy, but the reduction of cholesterol concentration is more difficult (Skřivan et al., 2000). In fact, cholesterol content in chicken meat can be altered by varying the composition of diet, age, and gender (Wang et al., 2005), as well as the use of different methodologies for cholesterol quantification or for sampling (Bragagnolo and Rodriguez-Amaya, 2002).

Chapter 15. Effect *in ovo* prebiotic and synbiotics administration on histopathological changes in pectoral muscle of broiler chickens

15.1 Histopatological changes evaluation

The effect of *in ovo* prebiotic and synbiotic administration on histopathological changes in the pectoral muscle is shown in Table 15.1. No statistically significant differences (P > 0.05) among groups were found for any of the pathologies analysed. The most histopathological changes were observed in the homemade synbiotic T2 and the least in the commercial synbiotic T4 group (overall means: C = 4.12%, T1 = 6.37%, T2 = 6.80%, T3 = 5.22%, T4 = 3.59%; SEM = 0.44).

Table. 15.1. Effect *in ovo* prebiotic and synbiotics administration on histopathological changes in pectoral muscle of broiler chickens

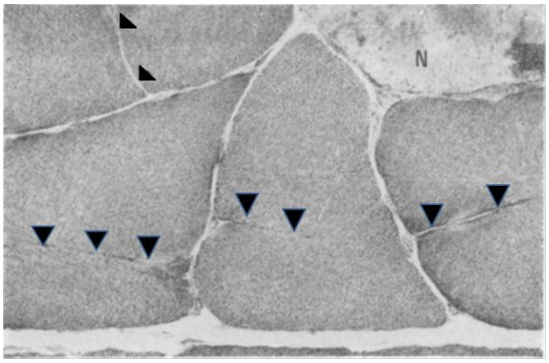
	GROUP[B]						
Item[A]	**C**	**T1**	**T2**	**T3**	**T4**	**SEM**	**P value**
n.	***12***	***12***	***12***	***12***	***12***		
Muscle fiber size							
- small diameter	2.71	3.91	4.80	3.82	2.43	0.31	0.133
- giant	0.08	0.42	0.39	0.13	0.23	0.05	0.156
Muscle fiber shape	0.65	0.30	0.14	0.13	0.10	0.08	0.116
Splitting of fibers	0.56	1.55	1.40	1.04	0.78	0.13	0.095
Necrosis of fibers	0.12	0.19	0.07	0.11	0.04	0.04	0.802
Total changes	4.12	6.37	6.80	5.22	3.59	0.44	0.076

[A]Small fiber diameter (reduced by more than 50 % compared to the average of normal fiber diameter); Muscle fibers shape = triangular, trapezoid, round and other.

[B]C = control group; T1 = prebiotic group; T2 = synbiotic group (prebiotic + 1000 CFU of *Lactococcus lactis subsp. lactis* SL1); T3 = synbiotic group (prebiotic + 1000 CFU of *Lactococcus lactis subsp. cremoris* IBB SC1); T4 = commercial synbiotic group (*Lactococcus acidophilus* + *Streptococcus faecium*).

Most of the incorrect size fibres were classified as small-diameters fibres .They ranged from 2.71 to 4.80% (SEM = 0.31) of the total number of fibres. Changes in the shape of fibres were less evident than the histopathological changes in all groups (ranging from 0.10 to 0.65%; SEM = 0.08). The splitting (Figure 15.1) of fibres was the second most common histopathological change. Splitting may consist only of a single central or lateral fissure; it may lead to the formation of two or more apparently separate fibres of identical histochemical type, each surrounded by a layer of basement membrane (Schwartz et al., 1976). Splitting affects type 1 fibres more commonly than type 2 fibres (Schwartz et al., 1976). In serial sections, these separate fibres may enlarge to attain a cross-sectional diameter similar to that of normal muscle fibres; others may fuse with each other, or insert into adjacent interstitial connective tissue (Swash and Schwartz, 1977).

Figure 15.1. Splitting of fibers



Splitting (arrows) affects several adjacent hypertrophied type I fibres. There is a necrotic fibre nearby (N).

In the present study, splitting occurred most frequently in the prebiotic (T1) group, and the least in the control group (overall means: C = 0.56%, T1 = 1.55%, T2 = 1.40%, T3 = 1.04%, T4 = 0.78%; SEM = 0.13). Split fibres may be an adaptive response to the metabolic stress associated with the increased diffusion distances for oxygen, metabolites and waste products in larger fibres (Macrae et al., 2006).

