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Influence of different prebiotics and mode of their administration on growth, carcass traits and meat quality in broiler chickens

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…to my brothers: rocks of my life

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ABSTRACT

The use of in-feed growth promoting antibiotic use in poultry production is no longer a tool legally available to producers to enhance productivity and control diseases on farms (Regulation EC No. 1831/2003). Since 2006, when the European Union banned the use of antibiotic growth promoters in poultry nutrition, many alternatives have been investigated to replace antimicrobials without any loss of productivity or negative influence on health. One possible way to solve this problem is to control the microbiota of the gastrointestinal tract. Two kinds of feed additives are of interest. The first method is the direct introduction of live bacteria into the digestive tract, the second is the creation of conditions for the development of beneficial bacteria. In the post-antibiotics era, probiotics, prebiotics and their combination (synbiotic) are proposed as a solution to the intestinal problem of poultry. Prebiotics have been used to improve chicken performance via direct impact on the microflora of host animals and in this way effect a reinforcement of the intestinal mucosal barrier against deleterious agents. There are different ways to deliver prebiotics into avian gastrointestinal tract; but, to achieve desired efficacy, prebiotics must be administered to an animal as early in life as possible. Conventionally, in-feed or in-water supplementation has been used at fist hours/days post hatching. However, this approach relies on amount of feed and/or water intake, the quality of water (chlorinated), and other experimental factors. As a consequence, consumed dose of prebiotics varies in the first hours/days after hatching. Also, during early post hatching period, infection of chicks by detrimental bacteria is also possible. Therefore, "*in ovo*" approach for injection of prebiotics directly to the incubating egg has been developed. It allows for a precise delivery of the bioactive substances to all embryos at the early stage of development, which unifies the effects of prebiotics across the flock and assures proper development of gut microflora in all chicks.

This research, which involves two studies, has aimed to evaluate the effects of prebiotics, "*in ovo*" administered, on growth performance, meat quality traits (physiochemical characteristics, intramuscular collagen properties, fiber measurement, cholesterol content, lipid oxidation), in the pectoral muscle (PS) of Ross 308 broiler chickens.

The first study aimed to compare *in ovo* method of prebiotic delivery with in-water supplementation and with both methods combined (*in ovo* + in-water) in broiler chickens. Two trials were conducted. Trial 1 was carried out to optimize the doses of two prebiotics, DiNovo® (DN), extract of beta-glucans, and Bi^{2}tos (BI), trans-galactooligosaccharides, for *in ovo* delivery. The estimated parameters were hatchability and bacteriological status of the

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newly hatched chicks. Prebiotics were dissolved in 0.2 mL of physiological saline, at the doses: 0.18, 0.88, 3.5 and 7.0 mg/embryo; control group (C) was injected *in ovo* with 0.2 mL of physiological saline. The results of the Trial 1 indicated that the optimal dose of DN and BI prebiotics delivered *in ovo*, that did not reduce chicks' hatchability, was 0.88 mg/embryo (DN) and 3.5 mg/embryo (BI). Both prebiotics numerically increased number of lactobacilli and bifidobacteria in chicken feces ($P > 0.05$). Trial 2 was conducted to evaluate effects of different prebiotics (DN, BI and RFO - raffinose family oligosaccharides) delivered *in ovo* (T1), in-water (T2) and in a combined way (*in ovo* + in-water) (T3) on broiler chickens performance. The results of Trial 2 showed that chickens from all prebiotic groups were heavier. In particular, significant differences ($P < 0.01$) were recorded between RFO and C group. Carcass weight and carcass yield were unaffected by prebiotics. Breast weight and breast yield were higher in prebiotic groups; however, significance differences ($P < 0.05$) were only observed between DN and C group. However, feed intake and feed conversion ratio were increased upon prebiotics delivery in spite of method used. IMC properties were not significantly affected by different prebiotics. The different modality of administration affected carcass weight and carcass yield, in particular birds from T1 group showed higher values of carcass weight ($P < 0.01$) and carcass yield ($P < 0.05$) than in T3 group. Breast weight was similar among the experimental groups, differently breast yield was higher in birds that received prebiotics only in water (T3) than in C group ($P < 0.01$).

The bioactives treatment did not affect ($P > 0.05$) pH_{24} and color coordinates, except for redness (a*) values at 45 min, which was higher ($P < 0.05$) in C group in comparison with DN and RFO group, and L* values at 24 hours, which was higher ($P < 0.05$) in RFO group in comparison with BI group. Water holding capacity was higher in all prebiotic groups compared with control group however, the study revealed no significant differences ($P < 0.05$) among the groups of chickens.

The way of prebiotic administration influenced the chemical-physical parameters. Breast meat pH values measured 45 minutes after slaughter was higher ($P < 0.01$) in T3 group compared with other groups. Differently, after 24 h the meat from T3 group showed the lowest value (5.78) that differed ($P < 0.05$) when compared with T2 and C group values (5.86).

Color (a*) after 45 minutes was also significant affected by modality of prebiotic supplementation: significant differences ($P < 0.01$) were recorded between T2 and T3 group and between C and T1 group ($P < 0.05$). Significant differences ($P < 0.05$) were also observed for redness (a*) after 24 hours between T1 and control group. Water holding capacity was

influenced by prebiotic way of administration: significant differences (P < 0.05) were observed between in water group and T2 and control group.

IMC properties were not significantly affected by way of prebiotic administration. Compared to control group, the bioactives treatment slightly increased intramuscular fat content, but differences between groups were not significant (P > 0.05). The breast muscle cholesterol content, ranging from 47.20 to 49.44 mg/100g, was found to be similar (P > 0.05) among experimental groups.

The way of prebiotic administration affected the fiber diameter of PM, birds from control group showed a lower (P < 0.05) value of this trait than those from T3 group (in-water). T1 and T2 had intermediate values (P > 0.05). Nevertheless, must be considered that at higher fiber diameter in prebiotics groups had corresponded a heavier breast muscles in prebiotics groups (Table 9.4). Compared with the control group, prebiotics groups had higher content of intramuscular fat; however, the differences were not statistically significant (P > 0.05). The way of prebiotic administration didn't affect the cholesterol content in the meat.

In conclusion, the injection of prebiotics *in ovo* combined with in-water supplementation did not express synergistic effects on broilers performance compared to *in ovo* injection only. This study has established an elegant protocol for stimulation of the intestinal microflora populations in broiler chickens. It was achieved using a single *in ovo* prebiotics delivery duting embryonic development. Has been demonstrated dose oprimization method using hatchability and microbiological screening. In basis of the results obtained from this study, it is possible to propose that *in ovo* route of prebiotic delivery can replace prolonged and costly in-water supplementation of the broiler chickens with those bioactive compounds.

The purpose of the second study was to examine the effect of *in ovo* injection of 2 different prebiotics, DN and BI, on growth performance, slaughter traits and lipid oxidation of meat in chickens reared under commercial condition. On d 12 of embryonic incubation, 350,560 Ross 308 crossbreed eggs were randomly divided into 3 experimental groups automatically injected *in ovo* with: physiological saline (control group – C), BI at dose of 3.5mg/egg and DN at dose of 0.88 mg/egg. Hatched chicks (males and females) were housed into 3 poultry houses with a stocking density of 21.2 - 21.5 chicks/m^2. Broilers were fed *ad libitum* commercial diets according to age. At 42 d of age, 15 birds (7 males and 8 females), randomly chosen per each treatment, were individually weighed and slaughtered. The final number of chickens/chicken house, the mortality recorded at 1st week of life and at the end of the experiment, BW/chicken house, stocking density (kg/m^2), feed intake, feed conversion rate (FCR) and European Broiler Index were not different (P > 0.05) among the 3

experimental groups. However, treatment with BI and DN were associated with slight increases (P > 0.05) in average BW and a slight reduction (P > 0.05) in the FCR compared to control group. Slaughter traits results showed that chickens from treated groups had significantly higher BW, carcass weight, carcass yield and breast muscle weight than those of control ones. Males had significant higher slaughter traits compared to females, except for breast muscle yield. Meat from *in ovo* treated birds displayed a higher lipid oxidation levels compared to that from untreated ones along the entire storage time.

Overall, the results obtained at the end of this study have provided interesting information for an effective application of the bioactives to be used in the future in breeding industry, with significant and positive impact on animal welfare and public health.

RIASSUNTO

Dal 2006, quando l'Unione Europea ha vietato l'utilizzo di antibiotici promotori della crescita nell' alimentazione avicola (Reg. CE n. 1831/2003), si è manifestata una maggiore incidenza di malattie enteriche che hanno determinato perdite di produttività, nonché un incremento della mortalità. A tale scopo, sono state studiate diverse alternative per sostituire gli antimicrobici al fine di evitare perdite di produttività e salvaguardare la salute dei consumatori. Uno dei metodi per risolvere tale problema riguarda il controllo della microflora del tratto gastrointestinale microbista. Ciò è possibile mediante due modi: il primo metodo riguarda l'introduzione diretta di batteri vivi nel tratto digestivo; il secondo riguarda la creazione di condizioni ottimali per lo sviluppo di batteri benefici. Nell'era post-antibiotici, i probiotici, i prebiotici e la loro combinazione (simbiotici) sono proposti come soluzione ai problemi intestinali dei polli. I prebiotici sono stati utilizzati per migliorare le performance dei polli da carne con un impatto diretto sulla microflora dell'ospite ed un'azione tesa a rafforzare la mucosa intestinale contro gli agenti nocivi. Vi sono diversi modi per fornire i prebiotici ai polli ma, per raggiungere l'efficacia desiderata, essi devono essere somministrati all'animale al più presto. Convenzionalmente, la somministrazione avviene nei mangimi o in acqua, alcune ore/giorni dopo la schiusa; tuttavia, questo approccio si basa sulla quantità di mangime e/o di acqua, la qualità dell'acqua, ed altri fattori (condizioni sperimentali e di campo). Di conseguenza, la dose consumata di prebiotici varia nelle prime ore/giorni dopo la schiusa; inoltre, durante le prime fasi, subito dopo la schiusa, è possibile che i pulcini vengano infettati da batteri nocivi. Per i motivi finora elencati, è stato sviluppato l'approccio "*in ovo*", che consiste nell'iniettare i prebiotici direttamente nell'uovo durante il periodo di incubazione. Tale metodo consente di somministrare una quantità precisa della sostanza bioattiva a tutti gli embrioni nella fase iniziale di sviluppo, che unifica gli effetti dei prebiotici all'intera popolazione e assicura il corretto sviluppo della microflora intestinale in tutti i pulcini.

La presente ricerca ha avuto lo scopo di valutare gli effetti di prebiotici, somministrati "*in ovo*", sulle performance di crescita e la qualità della carne (proprietà del collagene intramuscolare, misure delle fibre muscolari, contenuto di colesterolo, ossidazione dei lipidi), di polli da carne Ross 308.

Il primo studio ha avuto come obiettivo quello di valutare gli effetti della modalità di somministrazione dei prebiotici: *in ovo*, in acqua, *in ovo* + in acqua sulle performance produttive nel pollo da carne. A tal proposito, sono stati condotti due studi: il primo ha avuto l'obiettivo di ottimizzare le dosi di due prebiotici commerciali DiNovo® (DN), estratto di

beta-glucani e Bi2tos (BI), trans galattooligosaccaride da iniettare *in ovo*. I parametri studiati hanno riguardato la percentuale di schiusa e lo stato batteriologico dei pulcini appena nati. I prebiotici sono stati disciolti in 0,2 ml di soluzione fisiologica, alle dosi: 0,18, 0,88, 3,5 e 7,0 mg/embrione; al gruppo di controllo (C) sono stati iniettati *in ovo* 0,2 mL di soluzione fisiologica. I risultati di questo primo studio hanno indicato che le dosi ottimali di DN e BI che non riducono la percentuale di schiusa dei pulcini sono di 0,88 mg/embrione per il DN e 3,5 mg/embrione per il BI. Entrambi i prebiotici hanno consentito un aumento del numero di lattobacilli e bifidobatteri nelle feci di pollo (P > 0,05).

Il secondo studio è stato condotto per valutare gli effetti di differenti prebiotici (DN, BI e RFO - oligosaccaridi della famiglia del raffinosio) somministrati *in ovo* (T1), in acqua (T2) e in modo combinato (*in ovo* + in acqua) (T3) sulle performance dei broiler e sulle caratteristiche qualitative della carne. Il peso della carcassa, la resa alla macellazione ed il peso e la resa del petto sono risultati più elevati nei gruppi trattati con prebiotici, tuttavia, differenze di significatività (P < 0,05) sono state osservate soltanto tra il DN e il gruppo C. L'assunzione di cibo e l'indice di conversione alimentare sono stati più elevati nei gruppi trattati con prebiotici. I risultati del secondo studio hanno evidenziato che i polli dei gruppi trattati con i prebiotici sono risultati più pesanti (P < 0,05). Differenze significative (P < 0,05) sono state registrate tra il gruppo RFO ed il gruppo C.

Il trattamento con i bioattivi non ha avuto effetti (P > 0,05) sul pH_{24} ed il colore, ad eccezione del valore dell'indice del rosso (a*), misurato a 45 min dalla macellazione, che è stato più elevato (P < 0,05) nel gruppo C rispetto ai gruppi DN e RFO ed il valore L* 24 ore, che è stato più elevato (P < 0,05) nel gruppo RFO rispetto al gruppo BI. La capacità di ritenzione idrica è stata più elevata in tutti i gruppi di prebiotici rispetto al gruppo di controllo; tuttavia, le differenze non sono risultate significative (P > 0,05). Il trattamento con i prebiotici ha ridotto lievemente il contenuto di grasso intramuscolare e la quantità di colesterolo nella carne (da 47,20 a 49,44 mg/100gr) è risultata simile tra i gruppi sperimentali mentre non ha influenzato le proprietà del collagene intramuscolare. La diversa modalità di somministrazione ha influenzato il peso e la resa in carcassa, in particolare i broiler del gruppo T1 hanno mostrato valori più elevati del peso della carcassa (P < 0,01) e della resa in carcassa (P < 0,05) rispetto al gruppo T3. Il peso del petto è stato simile tra i gruppi sperimentali, mentre la resa del petto è stata maggiore nei polli che hanno ricevuto i prebiotici solo in acqua (T3) rispetto al gruppo C (P < 0,01).

La modalità di amministrazione dei prebiotici ha influenzato marginalmente i parametri chimico-fisici. Il pH del muscolo pettorale, misurato a 45 minuti dopo la macellazione, è stato

più elevato (P < 0,01) per il gruppo T3 rispetto agli altri gruppi. Diversamente, dopo 24 ore dalla macellazione la carne dal gruppo T3 ha registrato il valore più basso (5,78) che differiva (P < 0,05) rispetto al gruppo T2 ed al gruppo C (5,86). L'indice del rosso (a*) dopo 45 minuti è stato significativamente influenzato dalla modalità di supplementazione: differenze significative sono state registrate tra il gruppo T2 > T3 (P < 0,01) e tra il gruppo C ed il gruppo T1 (P < 0,05). Differenze significative sono state osservate anche per il colore (a*) dopo 24 ore tra il gruppo T1 > C (P < 0,05).

La capacità di ritenzione idrica è stata influenzata dal metodo di somministrazione: differenze significative (P < 0,05) sono state osservate tra il gruppo T2 ed il gruppo di controllo. La modalità di somministrazione dei prebiotici ha influenzato il diametro delle fibre; infatti, le fibre del gruppo controllo sono risultate di diametro inferiore (P < 0,05) rispetto a quelle del gruppo T3 (in acqua). Il valore del diametro delle fibre dei gruppi T1 e T2 è risultato intermedio (P > 0,05). Comunque, va considerato che ad un diametro delle fibre più elevato ha corrisposto un peso superiore del muscolo pettorale, risultato più pesante nei gruppi trattati con prebiotico. Le caratteristiche del collagene intramuscolare non sono state influenzate dalla modalità del trattamento. Il petto del gruppo controllo, rispetto al petto dei polli dei gruppi prebiotici ha evidenziato un contenuto di grasso intramuscolare più elevato anche se le differenze non sono risultate significative (P > 0,05). La modalità di somministrazione dei prebiotici non ha influenzato il contenuto di colesterolo nella carne.

In conclusione, l'iniezione di prebiotici *in ovo* combinati con la somministrazione in acqua non ha espresso effetti sinergici sulle performance dei broiler rispetto alla sola iniezione *in ovo*.

Lo scopo della seconda ricerca è stato quello di esaminare l'effetto dell'iniezione *in ovo* di 2 diversi prebiotici, DN e BI, sulle performance di crescita, le caratteristiche della carcassa e l'ossidazione dei lipidi della carne di polli allevati in condizioni commerciali. Al 12° giorno di incubazione embrionale, 350.560 uova della linea Ross 308 sono state divise a *random* in 3 gruppi sperimentali iniettati automaticamente *in ovo* con: soluzione fisiologica (gruppo di controllo - C), BI alla dose di 3,5 mg/uovo e DN alla dose di 0,88 mg/uovo. I pulcini nati (maschi e femmine) sono stati alloggiati in 3 ricoveri con una densità di 21,2 - 21,5 pulcini/m^2. I polli sono stati alimentati *ad libitum* con diete commerciali formulate in funzione della loro età. A 42 giorni di età, 15 polli da carne (7 maschi e 8 femmine), scelti a caso per ogni trattamento, sono stati pesati e macellati. Il numero finale di polli/pollaio, la mortalità registrata alla 1° settimana di vita e alla fine dell'esperimento, peso vivo/pollaio, densità (kg/m^2), l'assunzione di cibo, l'indice di conversione alimentare (FCR) e l' "European broiler

index" non sono risultati diversi tra i 3 gruppi sperimentali (P > 0,05). Tuttavia, il trattamento con BI e DN è stato associato con un leggero aumento (P > 0,05) del peso vivo medio e una lieve riduzione (P > 0,05) del FCR rispetto al gruppo di controllo. Alla macellazione, i broiler di ciascun trattamento hanno mostrato valori significativamente più alti in termini di peso vivo, peso della carcassa, resa in carcassa e peso del muscolo pettorale rispetto a quelli di controllo. Come atteso, i maschi sono risultati significativamente più pesanti e con rese alla macellazione più elevate rispetto alle femmine. Le carni dei polli trattati *in ovo* con i prebiotici hanno mostrato una maggiore ossidazione dei lipidi rispetto a quelle del gruppo controllo durante tutto il tempo di conservazione (0-6 giorni a 4°C).

Nel complesso, i risultati ottenuti con questo studio hanno fornito interessanti informazioni per un'applicazione efficace delle sostanze bioattive da utilizzare in futuro nell'industria avicola, con un impatto significativo e positivo sul benessere degli animali e sulla salute pubblica.

PART 1: INTRODUCTION

Chapter 1

1. Poultry meat production and consumption trends

Meat from chickens is considered a viable source of proteins to humans because it is easy and fast to produce and has a smaller environmental footprint than other types of meat (de Vries and de Boer, 2010). In addition, poultry meat has been widely accepted mainly because it is inexpensive compared to the meat from other species (e.g., beef, pork, lamb) and its nutritional profile (e.g., low energy level, cholesterol content, and high polyunsaturated fatty acid content) is suitable for human health.

For centuries chickens (Gallus gallus domesticus) have been raised for the production of eggs and meat for human consumption. From indigenous, and less productive, double-purpose genotypes, modern breeding programmes have emphasized productivity: either layers for the production of eggs or broilers for the production of meat (Tixer-Boichard et al., 2012). As a result of intensive selection, the production of broilers has seen an extraordinary rise in the last 40 years, and in 2010 more than 86 million tonnes of chicken meat was produced worldwide (FAOstat, 2012).

Due to the growing demand on poultry meats there is more pressure on producers to improve the production efficiency of live birds (e.g., growth rate and feed conversion), and at the same time, provide consumers with good-quality meat. Today, turkeys and chickens are maketed in about half the time and at about twice of body weight when compared to the early 1950s (Barbut et al., 2008) and the cost of production has been reduced. A typical broiler chicken today is ready for slaughter at around 35-40 days of age and will weigh around 2 kg. Due to their rapid growth rates the chickens require around 3.5-4 kg of feed during this growth period. In order to meet the high consumer demand for poultry meat, birds are grown under intensive farming conditions which can make them more susceptible to disease and enable a more rapid spread of disease due to the intimate living conditions (Lambie et al., 2000). However, with improved biosecurity and ventilation combined with vaccine usage and

improved knowledge of disease prevention and management, intensive farming remains an extremely successful operation.

Up until recent years the addition of growth promoting antimicrobials to the feed of food-producing animals as a means to enhance feed efficiency was common practice worldwide (Gustafson and Bowen, 1997). The mode of action of antibiotic growth promoters (AGPs) is thought to be due to the effects on the bacterial communities residing in the intestinal tract of all animals. The AGPs cause an overall reduction in bacterial numbers within the gastro intestinal (GI) tract of the birds, resulting in more nutrients being available for absorption by the host. The reduction in bacterial numbers also results in lower production of bacterial metabolites (which can suppress growth), thus a growth promoting effect is seen (Dibner and Richards, 2005). The first recorded evidence of these effects dates back to the 1940s where Moore et al. (1946) discovered that streptomycin, when added to feed, enhanced the growth of chicks with no toxic side effects. This discovery was echoed a few years later by Stokstad and Jukes (1949) while investigating inexpensive sources of vitamin B12 as a supplement for poultry feed. They were using the by-products of chlortetracycline fermentation as a source of vitamin B12 and found that the birds fed on this diet exhibited an increase in growth rate in comparison to birds fed on alternative diets. This increase could not be attributed to the vitamin B12 alone and later it became clear that this was due to traces of the antibiotic in the feed ration supplementing growth. This phenomenon was investigated further by Libby and Schiable (1955) where over a four year period they demonstrated that continuous feeding of antibiotics to birds resulted in an increase in weight compared to control birds. Subsequent studies with cattle and pigs showed similar effects (Cunha et al., 1950a, b; Loosli and Wallace, 1950). During this period farmers developed confinement rearing which was the first step towards intensive farming. This style of farming enhanced the risk of certain diseases, but the inclusion of AGPs in the diet helped to control these diseases by reducing or preventing the growth of deleterious bacteria in the gastrointestinal tract of the birds. A wide variety of antimicrobial preparations soon became available and as they became cheaper the usage of them became more widespread (Gustafson and Bowen, 1997).

In recent years however, there has been growing public concern that the medical and veterinary professions have been overusing antimicrobials and that this has contributed to the emergence of drug resistant bacteria (Gustafson and Bowen, 1997; Barton, 2000; Johnston, 2001). Investigations involving chickens administered with AGPs have highlighted the presence of drug resistant strains of *Enterococcus faecium* and other potentially pathogenic enterococci; the dominance of these drug resistant strains was shown to diminish once the

antimicrobials were removed from the birds' diet (Butaye et al., 2000; Donabedian et al., 2003; Hayes et al., 2003). In a move to combat the ever increasing risk of drug resistant bacteria, growth promoting antibiotics have been withdrawn from farming practices across the EU. The ban was initiated in 1999 and came into full force in 2006 (EC Regulation No. 1831/2003). This move has however been met with some criticism from veterinarians and animal health experts due to the adverse impact on the health and welfare of animals and on industry economics. Recent reports, from the EU and the US, have shown that therapeutic antibiotic usage has been on the increase since the removal of AGPs. There is a general consensus within the industry that the shift in legislation regarding AGPs has resulted in an increase in enteric problems. In order to pursue novel management strategies and develop natural alternatives to AGPs, a better understanding of the mechanisms involved in the development of enteric disease is required. Alternatives to AGPs should either reduce nutrient availability to the intestinal microbiota, improve host immunity against unfavourable organisms or enhance the dominance of the beneficial species of bacteria of the intestinal microbiota (Bedford, 2000). However, there is a need to first understand the dynamics of the relationship between the host, the intestinal microbiota and the diet.

1.1 European situation

Today, poultry meat is the second most important meat in the EU, both in production and consumption. All market analyses point to a bright future for poultry sector especially in developing countries and poultry meat will soon supplant pork as the world's most eaten meat. This strength creates opportunities for European producers. But a study of the competitiveness of the European poultry meat industry commissioned by A.V.E.C. shows that this sector has also weaknesses and is facing different threats. In particular, European producers have to meet much more complex and diversified consumer expectations than most of our competitors. This is particularly the case in the Western EU, where consumers are both quality and price sensitive in their purchases of meat. The Consumer Market Study on the Functioning of the meat market for consumers in the European Union, as reported by DG SANCO (2013), shows clearly that consumers' purchasing decisions are determined firstly by the appearance (freshness and presentation) of the product and that the price should be reasonable and affordable; while, much less important were animal welfare and origin of the product.

The poultry industry has witnessed significant improvements over the past several decades. During the past 60 years, the poultry meat has recorded impressive world growth

rates, both as regards the production and the consumption. In 1958 the global poultry production of meat industry was around 15 million tons, in 2013 it was recorded almost 104 million (AVEC, 2013). The global poultry meat production consists of chicken for 88%, of turkey for 5.5 % and of ducks for 4 % (Table 1.1). The European Union is an important poultry producer in the world. On average the EU is contributing 12 % of the global poultry meat production, but in turkey meat the EU has a share of 35 % (Table 1.1).

Table 1.1 *Poultry meat production in EU and in the world ('000 tons carcass weight)*

	2008	2009	2010	2011	2012	2013
Poultry						
EU-27	11,659	11,952	12,387	12,683	12,880	13,200
World	92,800	95,150	99,400	102,550	105,750	107,500
Broiler						
EU-27	8,531	8,923	9,445	9,612	9,843	10,166
World	80,744	83,366	87,286	90,100	92,730	94,000
Turkey						
EU-27	1,902	1,827	1,931	1,896	1,984	1,987
World	5,656	5,424	5,493	5,511	5,664	
Duck						
EU-27	512	488	496	508	503	480
World	3,798	3,782	4,073	4,214	4,378	

Source: AVEC Annual report, 2014

United Kingdom, Poland, Germany, France, Spain and Italy are producing about 95% of the broiler meat in the EU, moreover, USA and China are the most productive Countries in the world (Table 1.2).

Table 1.2 - *Broiler meat production in various contries ('000 tons carcass weight)*

Country/period	2008	2009	2010	2011	2012	2013
United kingdom	1,214	1,220	1,323	1,297	1,322	1,391
Poland	730	1,060	1,123	1,150	1,325	1,450
Germany	868	911	1,073	1,150	1,160	1,190
France	1,009	1,008	1,041	1,096	1,091	1,146
Spain	1,059	1,063	1,085	1,073	1,063	1,041
Italy	713	742	780	796	816	820
EU-27	**8,531**	**8,923**	**9,445**	**9,612**	**9,843**	**10,166**
USA	16,561	15,935	16,563	16,694	16,621	16,958
China	11,840	12,100	12,550	13,200	13,700	13,500
Brazil	11,033	11,021	12,312	12,863	12,645	12,308
Mexico	2,853	2,781	2,822	2,906	2,958	3,002
India	2,490	2,550	2,650	2,900	3,160	3,420
Russia	1,680	2,060	2,310	2,575	2,830	3,050
World	**80,744**	**83,366**	**87,286**	**90,100**	**92,730**	**94,000**

Source: AVEC Annual report, 2014

Chicken is by far the most popular poultry species consumed (Table 1.3); in particular, in the EU as a whole and mainly as cuts. Broiler processing in China or Russia is totally different

from what we can observe in UK or Germany. Especially breast meat is preferred by many consumers in the EU and USA, while the brown meat and cuts with bone and other parts are liked in Asia and Brazil. The value of the product is determined by the demand. But also the convenience market segment shows an increasing demand of chicken meat.

Table 1.3 - Broiler meat consumption in some European Countries and in some Third Countries (kg/per capita)

Country/period	2008	2009	2010	2011	2012	2013
Austria	11,8	12,3	12,6	13,1	13,3	13,4
France	14,2	14,6	14,8	15,2	15,8	16,2
Germany	10,3	10,9	10,9	11,4	11,1	11,7
Italy	11,0	11,4	11,5	11,6	11,7	11,7
Netherland	18,4	19,0	18,8	18,4	18,4	18,5
United kingdom	20,7	20,6	22,2	21,7	22,0	22,5
EU-27	16,9	17,1	17,4	17,6	17,6	17,8
Third Countries						
Argentina	32,1	33,2	36,5	38,2	42,0	41,0
Brazil	40,6	40,3	46,3	47,8	46,0	45,9
China	8,7	8,8	9,0	9,3	9,5	9,6
Mexico	28,5	28,0	28,5	29,1	29,5	30,0
Russia	19,8	20,8	20,6	21,0	23,2	24,7
South Africa	28,4	28,4	29,6	32,5	33,5	33,3
USA	43,8	41,8	43,1	43,4	42,0	42,7

Source: AVEC Annual report, 2014

Chapter 2

2. Food safety concerns in the poultry sector

2.1 Introduction

Recently, consumers of animal protein products have taken an interest in the animal agriculture practices used to meet the demands of producing these products. Particular attention has been placed on the health issues of the animals and the intense nature of the production systems. Commercial poultry production has been under tremendous scrutiny due to its dependence on chemotherapy to improve weight gain and feed efficiency, while avoiding enteric health problems common to confined animal feeding operations.

The world is experiencing a growing population and rising incomes. This has led to increasing demand for food products, especially meat, milk and eggs. Together with innovations on the supply side, this has caused rapid growth of the livestock sector as a whole. The process has been referred to as a "livestock revolution" comparable to the "green revolution" of the 1960s.

The search for the most viable protein sources has resulted in particularly rapid growth of industrial poultry production. Poultry does not need pastureland, and the food conversion rate of genetically superior poultry breeds is very good compared to other livestock such as cattle. Technical advances in the feed industry have added to the progress. Pork production has followed a similar pattern to poultry. Intensification has brought food-safety concerns into sharper focus (Blancou et al., 2005), and these concerns have been increasingly acknowledged, at least in developed countries, as information technology and medical science have advanced (FAO, 2005).