The necrotic lesions (Table 15.1) were observed only in individual cases and affecting individual muscle fibres. Their incidence ranged from 0.04% (T4 group) to 0.19% (T1 group; SEM = 0.04). No lymphocyte infiltration was found which leads to inflammation in the developing chicken pectoral muscle cells. The study of Elminowska-Wenda et al. (2004) indicated that histopathological changes are most extensive in birds with a fast growth rate and high meatiness. Hoving-Bolink et al. (2000) showed that many cases of myopathy in modern lines of chickens and turkeys with fast growth are caused by inadequate blood supply to the pectoral muscle. The increase in the diameter of muscle fibres, to which transportation of oxygen by capillaries is limited, may cause muscle cell hypoxia and necrosis (Mahon, 1999). Extensive histological studies in male commercial turkeys have shown that plasma activities of the intracellular enzyme marker creatine kinase and the incidence of structural muscle abnormalities increase with age (Mahon et al., 1995; Mills et al., 1998). Plasma creatine kinase activities have been shown to increase with age in chickens, with commercial broilers showing greater activity values than their genetic predecessors (Mitchell and Sandercock, 1994). The only histological study in the literature that has compared broiler and layer muscle pathology demonstrated a more frequent occurrence of structural muscle abnormalities in muscles of broilers compared to layers, but no alteration in incidence with age (Soike and Bergmann, 1998). At the onset of sexual maturity, a dramatic fall in plasma creatine kinase activity occurs in female broilers (Mitchell and Sandercock, 1996). However, it is not known whether this is accompanied by a reduction in the incidence of myopathology. Macrae et al. (2006) maintained the type of abnormal feature observed appeared to be related to both the type of muscle and the age of the bird. Features that were observed in most sections included “tiny” fibers (510 mm diameter), fiber size variation and fiber splitting. The abnormal features described include fibers that were necrotic (irreversible cell death induced by structural damage), basophilic (regenerative fibers) or hyaline (segmental hypercontraction stimulated by excessive

intracellular calcium), and fibers with NADH negative cores or NADH rich rims (indicative of either altered mitochondrial distribution or mitochondrial dysfunction).

In the light of the results obtained in the present study, the greater thickness of muscle fibres (not significant; Table 14.1) and the lower fibre density (statistically significant; Table 14.1), observed in treated birds in comparison with those of the control group, are not associated with histopathological changes in the pectoral muscle of broilers.

PART 5: CONCLUSION

In poultry farming, the intestinal microbiota and the "gut health" are topical subjects, especially since the use of antibiotics used usually as growth promoters (auxinic) has been banned by the EU (January, 2006), in order to avoid the onset of antibiotic-resistance and ensure the health of the consumer.
Nevertheless, these antibiotics exerted an important role in modulating the bacterial flora, checking subclinical infections and enteric diseases.
Because of their prohibition, increased incidence of enteric diseases is found in farms which provide a significant economic damage in the poultry industry: loss of productivity and increased mortality. In the post-antibiotics era, probiotics and prebiotics are proposed as a solution to the intestinal problems of poultry and to decrease the risk of food-borne infections in humans.

The aim of this research was to evaluate the effects of prebiotic used alone or in combination with strictly selected and characterized probiotic bacteria (synbiotics) *in ovo* administrated on growth, meat quality, and the presence of histopathological changes in the pectoral muscle of broiler chickens.

In ovo prebiotic and synbiotics administration (on the 12^{th} day of incubation) had little effect on most of the investigated traits, depending on the kind of bioactives administered. The combined application of probiotics and prebiotics (synbiotic applications) displayed a different effect than individual preparations but it does not simply result in additive or synergistic effects. The commercial synbiotic treatment, T4 (*Lactobacillus acidophilus* and *Streptococcus faecium*) reduced carcass yield percentage and, as observed even for T3 group (RFO enriched with of *Lactococcus lactis ssp. cremoris* IBB SC1), showed the higher FCR value in comparison with other groups.

The abdominal fat, the ultimate pH and the cholesterol content of the pectoral muscle were not affected by *in ovo* administration.

The effect of *in ovo* prebiotic and synbiotics injection had a partial influence on IMC properties. In general, the IMC concentration was notably reduced by the treatments and broiler chickens of treated groups, which showed both slightly large pectoral muscle and fiber diameter, had lower amount of collagen.

The *in ovo* prebiotic and synbiotics applications had a low effect on histopathological changes in the pectoral muscle, which did not affect the deterioration of meat quality obtained from these birds. The findings of the histopathological evaluation of the pectoral muscle were not associated with the fiber diameter and the fiber density.