Per capita demand for meat and fish products in developing countries has grown a rate of 3.7 percent over the last 20 years (FAO, 2003). At the same time, the new intensive production systems of the developing world are facing more and more pressure to comply with the regulations that prevail in the global market.

The various factors that influence production conditions (e.g. environment, infrastructure and culture) give rise to differing demands for food-safety standards in different

parts of the world. Food-borne diseases can also be related to demographic movements from rural areas to the cities, which cause overcrowding and, therefore, problems with hygiene, sanitation, housing conditions, etc., particularly in developing countries (Heath, 2006). Public health service systems are often unable to adapt to the rapid pace of urbanization. The urban lifestyle has also led to changes in consumption patterns, with more food products consumed outside the home, and to growing consumption of prepared foods (Stamoulis et al., 2004). Increased trade in food and feed across country borders, together with increased leisure and business travel, is contributing to the global character of the food-safety problematic (Table 2.1).

Table 2.1 - Some factors influencing the incidence of food-borne disease

Food supply system	Health and demographics	Social situation/lifestyle	Health system and Infrastructure	Environmental conditions
Mass production and distribution - larger outbreaks, etc	Population growth	Increased consumption outside	Decrease of resources and increase of food businesses	Pollution
Intensive agriculture - increased use of drugs and pesticides, etc.	Increase in vulnerable groups, e.g. the elderly, immunosuppression, malnourishment	Increased travel	Lack of water supply, sanitation and fuel for cooking	Changes in ecosystems - lack of water and resources
International trade	Increase in the number of displaced people	Changes in food preparation habits	Inadequate training of health workers	Climate change
More food service establishments - lack of training	Rapid urbanisation - lack of sanitation and water	Poverty and lack of education	Weak surveillance and monitoring systems	
Longer food chain		Lack of time	Lack of access to technologies	
		Changed social and cultural behaviour	Lack of consumer awareness	

Source: adapted from Motarjemi and Käferstein (1999).

Food safety can be defined as the system that keeps food and food products free from substances hazardous to human health. Food safety should be a part of governments' strategies to ensure secure food for the consumers. In this context, a "hazard" refers to any biological, chemical or physical property that may cause unacceptable risk (FAO, 1998). The emergence and discovery of new food-borne pathogens and other food-related hazards has increased the need for food-safety measures. The intensification of food production has also

changed food processing and handling systems and raised new challenges for food-safety institutions. Intensification has led to large amounts of potentially infectious material being concentrated at single sites, such as large industrial production establishments or processing plants, and has therefore contributed to the potential for large-scale outbreaks of infection.

2.2   Enteropathogen in poultry production

Poultry meat has been associated frequently and consistently with trasmission of enteric pathogens, including *Salmonella* and *Campylobacter* (Food and Agroculture Organisation of the United Nations and the World Health Organisation; FAO / WHO, 2002). More recently, other foods, such as fresh horticultural produce, have been recognized as significant vehicles of trasmission. However, human foodborne disease involving bacterial patogens, such as *Salmonella* and *Campylobacter*, is still often attributed to poultry (Batz et al., 2005). Callaway et al., (2008) stated that the 'link between human salmonellosis and host animals is most clear in poultry' and that raw eggs and undercooked poultry are considered by the entire community to be hazardous. Eggs have been implicated has vehicles in numerous outbreaks of salmonellosis; in particular, eggs are a major vehicle of transmission of strains of *Salmonella* serovar Enteritidis (Braden, 2006) although the incidence of disease associated with this particular mode of transmission has been decreased dramatically (Braden, 2006).

2.3   Enteric Disease

Disease can be defined as any deviation or interruption of the normal structure or function of any part, organ or system within the host (Hoerr, 1998). Enteric diseases are detrimental to poultry production due to the loss of productivity, increase in mortality, the potential for human health risks associated with food borne illness, and the increase in cost associated with disease prevention and treatment (Patterson and Burkholder, 2003). Pathogenic microorganisms have to elude many natural defenses of the host to cause disease. Low gastric pH, rapid transit through portions of the gastrointestinal tract, competitive intestinal microbiota, and immune defenses are in place to deny pathogenic microorganisms from colonizing the digestive tract and establishing disease. Factors such as stress, nutrition, and injury can leave the host more susceptible to disease. The severity and duration of stress factors, such as suboptimal temperatures, poor environmental conditions, and improper handling can influence the host's vulnerability to disease. Disease may also result from deficiency of vital nutrients or the ingestion of toxic substances from the feed. Nutritional deficiencies can lead to improper function of some natural defenses providing optimal

conditions for the establishment of disease; while some nutritional deficiencies may be reversible with supplementation of adequate nutrients, others are irreversible and leave the animal permanently disease prone. Injury, whether temporary or permanent, can lead to primary and secondary infections that result in disease (Bermudez and Stewart-Brown, 2008).

2.3.1 Disease prevention factors

Many management factors have an influence on disease control and prevention. Biosecurity practices are designed to prevent the spread of disease. Most poultry producers have adopted the practice of removing entire flocks from farms before any new replacements are added. This "all-in, all-out" concept decreases the spread of disease from one flock to the next. Furthermore, young poultry that is more diseasesensitive is reared in isolation from older poultry that is more disease-resistant. Poultry housing and environmental factors can influence the instance of disease. Modern poultry facilities have been constructed to minimize conditions conducive for harboring disease organisms. Advancements in ventilation have lead to a reduction of stressful conditions such as excess dust, high levels of ammonia, damp litter, and excessive draft over the birds. Advancements in poultry housing equipment has allowed for more favorable conditions. One example is the creation and adoption of the nipple drinking system.These drinking systems, when properly used, can decrease the amount of moisture being placed into the litter. Sanitation, mortality disposal, vaccination programs, and rodent control are other critical practices that must be addressed for proper biosecurity. While biosecurity practices are important in disease prevention, it cannot provide total protection against infection. To eliminate the threat of enteric disease and to promote the growth of the birds, producers have relied on the use of antibiotics at subtherapeutic levels.

2.4 Brief history of Antibiotic Growth Promoters (AGP)

During the 1940's, advancements made in poultry genetics, nutrition, housing, and marketing fueled the expansion of the poultry industry (Jones and Ricke, 2003). Poultry production became more of an intensive and confined production system in order to efficiently produce more birds in a shorter period of time. Along with increased production, came an increase in the occurrence of disease. During this time period, it was observed that animals fed dried mycelia of Streptomyces aureofaciens showed an increase in growth (Castanon, 2007). It was later discovered that the dried mycelia contained residues of chlorotetracycline, an effective broad spectrum antibiotic. Chlorotetracycline was the first of the tetracycline antibiotics. Moore et al. (1946) were the first to demonstrate the beneficial

effects of feeding antibiotics at subtherapeutic levels to improve performance in poultry. Streptothricin, streptomycin, sulfasuxidine or a combination of streptomycin and sulfasuxidine did not sterilize the intestinal tract of chickens, but showed a reduction of coliform bacteria in the ceca, while the combination of streptomycin and sulfasuxidine increased growth rates of the birds. In 1951, the United States Food and Drug Administration (FDA) approved the use of antibiotics as an animal feed additive without the prescription of a veterinarian (Jones and Ricke, 2003). The industry quickly adopted the use of antibiotic growth promoters and made it the industry standard for production.

2.4.1 Biological Aspects of AGP

Antibiotics fed at sub-therapeutic levels promote growth and feed efficiency in poultry and other animals (Dibner and Richards, 2005). The mechanism of action for AGP has been explained as an interaction between the antibiotics and the intestinal microbial population (Castanon, 2007). Four major mechanisms of action for AGP have been reviewed to explain their beneficial effects on performance (Gaskins et al., 2002; Dibner and Richards, 2005; Page, 2006). These mechanism consist of the following: 1) AGP inhibit endemic subclinical infection, thus reducing the metabolic cost of the innate immune system; 2) AGP reduce the growth-depressing metabolites produced by microbes, such as ammonia and bile degradation products; 3) AGP reduce microbial use of nutrients; and 4) AGP enhance the uptake and use of nutrients, due to the thinning of the intestinal wall in AGP-fed animals (Niewold, 2007). These mechanisms suggest that, either directly or indirectly, the intestinal microflora depresses the growth of the animal. The reduction of intestinal microbial population could be the underlying beneficial action of AGP.

2.4.2 AGP-associated problems and concerns

For over 70 years, the poultry industry has relied on the use of chemoprophylaxis with antibiotics, for the control and prevention of enteric diseases. Potential problems and concerns have resulted in global changes for the use of AGP, with the most drastic changes occurring in the European Union. One potential problem is the increased resistance of pathogenic bacteria to the approved antibiotics used for growth promotion (Bywater, 2005). An early sign of resistance to streptomycin in turkeys was reported shortly after the approval of AGP use by the FDA (Starr and Reynolds, 1951). Tetracycline resistance was associated with feeding growth-promoting levels to chickens in the late 1950's (Barnes, 1958; Elliott and Barnes, 1959). By the 1980's, resistance to numerous antimicrobial agents by pathogenic bacteria was

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reported worldwide (Aarestrup, 2003). The occurrence of AGP-associated resistant bacteria has lead to concerns of human health risks. The possibility of zoonotic bacteria with antibiotic resistance linked to animal use of AGP that could be contracted by human recipients has stimulated change (Bywater, 2005). The major driving force of these changes has been the pressure placed on the poultry industry by consumers of animal meat proteins that perceive AGP-associated human health risks to be serious (Singer and Hofacre, 2006). This consumer pressure has lead to major retailers and restaurant chains compelling poultry producers to voluntarily reduce the use of AGP.

2.4.3 Banning of AGP use in the European Union (EU)

In 1986, Sweden was the first country to ban the use of antimicrobials for growth-promoting purposes (Aarestrup, 2003). In 1995, Denmark banned the use of Avoparcin (a vancomycin-like compound) from use as an AGP in food animals due to reports of resistance in isolates from conventional and organic poultry farms. In 1997, the Commission of the EU banned Avoparcin in all EU member states (Dibner and Richards, 2005). After the banning of Avoparcin, the EU Commission launched an investigation into the use of all AGP approved for use in their member states. It was determined that the use of AGP could increase the instance of microbes with resistant genes and pose a potential for humans if they are transferred to persons. Therefore, the World Health Organization and the Economic and Social Commission of the EU concluded that the use of antimicrobials in food animals is a public health concern. From this conclusion, a plan was set forth to withdraw the use of all remaining approved AGP from all EU member states by January 1, 2006 (Castanon, 2007).

2.5   Campylobacteriosis

Human campylobacteriosis is a general term used to describe bacterial disease in humans caused by several members of the genus *Campylobacter spp.* The bacteria *Campylobacter* has been recognized as the main etiological agent causing human bacterial gastrointestinal disease (Friedman et al., 2000; Lindqvist et al., 2001; Adak et al., 2002; Lin, 2009; Hermans et al., 2012). Children and adults can be severely affected by *Campylobacter* and the socioeconomic costs can be very high (Samuel et al., 2004). The most common disease caused by *C.jejuni* in human is gastroenteritis (Altekruse et al., 1994). Human infections with *Campylobacter* pathogenic strains are characterized by nausea, vomiting, stomachache, malaise, profuse watery diarrhea, blood in feces and high fever (Blaser et al., 2008) and lethargy (Eberhart-Phillips et al., 1997). The incubation period is usually 4 days but

can vary from 2 to 10 days. Patients are advised to drink fluids and to follow antibiotic treatment when there is bacteremia or a serious underlying disease. In general, amoxicillin, tetracycline, erythromycin and fluoroquinolones are effective against campylobacteriosis if the pathogen is not resistant to these antibiotics (Moore et al., 2006; Wassenar et al., 2007).

The disease is usually self-limited but complications may occur. *C. jejuni* gastroenteritis is the commonest bacterial infection which precedes an acute, inflammatory, demyelinating polyneuritis characteristics by paralysis, pain and muscle wasting called Guillain-Barré syndrome (GBS) (Hadden and Gregson, 2001; Carter and Hudson, 2009; Shahrizaila and Yuki, 2011; Baker et al., 2012). The disease has a fatality rate of 3 to 8 percent (Smith, 2005). A variant of GBS, which is acute and self-limiting, called Miller-Fisher syndrome (MFS), causes paralysis of eye muscles (ophthalmoplegia), muscle inco-ordination (ataxia), loss of reflexes (areflexia), and facial weakness (Ropper, 1992).

Campylobacter is usually associated with sporadic human cases of disease, however, outbreaks could be more common than previously thought (Gillespie et al., 2003; Miller et al., 2004; Fussing et al., 2007). Outbreaks have been linked to contaminated chicken, water, milk and other food items (Allos, 2001; Frost et al., 2002; Black et al., 2006; Baker et al., 2007). The epidemiology of *Campylobacter* remains poorly understood partly due to its widespread prevalence in the environment. It is known that livestock, domestic and wild animals (birds in particular) constitute important reservoirs, in fact, they may carry *Campylobacter* without the development of clinical signs which leads to the hypothesis that *Campylobacter* may be part of their natural intestinal microbiota (Whyte et al., 2004; Young et al., 2007; Ogden et al., 2009; Garcia et al., 2010; Jokinen et al., 2011). There is increased evidence that, in many areas of the world, poultry, in particular broilers and chicken meat are the main contributors to human campylobacteriosis (Wingstrand et al., 2006; Wilson et al., 2008; Mullner et al., 2009; EFSA, 2010b; Friis et al., 2010; EFSA, 2011a; Hermans et al., 2012).

The poultry reservoir has been identified as one of the main sources for human campylobacteriosis, actually it may account for 50% to 80% of human cases. In particular, according to the expert panel in EFSA, preparation and consumption of chicken meat could be the source for 20-30% of human campylobacteriosis cases (EFSA, 2010b). Contaminated poultry meat has been implicated in human campylobacteriosis outbreak investigations (Pebody et al., 1997) and case-control studies (Studahl and Andersson, 2000; Kapperud et al., 2003; Neimann et al., 2003; Nielsen et al., 2006; Stafford et al., 2007; Doorduyn et al., 2010).

Efforts have been directed towards the control of *Campylobacter* in chickens as a strategy to reduce the risk of human campylobacteriosis. There is a general belief that

effective *Campylobacter* controls implemented throughout the food chain from poultry farms to the consumers will provide greater public health benefits than controls applied only later in the food chain because *Campylobacter* may infect humans via other pathways than chicken meat (EFSA, 2010a). However, despite a great number of research studies, it does not seem that an effective general strategy has been implemented in broiler farms to consistently produce *Campylobacter* free chickens (Hermans et al., 2011). The production of *Campylobacter*-free broiler flocks is possible but often expensive and difficult to achieve due to the fact that considerable investments in control strategies that are difficult to maintain might be necessary (Wassenaar, 2011; Hermans et al., 2012). Even when this aim is achieved, *Campylobacter*-free flocks might be contaminated at slaughter (Rivoal et al., 2005).

The identification of important risk factors for the introduction of *Campylobacter* in broiler flocks may assist on the implementation of efficient controls. Strict bio-security may result in a significant reduction of the probability of *Campylobacter* infection of poultry flocks. Some studies have found a clear correlation between the level of biosecurity and flock infection with *Campylobacter* (Cardinale et al., 2004; Johnsen et al., 2006). Nonetheless, strict biosecurity might be difficult to achieve and maintain throughout poultry production operations. In order to reduce the public health risk, controls against *Campylobacter* should be implemented during the farming period but also during the transport of poultry, at slaughter and during the production of poultry products and byproducts. Some control strategies will aim to prevent *Campylobacter* contamination of chickens and their products while other interventions such as vaccination will aim to reduce the numbers of *Campylobacter* in already contaminated animals, their products and by-products. Chickens might carry *Campylobacter* in numbers as high as 1010 Colony Forming Units (CFU) per gram of faeces (Stas et al., 1999; Sahin et al. 2002; Lütticken et al., 2007). Birds infected with *Campylobacter* will contaminate the food processing environment. The concentration of *Campylobacter* on chicken carcasses and *Campylobacter* numbers in caeca are positively correlated (Berrang et al., 2004; Reich et al., 2008). In alignment with this knowledge, it can be assumed that a reduction of the amount of *Campylobacter* in the intestinal tract of chickens will result in a decrease of the numbers of *Campylobacter* present in chicken meat. In addition, risk assessment models indicate that a 2 log reduction of *Campylobacter* in chicken carcasses may translate into a decrease of human campylobacteriosis cases by 30 times (Rosenquist et al., 2003; Reich et al., 2008).

Nowadays, consumers demand safer food putting pressure on governments and food industries all over the world to improve food safety and reduce the risk of food-borne

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illnesses. Risk analysis (risk assessment, management and risk communication) is used by governments and public health agencies worldwide as a structured, science-based, integrated tool to reduce the risk of foodborne illness (Taylor and Hoffman, 2001).

2.5.1 Campylobacter controls in poultry primary production

Campylobacter control strategies implemented during poultry primary production, defined as the on-farm rearing of poultry, are crucial for the control of this significant public health issue. The identification of important risk factors for the introduction of *Campylobacter* in broiler flocks will assist on the selection and implementation of efficient controls. It is interesting to notice that estimated *Campylobacter* prevalence in the environment around broiler houses from different farms seems to be quite similar independently of the biosecurity level (Hald et al., 2000; Hansson et al., 2007; Ridley et al., 2011a). Therefore, *Campylobacter* must be carried from the environment into chicken houses somehow and human traffic has been identified as an important vehicle for this transmission (Kapperud et al. 1993; Berndtson et al., 1996; Evans and Sayers, 2000; Cardinale et al., 2004; Hofshagen and Kruse, 2005). *Campylobacter* strains isolated from hands, boots and clothes of farm staff, catchers and farm managers have been associated with *Campylobacter* strains present in broiler flocks (Herman et al., 2003; Ramabu et al., 2004; Johnsen et al., 2006; Ridley et al., 2008, 2011a/b). The number of staff members and the number of human visits to the poultry houses have been found to increase the risk of introducing *Campylobacter* into poultry flocks (Refregier-Petton et al., 2001; Huneau-Salaun et al., 2007; Chowdhury et al., 2012).

Campylobacter can survive well in water (Blaser et al., 1980) and a close association between rainy weather and *Campylobacter* prevalence in puddles or standing water around chicken houses has been reported (Hansson et al., 2007). Additionally, *Campylobacter* strains isolated from soil and puddles around broiler houses in many cases can be identical to the strains isolated from the flocks supporting the hypothesis of *Campylobacter* transfer from the external environment into the broiler houses (Herman et al., 2003; Bull et al., 2006; Messens et al., 2009).

Campylobacter survives in poultry litter posing a risk for the infection of new flocks when poultry waste is stored on farm (Petersen et al., 2001; Rothrock et al., 2008). In fact, the risk of *Campylobacter* infection of flocks may increase significantly when the distance between the poultry house and used litter is less than 200 meters (Cardinale et al., 2004; Arsenault et al., 2007). Consequently, adequate removal and treatment of used litter from the

farm will potentially decrease the risk of *Campylobacter* infection in poultry. Removal of dead chickens from the house may also reduce the risk of a *Campylobacter* positive flock (Evans and Sayers, 2000).

The presence of other livestock on the same farm has been identified as a risk factor for the introduction of *Campylobacter* in poultry flocks in several studies (van de Giessen et al., 1996; Bouwknegt et al. 2004; Cardinale et al., 2004; Lyngstad et al., 2008; Ellis-Iversen et al., 2009). Recommendations have been made to minimize the presence of other livestock on poultry farms and/or to implement effective biosecurity barriers (Kapperud et al. 1993; Neubauer et al. 2005; Hald et al., 2007a,b). Biosecurity barriers should protect poultry by providing an effective physical separation between the "contaminated" environment outside the houses and the "protected" environment inside poultry houses. For example, an area at the entrance of a poultry house containing protective clothes, boots boot dips and hand washing facilities constitute a hygiene barrier. Nonetheless, the effectiveness of biosecurity barriers may vary between farms making the assessment of hygiene barriers as protective factors quite difficult (Neubauer et al., 2005). On the other hand, a significant reduction of the risk of *Campylobacter* infection of poultry flocks is possible to achieve by the effective use of biosecurity barriers specially when there are other animals on farm (van de Giessen et al., 1992; Berndtson et al., 1996; van de Giessen et al., 1998; Evans and Sayers, 2000; Hald et al., 2000).

The application of hygienic measures and general biosecurity barriers such as the use of separate boots between houses and footbath disinfection when entering broiler houses between many others may reduce the risk of *Campylobacter* infections in birds considerably (van de Giessen et al., 1996; Evans and Sayers, 2000).

2.6   Salmonellosis

Salmonellosis represents an important foodborne disease that continues to pose a major and unacceptable threat to human public health in both developed and developing countries (EFSA, 2010a). The dynamics of Salmonella infection is variable and may also be affected by human lifestyle and behavior, changes in industry, technology, commerce and travel. Salmonellosis is considered to be one of the major bacterial disease problems in the poultry industry world-wide, the second most often reported zoonotic disease in humans following campylobacteriosis. This illness is one of the most common and widely distributed foodborne diseases and is caused by the bacteria *Salmonella*. Currently, *Salmonella* spp. remains a

serious foodborne illness risk worldwide according to data of EFSA (2010a) and FAO/WHO (2002).

It is estimated that tens of millions of human cases occur worldwide every year and the disease results in more than hundred thousand deaths (WHO, 2013). In the European Union, over 100,000 human cases are reported each year. EFSA has estimated that the overall economic burden of human salmonellosis could be as high as EUR 3 billion a year (EFSA, 2011b).

For *Salmonella* species, over 2500 different strains (called "serotypes" or "serovars") have been identified to date. *Salmonella* is a ubiquitous and hardy bacteria that can survive several weeks in a dry environment and several months in water. *Salmonella enterica* subspecies *enterica* serovar Typhimurium is one serovar widely associated with human infections worldwide (Fashae et al., 2010; Hendriksen et al., 2011). In the EU, serotypes *Salmonella* Enteritidis and *Salmonella* Typhimurium are reported as the two major etiologic agents of salmonellosis that have adapted to humans (Ibrahim et al., 2013). While S. Enteritidis is mostly implicated in the consumption of poultry and eggs, S. Typhimurium is linked to a range of food-producing animals such as poultry, swine, cattle and sheep.
However, other serovars have been reported to be more prevalent in specific regions or within countries. *Salmonella spp.* in humans, are primarily a cause of self-limiting acute enteritis (diarrhea, abdominal pain, and fever, nausea, and sometimes vomiting with a typical duration of 4–7 days). In young children, the elderly and people with compromised immune systems the disease can be life threatening (WHO, 2005). However, invasive Salmonella spp. can spread beyond the gastrointestinal mucosa to infect other sites such as the blood stream, the meninges, bone or joint spaces (Crump et al., 2011). People generally acquire salmonellosis through food-borne exposure, although direct contact with infected animals is another possible route (Mead et al., 1999; L Plym and Wierup, 2006). A variety of investigations of outbreaks and sporadic cases have indicated that food vehicles identified as the most common source of S*almonella* infections in humans are poultry and poultry products, including raw and uncooked eggs (Hennessy et al., 2004). Due to its endemic nature, high morbidity and association with a wide range of foods, salmonellosis is of high public health concern (Aarestrup et al., 2007; Kottwitz et al., 2008).

2.7   Salmonella spp.

Salmonella is a gram negative bacterium with over 2500 identified serovars. *Salmonella* species are important food borne pathogens that cause gastroenteritis and enteric fever across

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a broad range of hosts. The genus *Salmonella* consists of 2 species, *Salmonella enterica* and *Salmonella bongori*. *S. enterica* represents a group of 6 subspecies with multiple serovars shown in Table 2.2. Typhimurium is one serovar within *Salmonella enterica*.

Table 2.2 - Salmonella subspecies and serovars (Grimont and Weill, 2007)

Species	Subspecies number	Subspecies name	Number of servovars
S. enterica			*2557*
	I	*enterica*	1531
	II	*salamae*	505
	IIIa	*arizonae*	99
	IIIb	*diarizonae*	336
	IV	*houtenae*	73
	VI	*indica*	13
S. bongori			22
Total number of serovars			**2579**

Salmonella enterica subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) is one serovar most widely associated with cases of human infection. Disease symptoms usually develop after between 12 and 72 hours after infection in humans. Infected individuals may develop a fever, abdominal cramps, and diarrhoea. Infection typically lasts between 4 and 7 days and the majority of people recover without the need for medical intervention. However, some individuals may require hospitalisation due to severe dehydration resulting from diarrhoea (WHO, 2012; CDC, 2012).

2.7.1 Livestock reservoirs and transmission of Salmonella species

Some *Salmonella* serovars are host restricted and may only infect one or two closely related species. For example, *S.* Choleraesuis, *S.* Dublin serotypes are rarely associated with species other than pigs and cattle, respectively. Data collected between 1958 and 1967 indicated that 99% of *S.* Choleraesuis cases detected were associated with pigs and 95% of *S.* Dublin cases detected were associated with cattle (Wallis and Barrow 2005). Host restricted strains like *S.* Choleraesuis and *S.* Dublin typically result in systemic disease. However, serovars *S.* Typhimurium and *S.* Enteritidis are not as host restricted and result in gastroenteritis in a broad range of unrelated hosts (Smith and Halls, 1968, Wallis and Barrow, 2005).

2.7.2 Salmonellosis in Poultry

In poultry, it has been speculated that more than 200 serovars of *Salmonella* have the ability to colonize the gastrointestinal (GI) tract (Gast, 2007; Foley et al., 2011). The outcomes of these infections can range from a subclinical infection that is not noticed by the producer to death (Park et al., 2013). None the less, because of the number of *Salmonella* serovars that are capable of colonizing the chicken gastrointestinal tract, poultry serve as an important vector for *Salmonella* in humans (Ricke 2003; Howard et al., 2012; Park et al., 2013). It is most commonly passed from poultry to humans through meat and egg products, posing a food safety risk for consumers and a challenge to keep *Salmonella* at reasonable levels for producers.

Salmonella enterica serovars Gallinarum and Pullorum cause fowl typhoid and pullorum disease respectively. These are systemic diseases of chickens and turkeys primarily but can also affect game birds. These serovars are host restricted and tend to only cause disease in birds. Following routine vaccination and strict control measures *S.* Gallinarum and *S.* Pullorum have largely been eradicated from the poultry industry of the Western world. However, these serovars still pose a threat to welfare and health of poultry in countries with less developed bio-security (Wallis and Barrow, 2005).

2.7.3 Control strategies against Salmonella

As a result of the profound health and economic impact salmonellosis has in the worldwide, it is imperative that precautions be taken to reduce the amount of *Salmonella* in poultry environments and eggs. One of the strategies being examined currently is the use of feed additives such as prebiotics, probiotics, essential oils, organic acids and others. Each of these feed additives has different properties that have been shown to be effective against *Salmonella*.

Prebiotics are nondigestible food ingredients for the host that selectively stimulates the growth or activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid, 1995; Gaggia et al., 2010). Probiotics are live microorganisms that when administered exhibit health benefits to the host, which include: regulation of bacterial homeostasis, stabilization of gastrointestinal barrier function (Salminen et al., 1996; Gaggia et al., 2010), expression of bacteriocins (Mazmanian et al., 2008; Gaggia et al., 2010), immunomodulatory effects (Salzman et al., 2003; Gaggia et al., 2010). The hypothesis for the use of prebiotics and probiotics in animal industry is similar, attempting to modulate the animal's gut microbiota of

the animal for better gut health and a reduction of pathogen invasion. The use of both essential oils and organic acids has gained recent attention due to the public pressure to stop antibiotics as growth promoters and also because of their antimicrobial properties (Van Immerseel et al., 2006). Essential oils are complex mixtures of plant metabolites consisting of low-boiling-phenylpropenes and terpenes (Brenes and Roura, 2010). Essential oils can be extracted from plant material such as: flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots (Brenes and Roura, 2010). Essential oils offer a potential natural method of reducing pathogens in the gastrointestinal tract of poultry. Organic acids are short chain or medium chain fatty acids that have a long history of being utilized in food production as an antimicrobial.

Organic acids can either be directly added to feed or are the result of fermentation of starter cultures. Both essential oils and organic acids have shown promising results in reducing *Salmonella in vitro* (Van Immerseel et al., 2003), however more studies need to be conducted *in vivo* to evaluate their efficacy.