In the light of the present results, it is believed *in ovo* prebiotic and synbiotics administration has a low impact under experimental conditions. The environmental and the stress status of the animals considered as the experimental setting are often too far from the farm conditions. However, probiotics, prebiotics and synbiotics for use in the poultry

industry could replace the antibiotic growth promoters as a non-antimicrobial enhancer additive.

Nevertheless, further research is needed to increase knowledge regarding the effect of *in ovo* prebiotic and synbiotics administration on growth and meat quality of broiler chickens in both experimental and field conditions. Additionally, future applications in field trials are necessary (i) to look for new combination in order to select the right probiotic, prebiotic or synbiotic, applying the knowledge of GIT health normal microbiota composition; (ii) to produce standard safe compositions at a high functional level, opening the opportunity of using, in technical scale, *in ovo* technology.

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List of Abbreviations

Abbreviations	Meaning
PS	*Pectoralis Superficialis*
RFOs	Raffinose Family Oligosaccharides
FCR	Feed Conversion Ratio
IMC	Intramuscular collagen
US	United States
EU	Europe Union
EFTA	European Fair Trade Association
CIS	Commonwealth of Independent State
BC	before Christ
AD	after death
GGP	great grandparent generation
GP	grandparent generation
VLDL	very low density lipoproteins
HDL	high density lipoproteins
GIT	gastrointestinal tract
DNA	deoxyribonucleic acid
G+C	guanine + cytosine
RNA	ribonucleic acid
IBD	intestinal bowel disease
AGPs	Antibiotics growth promoters
EFSA	European Food Safety Authority
NE	necrotic enteritis
GRAS status	Generally Regarded As Safe status
QPS	Qualified Presumption of Safety
Ig	immunoglobulin
LAB	Lactic acid bacteria
GALT	Gut-Associated Lymphoid Tissue
CE	Competitive exclusion
OS	oligosaccharides
NDOs	non-digestible oligosaccharides
FOS	fructo-oligosaccharides
GOS	galacto-oligosaccharides
TOS	transgalacto-oligosaccharides
MOS	mannano-oligosaccharides
SCFOS	short-chain fructooligosaccharide
SCFA	short chain fatty acids
IMO	isomaltooligosaccharide
IOF	*in ovo* feeding
HMB	β-hydroxy-β-methylbutyrate
ED	embryonic day
ATP	adenosine triphosphate
PSE	pale soft exudative
ECM	extracellular matrix
PGs	proteoglycans

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TNF	total number of fibers
CSAF	cross-sectional area of muscle fiber
SR	Sarcoplasmic Reticulum
MFN	muscle fiber number
DPM	Deep pectoral disease
IMCT	intramuscular connective tissue
WHC	water-holding capacity
IMF	Intramuscular fat
EPA	eicosapentaenoic
DHA	docosahexaenoic
BSE	Bovine Spongiform Encephalopathy
CLA	conjugated linoleic acid
PUFA	polyunsaturated fatty acids
HLKNL	hydroxylysinoketonorleucine
PYR	pyridinoline
PC	protein concentrate
HDFP	high dietary fiber product
LE	lupin extract
HPLC	high pressure liquid chromatography
TLC	thin layer chromatography
FH	hydrophobic
GM17	glucose medium 17
CF1	fibrous cellulose
cfu	colony-forming unit
HLP	Hydroxylysylpyridinoline
BW	body weight
COS	chitooligosaccarides
SFA	saturated fatty acid
UFA	unsaturated fatty acid
NADH	nicotinammide adenina dinucleotide dehydrogenase

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2. **D. Cianciullo**, A. Sławińska, G. Elminowska-Wenda, M. Bednarczyk, G. Maiorano. 2010. "*In ovo* prebiotic, probiotic and synbiotic administrations: effects on carcass traits and meat quality in Ross breed broilers". **International PhD Workshop on "Welfare, Biotechnology and Quality of Animal Production" Zielonka (Poland)**, 3rd-7th July, 2010;

2. G. Maiorano, S. Tavaniello, **D. Cianciullo**, M. Gambacorta. 2011."Effects of slaughter weight on carcass traits and meat quality of Casertana pigs". **XIX ASPA National Congress, Cremona (Italy)** 7th –10th June, 2011 (Poster);

3. G. Maiorano, **D. Cianciullo**, S. Tavaniello, L. Morbidini, A. Manchisi. 2011."Effect of sex on meat quality of Maremmano wild boar (Sus scrofa majori) x Duroc sow reared outdoors". **XIX ASPA National Congress, Cremona (Italy)** 7th –10th Giugno, 2011;

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