Chapter 3

3. The avian gastrointestinal tract

3.1 Introduction

The gastrointestinal (GI) tract of animals and birds is a specialised tube running from the mouth to the anus, of which the primary purpose is the conversion and digestion of food into its basic components for absorption and utilisation by the host (Zoetendal et al., 2004). The early embryological development of the different regions of the GI tract is fundamentally similar developing cranially to caudally giving rise to a common gross structure of an inner mucous membrane lining and an outer muscular layers separated by connective tissue (Grand et al., 1976; Sell et al., 1991). However, once the adult alimentary tract has fully developed it is a highly organised and segregated structure (Figure 3.1) comprising of distinct regions namely the oesophagus, stomach, small intestine (duodenum, jejunum and ileum), and large intestine (caecum, colon and rectum). Each of these regions has a varying histological and anatomical structure designed for their individual functions in the digestive process (Chivers and Hladik, 1980; Van de Graaff, 1986).
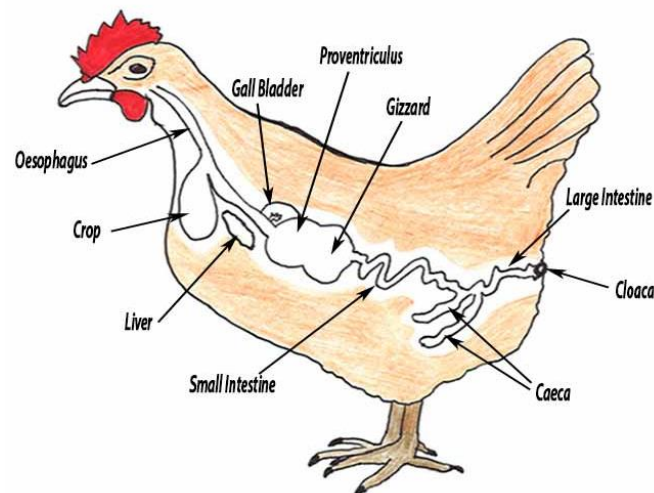

Figure 3.1 - The digestive system of the chickens

https://poultrykeeper.com/digestive-system-problems/digestive-system-chicken/
http://www.avianaquamiser.com/archives/2011/07/ - viewed November 30th 2015

The anatomy of the avian **GI tract** (Figure 3.1) demonstrates a variety of evolutionary adaptations to reduce body mass for flight (Duke, 1997), and these adaptations make the avian GI tract different to that of mammals. Firstly, birds have a lightweight beak which is used in the prehension of food and as they lack teeth they are unable to masticate and as a result food is swallowed whole. On swallowing, the food is stored in the crop before passing into the stomach (Grist, 2006). The crop is an extension of the oesophagus and the inner surface is covered with a thick layer of non-secretory stratified squamous epithelium (Fuller and Brooker, 1974; Mead, 1997). The food remains in the crop for up to 6 hours where it undergoes bacterial fermentation predominantly by members of the Lactobacillus genus; this marks the beginning of the digestion process (Barnes et al., 1980; Mead, 1997). From the crop the food passes into the proventriculus and then swiftly into the gizzard. These regions make up two independent regions forming the glandular and muscular parts of the avian stomach respectively (Grist, 2006). The mucosa of the proventriculus secretes acid resulting in a low pH environment in both the proventriculus and the gizzard (Smith and Macfarlane, 1996). The inner surface of the gizzard is lined by a very tough koilin layer and the outer surface is comprised of thick muscle which lends itself to the function of mechanically grinding the digesta (Barnes, 1979). It is not uncommon for wild birds to selectively include pieces of grit or stone in their diets to aid the grinding in the gizzard (Norris et al., 1975). On leaving the gizzard the digesta passes into the small intestine which is comprised of the duodenum, jejunum and the ileum. The regions of the small intestine are not as clearly defined as those of the foregut. The duodenum and jejunum meet at the caudal aspect of the duodenal loop and it is widely accepted that the region defined by Meckel's Diverticulum marks the junction of the ileum and jejunum (Grist, 2006). Meckel's Diverticulum is a vestigial remnant of the attachment of the yolk sac during development forming a distinctive and easily recognisable landmark along the small intestine (Besoluk et al., 2002).

Within the small intestine the digesta is mixed with bile salts from the gall bladder and enzymic secretions from the pancreas consisting of proteinases, amylases and lipases. In addition, the mucosa of the small intestine is secretory, producing mucus and digestive enzymes which when combined with its high surface area, due to the presence of villi and microvilli, makes the small intestine the major site of chemical digestion and nutrient absorption. The digesta then passes through the ileocaecal junction into the large intestine; at this point birds have two enlarged caeca which branch out forming two separate blind ended compartments (McLelland, 1989). The caeca are thought to be involved in the breakdown of otherwise indigestible plant material and the absorption of water, glucose and volatile fatty

acids. The caeca empty every 24-48 hours at which point they are refilled (McNab, 1973), originally it was thought that the caeca drew up contents from the ileum (Barnes, 1979); however, it has been discovered that caecal filling occurs by retrograde peristalsis where colonic contents are pushed backwards against a meshwork of villi at the opening of the caeca which results in the caecal contents being made up of the finer more particulate matter of the colonic contents (Clench and Mathias, 1995). From the ileocaecal junction the digesta enters the colon which is very short in comparison to mammals and hence there is very little absorption or digestion; from here the faecal pellet passes into the cloaca where it is mixed with uric acid (the avian product of protein breakdown) and expelled via the vent.

3.2 Intestinal Microbiota

Bacteria are one of the most diverse group of organisms on the planet existing ubiquitously in interdependent communities in any given environment (Whitman et al., 1998). These communities are found virtually everywhere in and around our environment, such as in soil, seas, rivers, and both in and on our bodies. When faced with the extremes of conditions (such as temperature, pressure and pH), evolution and adaptation have enabled bacteria to prevail and exploit the advantages of living in an environment too extreme for other organisms (Hugenholtz and Pace, 1996; Schlegel, 2004; von Mering et al., 2007). Consequently, higher organisms such as birds and mammals had to adapt to a world full of microbes and interact with many different bacteria on a daily basis resulting in symbiotic, competitive and pathogenic relationships (Hooper and Gordon, 2001). All of these interactions are exhibited in the GI tract (Zoetendal et al., 2004). Along the entire GI tract there is a diverse microbial community comprised of bacteria, yeasts, archaea, ciliate protozoa, anaerobic fungi and bacteriophages (Mackie, 2002), commonly referred to as the intestinal microbiota (Guan et al., 2003). The composition of the intestinal microbiota is highly dynamic and there are spatial shifts in population along each region of the GI tract in relation to the change in environmental conditions of each compartment (Hooper and Gordon, 2001). The intestinal microbiota consists predominately of bacteria, and it has been estimated that the bacterial cells outnumber the host cells by approximately ten to one (Guarner and Malagelada, 2003).

3.2.1 Intestinal microbiota of the chicken

Despite the presence of a monogastric digestive system the intestinal microbiota of a chicken differs to that of a human; these differences can be explained by differences in gut

physiology and dietary intake. Recent studies focusing on poultry have proposed that the GI tract of a broiler chicken is colonised by an estimated 640 species of bacteria from 140 different genera (Apajalahti et al., 2004). The abundance and diversity of the microbiota varies along the GI tract and predictably the regions which have less tolerable conditions and faster passage of contents have lower numbers of bacteria. It is generally considered that the digestive tract of a newly hatched chicken is sterile and that colonisation begins through contact with environmental microbes. However, the presence of bacteria in the caeca of unhatched embryos has been demonstrated (Binek et al., 2000). It has been suggested that the bacteria originated from the mother or that the bacteria penetrated the egg shell and then into the developing intestine of the embryo, as microbes have been shown to pass through the egg shell shortly after laying while the shell is still moist (Deeming, 1996; Berrang et al., 1999;
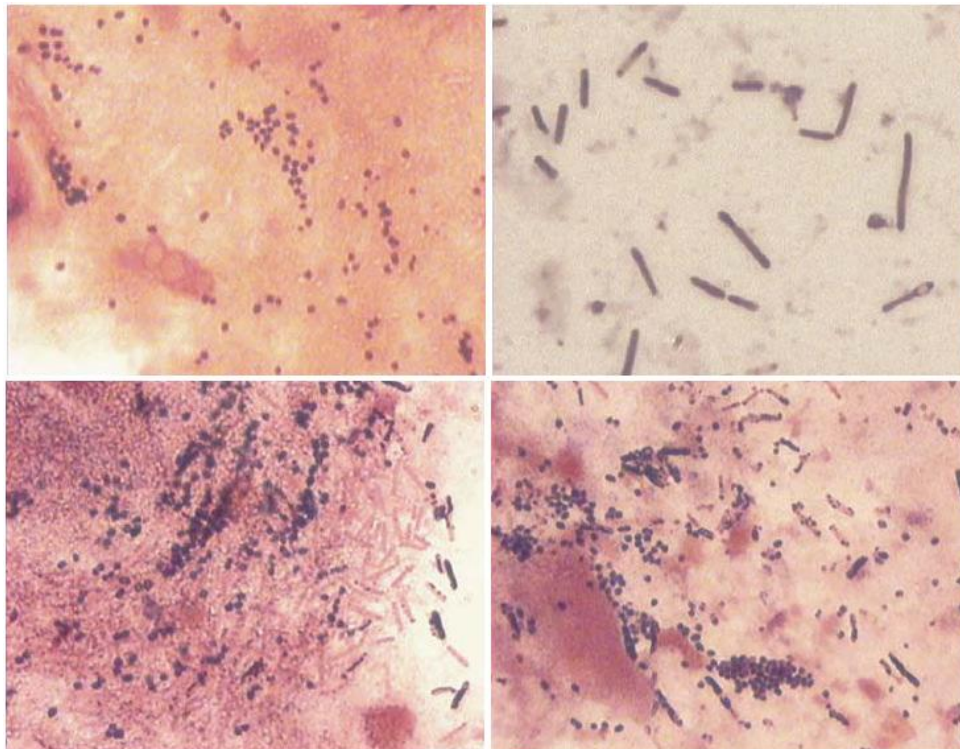
Figure 3.2 - Microscope slide images taken from Kizerwetter-Świda and Binek (2008) showing presence of bacteria in direct smears prepared from caecal contents of 20 day old chicken embryo (top two images) and newly hatched chicken (bottom two images). Gram stain at 1000x magnification.

Labaque et al., 2003). A recent study (Kizerwetter-Świda and Binek, 2008) demonstrated the presence of bacteria in the caeca, liver and yolk sac of 18 and 20 day old embryos. Figure 3.2 shows microscope images from the study indicating the presence of bacterial cells in the caeca of a 20 day embryo and a newly hatched chick.

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Despite these findings it is generally considered that the development of the adult intestinal microbiota begins on hatching where bacteria are picked up from the environment, the feed and people handling them post-hatch (Vahjen et al., 1998; van der Wielen et al., 2002). After one day post-hatch the ileum and caeca are both dominated by bacteria with densities around 108 and 1010 cells g^{-1} digesta respectively, and after three days these levels increase to 109 and 1011 cells g^{-1} of digesta respectively (Apajalahti et al., 2004). Within two weeks the typical adult small intestinal microbiota will be well established and after 30 days the caecal flora will have also developed (Amit-Romach et al., 2004).

During the early life of the bird the caeca are dominated by lactobacilli, coliforms and enterococci (Mead and Adams, 1975). However, due to the low rate of peristaltic flow of the caeca a more stable and complex microbiota develops over a period of time. By four weeks of age the adult caecal flora will have established and the species found include members of the bacteroides, Eubacteria, Bifidobacteria, lactobacilli and clostridia (Barnes et al., 1972; Salanitro et al., 1978).

3.2.2 Role of the microbiota in health and disease

Within the GI tract there are multiple interactions between the host cells, the intestinal environment, bacterial cells and digesta (Zoetendal et al., 2004; Bjerrum et al., 2006). These interactions emphasise the extremely important role of intestinal microbiota in the health and well being of the host; the manner in which this is achieved is multifactorial and not yet fully understood. The intestinal microbiota dominate the mucosa along the GI tract, forming a protective barrier competing against pathogenic bacteria for adhesion (Lloyd et al., 1977; Hooper, 2004). This principle has a variety of names but is most commonly known as competitive exclusion and has been exploited successfully in human nutrition by including commensal bacteria as probiotic agents in foodstuffs (Nomoto, 2005). Similar commensal agents have been used as competitive exclusion agents for serious enteric pathogens such as Salmonella spp., Campylobacter jejuni and other food borne pathogens in poultry (Mead, 2000; La Ragione et al., 2004; Schneitz, 2005). The means by which the intestinal microbiota inhibits the colonisation of pathogenic bacteria is not fully understood but one theory suggests that the microbiota dominate the attachment sites on the mucosal epithelium reducing the opportunity for attachment and colonisation by pathogens (Bernet et al., 1993; Bernet et al., 1994). Another proposed mechanism is that the intestinal microbiota are able to secrete compounds, including volatile fatty acids and bacteriocins, that either inhibit growth or make

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the local environment unsuitable for other potential colonisation suitors (van der Wielen et al., 2002; Servin, 2004).

Studies using germ-free and gnotobiotic animals have shown that the intestinal microbiota are important in the stimulation and development of the immune system. Not only are the commensal microbiota thought to maintain the gut enteric system in a state of "alert" by inducing a base level of inflammation but they are also considered to be an important factor in the development of the humoral and cellular immune systems during early life (Cebra, 1999). Studies have shown that animals lacking an intestinal microbiota are more susceptible to disease and have poorly developed secondary lymphoid tissues, slower lymphoid cell development and general deficiencies in immunological mechanisms when compared to their wild type counterparts (Umesaki et al., 1993; Hooper, 2004). In addition, to protection against disease and stimulation of the immune system the intestinal microbiota can influence host growth rates by producing extra nutrients such as short chain fatty acids (SCFA) and vitamins through the fermentation of non-digestible components of the digesta and the endogenous mucus produced by goblet cells (Hooper and Gordon, 2001; Guarner and Malagelada, 2003; Backhed et al., 2005; Simpson et al., 2005).

The gastrointestinal microbiota is a highly metabolic entity, as powerful as any organ in the animal body (Apajalahti and Bedford, 2000), and it has the potential to elicit deleterious effects on the growth rates and performance of the host animal. Firstly there is competition between the host cells and the microbiota for nutrients such as glucose and amino acids resulting in reduced availability of nutrients from the feed (Coates et al., 1963; Engberg et al., 2000).

Additionally, bacteria such as lactobacilli deconjugate bile acids leading to poor fat emulsification and thus reduced fat absorption which reduces the nutrients available for the host (Furuse and Yokota, 1985; Engberg et al., 2000). The products of bacterial metabolism can also have a negative effect on growth due to the production of ammonia and toxic amines from the fermentation of peptides (Ewing and Cole, 1994; Gaskins, 2001). The intestinal microbiota also causes a thickening of the intestinal wall and as a result the rate of nutrient absorption is reduced (Butcher and Miles, 1995). AGPs reduce the population of bacteria within the intestinal lumen, thus reducing these negative effects leading to less competition for nutrients (Gustafon and Bowen, 1997), reduced thickening of the intestinal wall (Miles and Harms, 1984) and it is believed that some of the metabolic pathways which produce toxic amines are inhibited (Eyssen and Cole, 1994), thus giving the observed growth promotion due to more energy being available to the host for growth (Bedford, 2000).

3.2.3 Factors affecting the gastrointestinal tract microbiota

There is a delicate balance between the host, the intestinal microbiota, the intestinal environment and dietary compounds. If there is an imbalance in this relationship then disease can occur (Apajalahti and Bedford, 2000). Microbial populations can be positively or negatively influenced based on the environmental conditions, the age and health state and the dietary intake of the host (Lu et al., 2003; Apajalahti et al., 2004; Gabriel et al., 2006;). Intensive farming practices included subtherapeutic doses of antibiotics in the diets of animals for two reasons, they promote growth and they can prevent the onset of endemic diseases (Gustafon and Bowen, 1997). However, the emergence of drug resistant bacteria along with pressure from consumers, supermarkets and government bodies to reduce the drugs used in food-producing animals has created a need for "natural" alternatives to boost performance and prevent disease spread (Barton, 2000; Emborg et al., 2003). In the hunt to find alternatives to AGPs there have been many attempts to manipulate the intestinal microbiota with varying degrees of success. Prebiotics and probiotics are popular methods for manipulating the intestinal microbiota. They are defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" (Gibson and Roberfroid, 1995) and "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Fuller, 1989), respectively. Many studies have shown the benefits to the health of broilers administered with either probiotics, prebiotics or a mixture of both (synbiotics) (Higgins et al., 2008; Awad et al., 2009a, b; Chen et al., 2009; Li et al., 2009). Despite probiotic supplements showing potential in laboratory trials, success varies in the "real world" of a commercial broiler unit. This is likely due to the complexity of the relationship between the host and the intestinal microbiota and the wide array of factors which can influence the composition of the intestinal microbiota and these must be appreciated when trying to manipulate the intestinal microbiota.

Intestinal bacteria derive most of their energy from dietary compounds and thus diet has a major influence over the bacterial populations (Craven, 2000; Apajalahti et al., 2004). As individual bacterial species have different nutritional demands and preferred substrates it is possible to influence the gut microbiota through dietary change (Mead, 1989; Macfarlane et al., 1998). Various studies have looked at the effects of dietary components on gut bacterial populations. Using method based on the guanine and cytosine (% G+C) content of the component population, Apajalahti et al. (2001) demonstrated the effects of different diets on

the intestinal microbiota of birds. The results indicated that the addition of wheat to the diet stimulated the growth of bacteria with 50%-55% G+C content and suppressed those with a G+C content of 60%-69%, indicating a shift in microbial populations. Populations of lactobacilli and coliforms were shown to increase in response to diets containing wheat and barley in contrast to maize based diets (Mathlouthi et al., 2002). A study by Knarreborg et al. 2002 showed that dietary fat source influenced the ileal microbiota with the lactobacilli population and *Clostridium perfringens* being most strongly affected by the changes. Furthermore, changing the dietary fat source from soy oil to tallow resulted in an increase in the total number of anaerobes in the intestinal microbiota and this result correlated with an increase in gut transit time (Danicke et al., 1999). This allows the microbiota of chickens to be modulated by altering the diet and including specific components (essential oils, oligosaccharides, enzymes and specific carbohydrate sources), designed to boost growth and make the conditions within the intestinal tract more favourable for specific commensal bacterial groups (Wu et al., 2004; Solis de los Santos et al., 2005; Oviedo-Rondon et al., 2006).

Another major factor affecting the gastrointestinal microbiota is the environment in which the birds are kept and the management associated with them. Poor hygiene predictably results in an increase in enteric disease and associated problems with ensuing wet litter (Butcher and Miles, 1995; Stamp Dawkins et al., 2004; Hermans et al., 2006). The litter on which the birds are placed is undoubtedly a source of bacteria for the birds, and thus is a potential source of pathogenic bacteria; therefore, it is essential that good litter management is followed on farms (Fries et al., 2005).

The health status of the birds plays a vital role in the modulation of intestnal microbiota. The immune system of the host and integrity of the intestinal wall (Guarner and Malagelada, 2003) prevent the entrance of bacteria into the sterile tissues around the intestinal tract. In the event of these mechanisms failing or being put under pressure from a disease challenge, opportunistic pathogens are able to invade tissues and cause infection (Apajalahti and Bedford, 2000). The immunocompetence of an animal can be diminished if the animal is exposed to stress factors such as heat, handling and transportation, overcrowding or placement in new housing. One response to stress is an increase in the release of compounds such as cortisol, adrenaline and noradrenaline (Mayer, 2000). These hormones have a dampening effect on the immune system which can result in an opportunity for infection to occur (Siegel and Gross, 2000). It has been found that the growth of certain pathogenic

enteric bacteria, such as Escherichia coli O157:H7, Salmonella enterica and Yersinia enterocoliticia, is stimulated by noradrenaline (Freestone et al., 2007).

Suzuki et al. (1983) demonstrated that overcrowding and heat stress, very commonly seen in intensive poultry farming, have a significant impact of the microbiota of chickens. Host genotype appears to play a vital role in the maintenance of a healthy intestinal environment. This phenomenon is still not fully understood but it is thought to be related to host mediated factors influencing the enteric environment and the inhabitants therein. An extensive study in poultry, by van der Wielen et al. (2002), demonstrated that individual birds had a unique microbial composition. These findings have been mirrored in studies performed in humans (Zoetendal et al., 1998, 2001), pigs (Simpson et al., 2000) and mice (Toivanen et al., 2001; Vaahtovuo et al., 2003). Within the animal industry a great deal of research is focused on host resistance to disease, and animals found to have increased resistance to certain enteric diseases are included into selective breeding programs (reviewed in Stear et al., 2001).

In addition, to the influences of host genotype, age has been indicated as a factor influencing the composition of the intestinal microbiota (Hopkins et al., 2001). Culture-independent molecular profiling techniques have shown that as birds age there is an increase in the diversity and complexity of the bacterial populations within the intestinal microbiota where older and younger birds are kept under the same conditions (van der Wielen et al., 2002). Lu et al. (2003) illustrated that ileal and caecal microbiota of broiler chickens had a significantly similar microbiota at three days of age but after two weeks these subpopulations had progressed into significantly different communities. The changes of the gut microbiota with age is likely to be associated with a variety of factors including a change in diet as the animal ages, maturation of the immune system, changes in environmental influences through time and an increase in the interplay with other animals, which would expose individuals to a greater repertoire of bacteria.

Chapter 4

4. Growth promoter and novel feed additives

There has been a growth in poultry production during the last decade, which has been largely excluded to the large and small scale organized poultry industries. This has been primarily achieved due to the utilization of various modern growth promoting strategies and appropriate disease prevention measures (Kataria et al., 2005; Angelakis et al., 2013). The main restriction for an economic poultry rearing in especially the un-organized poultry sector is the threat posed by the pathogens that are present in and around the poultry houses. This has lead to high cost treatment using antibiotics which has reduced the profitability of small scale poultry farming. Therefore, it has been thought to evolve newer trends and strategies to minimize the alarming farm inputs and maximize the outputs. For achieving the objective, several approaches have to be followed like genetic improvement by selective breeding, effective application of immunoprophylactic measures and better health cover with low cost growth promoting agents. The demand for cheap and quality food is continuously increasing due to the growing world population and this highlights the importance of maximizing the efficiency of poultry production in a cost effective manner, through the application of growth promoters, which are non-nutrients aimed to maximize utilization of the nutrients present in feed (Patterson and Burkholder, 2003; Kocher, 2006; Akinleye et al., 2008; Huyghebaert et al., 2011).

Growth promoters are the substances that are added to a nutritionally balanced diet which provoke response towards the exploitation of maximum genetic potential of the host, in terms of growth as well as improvement in feed conversion efficiency. There are different types of growth promoters which are used to exploit the broiler industry like antibiotics, probiotics (bio-growth promoters), prebiotics, exogenous enzymes, antioxidants, coccidiostats etc. (Allen, 1999; Walker and Duffy, 1998; Dhama et al., 2007, 2011; Angelakis et al., 2013). Many other novel growth promoters include herbs and certain other nutritional substances (Mahima et al., 2012, 2013; Dhama et al., 2013a). The use of growth promoters has been accepted in the broiler industry and they are usually included in the feed in very small quantities. Many antibiotics are used in animal and poultry feeds as growth promoters to

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improve the health and well being of animals and as a prophylactic agent for promoting growth. The withdrawal of these antibiotic growth promoters (AGP) not only affect or reduce productive performance, but also increases morbidity and mortality in poorly maintained flocks (Dibner and Richards, 2005). Research has been conducted in an intensified manner in the last two decades for developing antibiotic alternative for maintaining health as well as performance of animals. Probiotics and prebiotics, acidifiers as well as extracts of plants and neutraceuticals like copper as well as zinc, are the alternatives of antibiotics. At the same time the potential of antimicrobial peptides, clay minerals, antibodies from chicken egg yolk, essential oils, medium chain fatty acids (from eucalyptus oil), rare earth elements, as well as enzymes (recombinant) have been tested for their ability of replacing antibiotics as dietary feed additives (Han and Thacker, 2010; Jones et al., 2010; Thacker, 2013). The cost factor and the possibility of evolution of antibiotic resistant microbes has made it necessary for the feed industry to use alternatives to antibiotics, such as probiotics, prebiotics, organic acids to maintain good production and health of poultry as well as livestock. High environmental temperature is responsible for imbalance of homeostasis in poultry resulting in reduction in plasma protein as well as elevation of concentration of blood glucose.

4.1   Types of growth promoters and feed additives

During the last 2-3 decades, a remarkable growth in poultry industry has been observed, mainly due to exploitation of various modern growth promoting strategies and appropriate disease preventive and control measures. Many antibiotics are used in poultry feeds as growth promoters for improving health of animals. Others growth promoting agents and feed additives comprises of probiotics, prebiotics, synbiotics, organic acids, vitamins and minerals and herbs which have been used widely to promote poultry health and production (Dhama et al., 2011, 2013a; Mahima et al., 2012; 2013; Angelakis et al., 2013). Antimicrobial compounds are commonly included in poultry diets at sub therapeutic dosage for stabilization of the microflora of intestine and for improving the performance in general along with prevention of certain specific pathological conditions of the intestine (Dibner and Richards, 2005; Hassan et al., 2010). For neutralization of entero-toxins, probiotics play an important role. Supplementation of the diet with small fragments of carbohydrates manipulates the gut ecosystem (NRC, 1994; Patterson and Burkholder, 2003; Ashayerizadeh et al., 2009). The organic acids have got bacteriostatic properties as well as anti-mycotoxic effects (Gaglo-Disse et al., 2010; Wang et al., 2010; Cengiz et al., 2012). As premix in feed of poultry, multivitamin-minerals have been used for improving broiler growth and feed utilization

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thereby helping in realization of better return on production as well as economy (Peric et al., 2009). Various herbal products have got enhanced digestive activity, antimicrobial, antioxidant and immunomodulatory properties and can be used as alternative to chemotherapeutic agents (Fallah et al., 2013). The use of natural feed additive as substitute for antibiotics in poultry production has become an area of great interest (reviewed by Abaza et al., 2008).

4.2 Antibiotic as growth promoter

The AGP are the antibiotics that are used in poultry feed continuously at a lower level to improve growth and feed conversion and not for the purpose of any therapeutic reasons. Antibiotic compounds commonly used as growth promoters include Bacitracin, Penicillin, Virginiamycin, Flavomycin, Chlortetracycline, Oxyteracycline, Colistin sulphate, Doxycycline, Erythromycin, Aureomycin, Avilamycin, Tiamulin, Furazolidone, Lincomycin, Enrofloxacin and Neomycin sulphate (Chowdhury et al., 2009). In germ-free birds, the use of antibiotics as growth promoters are not of much value regarding performance enhancement, which reflects that their mode of action is primarily due to antibacterial activity instead of having any direct effect on physiology of the birds (Stanley et al., 2004; Dibner and Richards, 2005).

Principal mode of action of antibiotics is the regulation and maintenance of optimal balance of avian intestinal microflora (between gram-negative and gram positive organisms). The well balanced intestinal microflora contains more than 90% Gram positive bacteria (mainly *Lactobacillus*). During stress or digestive disturbances the number of pathogenic organisms, like *Escherichia coli* or other gram-negative organisms, is increased, leading to an imbalance of microflora. The gram-negative bacteria further colonize in the intestines, adhere to intestinal epithelium and cause inflammation of intestinal mucosa thus reducing the absorption of nutrients and in turn retarding the growth and productivity of birds. The AGP also has the ability to alter processes of mucin biosynthesis; and modifications in mucin dynamics influence the gut function and health and may change nutrient uptake. Hence, for eliminating the chances of imbalance under practical conditions, antibiotics are added to the feed or water as a prophylactic measure (Jones and Ricke, 2003; WHO, 2004). Use of anticoccidial drugs along with antibiotics as growth promoter increased the growth and immune status of chicken in contaminated environment (Lee et al., 2012). Antibiotics are given at sub therapeutic dosage for stabilization of the microflora of intestine and for improving the performance in general along with prevention of certain specific pathological

conditions of the intestine. However, while using antibiotics as growth promoters it must be kept in mind that their use on long term basis must be avoided as it can give rise to resistance group of microorganisms i.e., gram negative bacteria (*E. coli* and *Salmonella* spp.) (Gustafson and Bowen, 1997; Dibner and Richards, 2005; Hassan et al., 2010; Seniya et al., 2012).

Salient features of AGPs:

✓ modifies intestinal microflora and help to improve bird's performance and health status;

✓ have inhibitory effect on enzymes released by microorganisms and also on enzymes involved in microbial metabolism;

✓ reduces the growth-depressing metabolites produced by microorganisms (Feighner and Dashkevicz, 1987; Huyghebaert et al., 2011);

✓ addition of antibiotics to feed results in increased amino acid levels in the gut and improved nitrogen balance;

✓ improves absorption of feed nutrients because of thinning of intestinal wall, feed conversion ratio, weight gain, performance and productivity. Increases egg production and hatchability;

✓ reduces the damage caused by dietary fluctuations and destroy the harmful bacteria, keeping and minimize the adverse effects of dietary changes;

✓ prevents exponential multiplication of common pathogenic bacteria (*E. coli*, *Salmonella* spp., *Streptococcus* spp., *Hemophilus* etc.), reduces the incidences of non-specific diarrhoea or enteritis of chicken (George et al., 1982; Brennan et al., 2003; Huyghebaert et al., 2011);

✓ reduces stress and mortality in chicks by boosting body defense;

✓ reduces the microbial use of nutrients (Snyder and Wostmann, 1987; Huyghebaert et al., 2011).

The use of antibiotics through feed or drinking water when the birds are not infected can be disadvantageous, in certain aspects. It can lead to the development of antibiotic resistant strains of pathogenic microorganisms like *Staphylococcus aureus* and *Streptococcus* spp. of bacteria. The continued use of antibiotics as routine feed additives may also result in the presence of antibiotic residues in poultry products. Certain antibiotics even as residues can cause allergic or hypersensitive reactions in consumers. The continuous application of antibiotics can suppress sensitive natural microflora in the gastrointestinal tract like

saprophytes, commensals, non-pathogenic bacteria, fungi and yeasts or can show a compensatory growth and few can even increase their virulence.

Since 1999, some of the antibiotics growth promoters like spiramycin, tylosin, bacitracin and virginiamycin are banned in European Union due to development of resistance among bacteria of man and animals (McNamee et al., 2013). Apart from that, many other commonly used feed antibiotics had been banned by European commission due to development of resistant bacteria (Huyghebaert et al., 2011; Devirgiliis et al., 2013; Koluman and Dikici, 2013). At present use of antibiotics as growth promoter in food animals has become a great concern (Devirgiliis et al., 2013). World health organization along with World Organization for Animal Health (WOAH) encourages the health, agriculture, veterinary sector for reducing the injudicious use of antibiotics as growth promoters and further to decrease the spread of resistant bacteria (Aidara-Kane, 2012). Therefore, other non-therapeutic alternatives such as probiotics, prebiotics, symbiotics, antimicrobial peptides, enzymes, etheric oils, essential oils, eucalyptus oil, medium chain fatty acids, clay minerals, egg yolk antibodies, rare earth elements, recombinant enzymes and immunostimulants have been introduced as an alternative to the antibiotic growth promoters (Yang et al., 2008; Windisch et al., 2008; Nava et al., 2009; Huyghebaert et al., 2011; Wen and He, 2012; Tellez et al., 2012; Thacker, 2013; Mookiah et al., 2014).

Studies conducted concerning antimicrobials that are safer as alternatives for antibiotic replacement for interaction with the microflora of intestine, include prebiotics and probiotics, enzymes or dietary acidifications (Bedford, 2000; Patterson and Burkholder, 2003; Ricke, 2003; Diebold and Eidelsburger, 2006; Kocher, 2006; Niewold, 2007). Pedroso et al. (2013) reported that withdrawal of antibiotics or using alternatives to antibiotic growth promoter could alter the microbial flora of body.

4.3 Probiotics

Extensive use of antibiotics has led to imbalance between pathogenic and normal microflora as well as emergence of antibiotic resistant strains of bacteria. So, there is an increased interest in finding antibiotic alternatives for production of poultry. In poultry natural feed additives, like live probiotics, have got the potential to reduce in poultry the enteric diseases subsequently causing contamination of poultry products (Dhama and Singh, 2010; Gupta and Das, 2013). Probiotics are the live microbial feed supplements which are used for balancing the microbial population in the intestine through the production of various compounds, competitive exclusion and displacement of pathogens from enterocytes, as well

as maintenance of gut pH and thereby improving the health and immune status of the birds. Along with this, the broiler production factors are also improved. This helps in production of healthy meat without having any drug residues (Alavi et al., 2012). From a practical point of view, knowledge on the potential use of probiotics to optimize the balance of microflora in avian gut is essential. The idea that intestinal bacteria play a role in maintenance of health was originated by Metchnikoff in 1907, when he studied "lactic acid bacteria" in fermented milk products and their use to increase longevity and maintenance of youthful vigor in humans. The gastro-intestinal microflora of the host is responsible for the natural resistance of animals against infection (Fuller, 1989; Bengmark, 1998). Soon after birth, young ones acquire microflora from the surrounding environment. With the increase in the age, the microflora stabilizes themselves in the intestinal environment and a balance comes into existence between host's favorable and harmful microflora (Gibson and Roberfroid, 1995; Fuller, 2001; Dhama et al., 2007, 2008). If the microbes, which contribute to the proper microbial balance, are added to the feed, then the host receives a 'boost' to establish a proper microbial population in its gut and beneficially affect the host by improving the properties of the indigenous gastrointestinal microbiota. This finding has given rise to the concept and development of probiotic mediated growth promoters. Apart from establishing a balance in the gut environment/microflora, they specifically generate antibacterial substances (e.g., bacteriocins or colicins, lactoferrin, hydrogen peroxide, lactoperoxidase), competes for nutrients and make nutrients non-available to pathogens, modulate immune responses and compete with pathogenic bacteria (competitive exclusion) for adhesion receptors to intestinal epithelium (Jin et al., 1997; Balevi et al., 2001; Haghighi et al., 2006; Mountzouris et al., 2007; Gao et al., 2008; Modesto et al., 2009; Dhama et al., 2011; Tellezet al., 2012). Probiotics have also been found to improve digestion and utilization of nutrients and help in metabolism of minerals and synthesis of vitamins (e.g. Biotin, Vitamin-B_1, B_2, B_{12} and K), which are responsible for proper growth and metabolism. These can neutralize toxins released by pathogenic bacteria by releasing anti-enterotoxin substances (acidolin, acidophilin and lactin) and are also proved to bind mycotoxins present in feed. This has been found useful in reducing ammonia production in litter by their antagonistic action towards ammonifying bacteria and reducing urease activity and thus prevents developing of keratoconjunctivitis (NRC, 1994; Patterson and Burkholder, 2003; Ashayerizadeh et al., 2009). The most commonly used probiotics contain one or a mixture of harmless microbes. The microbes generally considered for developing probiotic growth promoters are *Lactobacillus acidophilus*, *L. sporogenes*, *L. bulgaricus*, *L. casei*, *L. plantarum*, *L. cellobiosus*, *L. salivarius*;

Streptococcus faecium, *S. thermophilus*; *Bacillus coagulans*; *Bifidobacterium bifidum*; *Saccharomyces cerevisiae*, *Enterococcus faecium*, *Torulopsis* spp. *Aspergillus oryzae* and *Bacillus licheniformis* (Owens and McCracken, 2007; Dhama et al., 2011; Liu et al., 2012; Lv et al., 2012; Tellez et al., 2012; Mookiah et al., 2014).

Probiotic supplementation plays a crucial role in countering enteric bacterial infections, especially inhibiting pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis*, *S. typhimurium*, *Clostridium perfringens*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Candida albicans* and the coccidian parasites *Eimeria* sp. (Van Coillie et al., 2007; Vicente et al., 2007; Willis and Reid, 2008; Nava et al., 2009; Dhama et al., 2011, 2013b, 2013c; Tellez et al., 2012). Dietary probiotics have been found beneficial in increasing the performance of broiler chickens experimentally challenged with *E. tenella* (Giannenas et al., 2012) as well as have a positive effects on raw chicken meat against *Listeria monocytogenes* and *Salmonella enteriditis*.

In broiler chick's diet, probiotics improved the immune response significantly (Cotter et al., 2000). Probiotics feeding also have been reported to improve antibody titres against viral diseases like Newcastle Disease (ND) and Infectious Bursal Disease (IBD) (Talebi et al., 2008). Probiotics augment the bird's resistance to fight off infectious pathogens and limit the negative growth effects of pathogenic microbes. By reducing the intestinal pathogenic microbial load, probiotics lower the pathogen spread in the poultry house via fecal contamination. A multi strain probiotic need to be used timely and regularly in feed for preventing various infectious agents including bacterial, fungal, protozoan and viral agents. Probiotics can reduce the flock mortality occurring due to immunosuppressive diseases (IBD, chicken infectious anemia, reoviral infections, Marek's disease, mycotoxins etc) (Dhama et al., 2011).

Use of probiotics is recommended in newly hatched chicks to establish gut microbial balance and prevent early chick mortality; stressful conditions like during de-worming, overcrowding, vaccination, temperature and environmental stresses, change of feed/ingredients, management (shifting/transportation, contamination), gastro-intestinal disturbances (scouring, loss of appetite, poor digestion and absorption of nutrients). Nowadays, application of probiotics is being recommended during antibiotic therapy for maintaining the required intestinal balance of microflora and reducing diarrhea, without affecting the efficacy of antibiotics. Probiotics have been considered as good alternatives to antibiotic growth promoters which help in limiting antibiotic residues in poultry products and the development of drug resistant microorganisms (Dhama et al., 2011). Probiotics

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formulations include bacteria, fungi and yeast, therefore the use of term "Direct Feed Microbials (DFM)" has been preferred now-a-days than the former. The addition of probiotics to poultry diet has been found to improve growth performance and feed conversion in broilers, egg production in layers and modulates the immune system of the birds to fight against various pathogens (Salim et al., 2013). Seeing the residual effect of antibiotics observed in poultry products and generation of antibiotic resistant strains, both having public health significance. Nowadays, the use of probiotics as substitute for antibiotics in poultry production has become an area of great interest (Yang et al., 2009; Dhama et al., 2011; Salim et al., 2013).

Advantages of probiotics:

- ✓ inhibits growth of diseases producing organisms;
- ✓ prevents digestive upsets and diarrhoea due to bacterial invasions;
- ✓ improves intestinal ecology by creating a balance in the gut microbial population;
- ✓ harmonize functions of digestive system and improves absorption of nutrients;
- ✓ improves feed intake and feed conversion efficiency;
- ✓ increases growth rate, body weight gain and productivity;
- ✓ regulates the lipid metabolism and reduces the body cholesterol content;
- ✓ improves fertility, egg quality and reduces cholesterol concentration;
- ✓ enhances survival and significantly help lowering of chick mortality;
- ✓ helps to maintain healthy gastrointestinal tract after antibiotic therapy;
- ✓ reduces stress after vaccination, antibiotic therapy, transportation, change of feed etc;
- ✓ stimulates immunity and also augment the effects of drugs and vaccines;
- ✓ helps in quicker detoxification of mycotoxins;
- ✓ improves litter condition by reducing ammonia and faecal water contents;
- ✓ synthesis of the vital B group vitamins;
- ✓ production of short chain fatty acids. Much safer without any side effects;
- ✓ no residue carry-over in meat or eggs;
- ✓ cost effective and reduces expenditure on antibiotics.

(Bengmark, 1998; Dhama et al., 2011; Mookiah et al., 2014).

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4.4   Prebiotics

Supplementation of the diet with small fragments of carbohydrates (such as oligosaccharides) is another method used to manipulate the gut ecosystem. Prebiotics selectively fermented by beneficial microflora into SCFA which effectively excludes the pathogenic microbes due to a lowered pH in GI tract through lactic acid production and thus inhibiting colonization of pathogenic bacteria. But the effect is specific for the type and dose of carbohydrate and also the rate of fermentation by the beneficial organisms. Aside to this, the SCFA produced by fermented prebiotics have strong effect on the metabolism of the host. The acetate and propionate are having gluconeogenic effects while butyrate is a major source of energy for intestinal epithelial cells. Also, the non-digestible oligosaccharides have been found to stimulate absorption of several minerals like calcium, magnesium, zinc and iron (Fallah and Rezaei, 2013).

The term 'prebiotics' was introduced by Gibson and Roberfroid (1995), who defined them as "a non-digestible food ingredient/supplement that beneficially affects the host by selectively stimulating the growth of some or all of the non-pathogenic organisms (bacteria) in the gut/colon." The commercially available prebiotic products mainly including oligosaccharides of galactose, fructose or mannose viz., galacto-oligosaccharides (GOS), mannan oligosaccharides (MOS) and fructo-oligosaccharides (FOS) have been tried in poultry with much success (Gibson and Roberfroid, 1995; Dhama et al., 2007, 2008; Roberfroid, 2007). A bioactive mixture of galactooligosaccharides (GOS) produced through the enzymatic activity of β-galactosidase from *Bifidobacterium bifidum* NCIMB 41171 was earlier proposed as B-GOS prebiotic for human treatment (Clasado Biosciences Ltd., Jersey, United Kingdom). B-GOS possess bifidogenic and immunomodulatory properties which were proven in monogastric model earlier (Tzortzis, 2009), thus a derivative candidate non-digestive trans-galactooligosaccharides in the novel formulation Bi^{2}tos (Clasado BioSciences Ltd., Jersey, United Kingdom) has been proposed for a routine treatment in poultry.

Studies have shown that dietary provision of a *Laminaria* spp. derived seaweed extract, containing laminarin and fucoidan, promotes improved growth and feed efficiency of pigs in the absence of in-feed antibiotics (Gahan et al., 2009; Figat et al., 2010; McDonnell et al., 2010). Furthermore, previous investigations indicated that the supplementation of a seaweed extract to pigs suppressed enteric *Enterbacteriaceae* populations (Reilly et al., 2008; Lynch et al., 2010a) and increased lactobacilli numbers (Lynch et al., 2010a; McDonnell et al., 2010).

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This suggests that seaweed extracts may provide a means to improve productivity and gut health in monogastric animals.

These non-digestible substrates (oligosaccharides) are considered as 'food for beneficial microbes', which on fermentation inhibit pathogens while simultaneously stimulating the absorption of several minerals in intestine. Prebiotics exert their beneficial effects on the host by selectively feeding the harmless bacteria at the expense of the harmful ones. *Salmonella*, *E. coli* and many other gram-negative harmful microbes are unable to utilize these oligosaccharides and therefore their growth is inhibited (Nisbet et al., 1993). Bacteria use lectins on their cell surface to bind to mannan on the intestinal epithelial cells to initiate attachment and colonization and during such a scenario, the MOS itself binds to receptors on bacterial pathogens and prevent their attachment to epithelial cells and prevent their colonization. Later the MOS pass through the gut with the pathogens attached, thus assisting in their effective clean up. Prebiotics such as FOS on the other hand, serves as a fiber source for certain microbial populations and enhance production of organic acids in the gut (Dhama et al., 2007). Feeding of MOS and FOS was found to increase immune status and enhance the macrophage activity along with T-helper cell activities (Hofacre et al., 2003; Shohani et al., 2013). It has been reported that mannan oligosaccharides from yeast cell wall works by providing specific binding sites to enteric pathogens, thus reduces their chances to attach to the intestinal tract and these oligosaccharides being not digested by the endogenous enzymes of the bird pass through the gut with the pathogens attached thus producing a cleaning up effect (Zopf and Roth, 1996). The mannans also help in changing the acidity of intestine via increasing lactic acid density as well as decreasing activities of harmful bacteria of intestine (that include: *Escherichia coli*, *Salmonella*, *Clostridium* etc). They also help in colonization of *Lactobacillus* thus improve their activities (Elwinger et al., 1998; Al-Ghazzewi and Tester, 2012; Khan et al., 2012).

Salient features of prebiotics:

✓ beneficially affects the host by stimulating the growth/activity of harmless bacteria, indicating a synergistic effect with probiotics;

✓ provides substrates for the bacterial fermentation in colon or caecum to produce vitamins and antioxidants that further benefit the host animal by indirectly providing energy, metabolic substrates and essential micro nutrients;

✓ prebiotics can provide energy and other limiting nutrients to the intestinal mucosa;

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✓ simultaneously produce systemic effect on utilization of feed ingredient, stimulation of immunity and neutralization of toxins;

✓ prebiotics help inhibiting colonization of pathogenic bacteria;

✓ because of their organ as well as organism specific action, the prebiotics are potential candidates for incorporation in diet of chicken;

✓ additionally, some prebiotics can provide specific members of the native microflora such as *Bifidobacteria* and *Lactobacillus* (probiotics), a competitive advantage that can exclude pathogenic bacteria from the intestine (Dhama et al., 2007).

Nowadays, combination of probiotics and prebiotics (synbiotics), having shown synergistic effects, are effectively used to counter the impact of stress factors or pathogens in poultry production systems (Mookiah et al., 2014).

4.5   Synbiotics

Synbiotics are defined as a mixture of probiotics and prebiotics that beneficially affects the host by activating the metabolism of one or a limited number of health promoting bacteria and/or by selectively stimulating their growth improving the host's welfare. Such an approach could ultimately produce the following nutritional benefits: improved survival of live bacteria in food products with, as a consequence, prolonged shelf life; an increased number of ingested bacteria reaching the colon in a viable form; stimulation in the colon of the growth and implantation of both exogenous and endogenous bacteria; and activation of the metabolism of these bacteria. (Tanaka et al. 1983; reviewed by Gibson and Roberfroid, 1995).

Synbiotics bringing in positive effect on growth, production and health. The live microbial additions (probiotics) may be used in conjunction with specific substrates (prebiotics) for growth; an example could be fructooligosaccharide in conjunction with a bifidobacterial strain. This combination of prebiotics and probiotics could improve the survival of the probiotics organism because its specific substrate readily available for its fermentation, and result in advantages to the host that the live microrganism and prebiotic offer.

Beneficial effects of combination of probiotics and prebiotics: (adapted from Patterson and Burkholder, 2003)

✓   modify intestinal microbiota;

✓   stimulate immune system;

- ✓ reduce inflammatory reactions;

- ✓ prevent pathogen colonization;

- ✓ enhance animal performance;

- ✓ decrease carcass contamination;

- ✓ decrease ammonia and urea excretion;

- ✓ increase production of volatile fatty acids;

- ✓ increase biomass and stool bulking;

- ✓ increase B vitamin synthesis;

- ✓ improve mineral absorption.

4.6 Organic acids

In recent years, the use of acidifiers has been increased many fold and are found to have the ability to reduce many pathogenic and spoilage organisms by lowering the gut pH. Because of the development and emergence of antibiotic resistant microbes (Tiwari et al., 2013), the utilization of organic acids has been increased as growth promoters in animal agriculture, which could help in providing protection from adverse human health implications. In poultry diets, the use of organic acids elicits a positive response in performance of broiler growth. In order to inhibit growth of bacteria of intestine (those which compete with host for the nutrients that are available) there is requirement of dietary acidification thereby causing reduced possibility of availability of bacterial metabolites which are toxic in nature. In the ceaca as well as small intestine it has been suggested by a number of studies that organic acids affect the bacterial concentration. In the crop of the poultry birds they are bactericidal for *Salmonellae* (Gaglo-Disse et al., 2010; Wang et al., 2010; Cengiz et al., 2012). In the young ones, acid production in the gut is insufficient and acidifiers are sometimes used in feeds to compensate it. The use of organic acids such as formic, lactic, propionic, citric, sorbic and phosphoric acids optimizes the balance of the microflora of the gastrointestinal tract (Byrd et al., 2001; Griggs and Jacob, 2005; Van Immerseel et al., 2006; Nava et al., 2009; Emami et al., 2013). They lower the pH, at which the activity of proteases and beneficial bacteria is optimized and proliferation of pathogenic bacteria is minimized by a direct antibacterial effect destroying their cell membranes (Partanen and Mroz, 1999; Chowdhury et al., 2009; Nava et al., 2009). In experimental studies, organic acids have been found suitable growth promoters in pigs (Partanen and Mroz, 1999; Overland et al., 2000; Partanen et al., 2002) and poultry (Gauthier, 2005). Supplementation of organic acid also

increases intestinal colonization of *Lactobacillus* spp. in chicks (Nava et al., 2009). They are widely used to inhibit pathogens, like salmonellae and in their undissociated forms are able to pass through their cell membrane. Inside the bacterial cell, the acid dissociates to produce H^+ions, which lower the pH causing the organism to use its energy in trying to restore the normal balance. It also disrupts DNA and protein synthesis and thus the bacteria are unable to replicate or its replication slows down. Lower pH conditions thus protect the bird from infection especially at young ages. In addition to direct microbial action, recent studies have shown that salt form of organic acids including butyrate, propionate and acetate have shown their ability in reducing *Salmonella* colonization in chicken cecum by enhancing innate immune defense via increased synthesis of host defense peptides (Sunkara et al., 2011). Furthermore, organic acids also reduce the contamination of litter with the harmful microorganisms, neutralize ammonia production and diminish the risk of re-infection. The effectiveness of organic acids in poultry may also depend on the composition of the diet and its buffering capacity. However, the bacteriostatic effects of organic acids are well known and as *Salmonella* control agents they have been used in the feed as well as water supply of poultry (Ricke, 2003). Explanation has been given by Brul and Coote (1999) regarding the salient basic principle of the mechanism of action of organic acids on bacteria. They noted that organic acids are non-dissociated undergo penetration of the cell wall of bacteria, what means that a wide margin of external as well as internal pH can not be tolerated. Various organic acids as formic, fumaric, propionic, lactic as well ascorbic acids acidify the diet and help to decrease pathogen colonization as well as toxic metabolite production; digestibility of protein as well as calcium and phosphorus and magnesium and zinc are improved. Various studies have demonstrated that organic acid supplementation in diet of broilers improves the growth performance and reduces diseases as well as problems associated with management (Vlademirova and Sourdjiyaska, 1996; Runho et al., 1997; Gunal et al., 2006; Islam et al., 2008; Ao et al., 2009b).

The more positive effects of acidifiers are noticed when they are either partially protected as sodium butyrate or as gastric coated form. Fernandez-Rubio et al. (2009) reported a significant reduction in shedding of *Salmonella* through the feaces in *Salmonella enteriditis* infected birds fed partially protected butyrate.

The selection of appropriate acidifiers plays an important role in its usage as an alternate to antibiotics and the pKa value in range of 3-5 was found optimal. The pKa values of different organic acids were presented in Table 4.1.

Table 4.1 - Organic acids and their pKa value

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Acid	Chemical name	Formula	Molecular weight	pKa
Formic	Formic acid	$HCOOH$	46.03	3.75
Acetic	Acetic acid	CH_3COOH	60.05	4.76
Propionic	2-propionic acid	CH_3CH_2COOH	74.08	4.88
Butyric	Butanoic acid	$CH_3CH_2CH_2COOH$	88.12	4.82
Lactic	2-hydroxypropanoic acid	$CH_3CH(OH)COOH$	90.08	3.83
Sorbic	2,4hHexandienoic acid	$CH_3CH:CHCH:CHCOOH$	112.14	4.76
Fumaric	2-butenedienoic acid	$COOHCH: CHCOOH$	116.07	3.02
HMB	2-hydroxy-4-methylthio butanoic acid	$CH_3SCH_3CH_2CH(OH)COOH$	149.00	3.86
Malic	Hydroxybutanedienoic acid	$COOHCH_2CH(OH)COOH$	134.09	3.40
Tartaric	2,3-dihydroxy-butanedioic acid	$COOHCH(OH)CH(OH)COOH$	150.09	2.93
Citric	2-hydroxy-1,2,3-propanetricarboxylic acid	$COOHCH_2C(OH)(COOH)CH_2COOH$	192.14	3.13

4.7   Vitamins and minerals

As premix in feed of poultry (particularly in feed of broilers), multivitamin-minerals have been used for improving the broiler growth as well as feed utilization thereby helps in realization of better return of production as well as economy. Their performance level is optimum when there is poor health condition of the birds (Peric et al., 2009). They also exert beneficial effect on health of gut as well as immunity along with immune performance. Positive effect is exerted via better appetite even though there is variation in mechanism of action. Along with this, there is improved conversion of feed, immune system stimulation, growing vitality as well as regulation of the microflora of intestine. In terms of improvement of utilization of feed, as well as metabolism and minimization of various stresses, all the vitamins (especially vitamin C) have essential roles to play (Sahin et al., 2003). Ascorbic acid or vitamin C is also having the ability to reduce the weight loss in birds due to heat stress. It resulted in enhanced performance in broiler chicks exposed to multiple concurrent environmental stressors (McKee and Harrison, 1995). In addition to ascorbic acid, tocopherols or vitamin E-supplemented diets resulted in better growth performance, by improving the feed conversion efficiency. Vitamin E is available in two forms: tocopherols and tocotrienols. After absorption, vitamin E is hydrolyzed in its unesterified form. L-arginine can also be supplemented with vitamin C for obtaining better meat quality. There is reduction in the effect of diet that is modified on the iron concentration in the liver as well as spleen and in heart of copper (Al-Darajih and Salih, 2012a, b; Suliburska et al., 2014).

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4.8   Organic minerals

The efficacy of the use of microelements is an important issue in modern poultry nutrition. Microelements are essential for normal growth and many metabolic processes in living organisms, as they are catalysts or constituents of the enxymatic system of many cells.

Supplementation of zinc (Zn) and manganese (Mn) in broiler diets is of particular interest because they function predominantly as catalysts in many enzyme and hormone systems that are associated with growth, skeletal soundness and immune response (McDowell, 1992). Supplementation of Zn and Mn at 40 and 60 ppm, respectively was recommended in broiler diets by National Research Council (1994), considering growth as the primary response criterion. However, modern broiler chickens have undergne considerable genetic transformation necessitating re-evaluation of mineral requirements in the context of higher growth rate, skeletal demands and immune response (Sunder et al., 2013). Each of these criteria responded differently to Zn and Mn levels in diets, suggesting the existence of variability in mineral requirements for individual parameters (Wedekind et al., 1992; Sunder et al., 2006, 2011; Huang et al., 2007). Equally important is the source of minerals and mineral interactions that determine the mineral bioavailability and mineral excretion (Ao et al., 2009a; Sunder et al., 2011, 2013).

Conventionally, inorganic minerals (as oxides and sulfates) are used in chicken diets, because they are cost-effective and readily available, but are relatively inferior to organic minerals due to poor bioavailability (Virden et al., 2004). In the gastro-intestinal tract, the inorganic minerals chelate with phytic acid complex allows a decrease of minerals rate of absorption and consequently affect the tissue uptake of minerals (Linares et al., 2007). Inorganic minerals were found to compete with each other for binding ligands and for the uptake sites in gut mucosa, reducing their absorption (Santon et al., 2002). However, the extent of mineral absorption varies significantly with the interaction between the minerals which could either be synergistic (Zn and Mn) or antagonistic (Zn and Cu) based on their compatibilities (Ao et al., 2009a; Sunder et al., 2011, 2013). In contrast, organic minerals complexed with amino-acids are devoid of free divalent cations for chelation in the intestinal lumen with phytic acid (Kidd et al., 1996) and hence, they are differently metabolized facilitating enhanced absorption (Burrell et al., 2004). It is in this context that organic minerals could be advantageously incorporated in diets at lower levels than the inorganic sources for realizing higher mineral bio availability and lower excretion to address the environmental concerns (Nollet et al., 2008).

As it was indicated in a comprehensive review by Aksu et al. (2012), use of organic minerals at relatively low levels in poultry diets has become widespread, especially due their ecological and physiological contributions; however there still is not enough experimental data on metabolic response of birds to the reduced dietary levels of these minerals (rewieved by Świątkiewicz et al., 2014; Sirri et al. 2016).

Chapter 5

5. *In ovo* feeding

5.1 Introduction

In the poultry industry, it is usual to hold chicks without feed and water for many hours after hatch and birds they may remain for up to 36-48 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). 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. Early feeding stimulates gastrointestinal motility and use of yolk sac nutrients necessary for growth (Noy and Sklan, 1998a, 1998b, 1999, 2001). 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.

There is great interest in ways to aid poult nutrition before placement; 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.

One of the new technologies recently introduced to poultry industry is *in ovo* feeding. This method is a mechanism for inject liquid nutrients into the embryos amniotic fluid (Herfiana, 2007). This mechanism of injection is useful for embryo development because protein and energy are first obtained from the yolk, and derive from the albumen only after 14 days of incubation (Vieira, 2007).

Use of *in ovo* feeding could enhance the nutritional status of the embryo and hatched chicken. The previous studies (Uni and Ferket, 2004; Uni et al., 2005) indicated muscle improvement and immune development, breast meat yield, health status and showed enhance jejunal nutrient uptake, increase in activity of the intestinal enzymes and post hatch growth (Foye et al., 2007) by using this method. The other researches showed the role of *in ovo* feeding to increase in size and surface area of the intestinal villi (Uni et al., 1998; Geyra et al., 2001; Tako et al., 2004), enhanced intestinal function and maturation prior to hatching (Foye et al., 2007) and to improve hatching weight and final weight of chickens, as well as to reduce FCR and abdominal fat percentage (Gholami et al., 2015).

The first research on the *in ovo* administration of vaccines was completed in the early 1980's by Sharma and Witter (1983). Subsequent work demonstrated that *in ovo* administration of Marek's disease (MD) vaccines HVT (Sharma and Burmester, 1982; Sharma and Witter, 1983), SB-1 (Sharma and Witter, 1983), and CVI988 (Zhang and Sharma, 2001) to late-stage chicken embryos was safe and would induce earlier immunity than post-hatch administration (Sharma, 1984; Zhang and Sharma, 2001). The concept of *in ovo* MD vaccination was moved from the laboratory to the field by the development of an automated *in ovo* injection machine (Sarma et al., 1995). Commercial *in ovo* vaccination began in the United States in late 1992 and today occurs routinely in many commercial broiler hatcheries in more than 30 countries (Avakian et al., 2002). The process and technique used to administer *in ovo* vaccines is critical. Efficacy of MD vaccine may depend upon the *in ovo* site in which the vaccine is delivered. Delivering vaccine to an incorrect *in ovo* site can lead to an ineffective vaccination, thus reducing the benefits normally seen by vaccinating *in ovo*. The normal fertile egg on day 18 of incubation has five sites that are large enough to be accessed by the needle of the commonly used *in ovo* injection machine and manual *in ovo* injection using a syringe and needle. These compartments are the air cell, allantois, amniotic fluid surrounding the embryo, the embryo body and the yolk sac. The efficacy of a vaccine and the safety to the embryo can be affected by the embryonic compartment into which it is deposited (Williams and Hopkins, 2011).

Review of literature reveals that there is no unique idea about time for injection *in ovo* feeding method and each researcher has introduced specific time for this purpose: flock age, genetics, incubation conditions and size of egg affects *in ovo* feeding (Ferket, 2005).

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 5.1).

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Figure 5.1 *- Schematic representation of in ovo injection*

The *in ovo* studies presented on Table 5.1 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 (2006) 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 *in ovo* feeding 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.

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* 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. A summary of research papers mentioning *in ovo* administration is presented on Table 5.1.

Table 5.1 *- Summary of research papers mentioning in ovo administration of substances*

Authors	*In ovo* injected substance	Target
Balaban and Hill, 1971	L-hyroxine, thiourea	embryo
Al-Murrani, 1982	amino acids	yolk
Decuypere et al., 1982	Iopanoic acid	allanotic circulation
Sharma and Burmester 1982, 1984	vaccine	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
Sarma et al., 1995	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)
Pedroso et al., 2006	glucose	amnion
Kim et al., 2007	antibodies against myostatin	yolk

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5.2 The application of prebiotics *in ovo*

Actually, around 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. Many vaccine manufacturers, nowadays, want 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 (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; thus, 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 use of bioactives in poultry production could be a valid alternative to the use of antibiotics, but their exact mode of action is still not clear. There are several hypothesis: initially some researcher proposed that administration of probiotic bacteria on 1-day old chicks, reduces infection by pathogens thanks to competitive exclusion mechanism between bacteria. Nowadays, it is known that the commensal bacteria can prevent infection by pathogens throught maintenance of the integrity of the gastrointestinal tract and regulation of immune system.

Prebiotics have been shown to alter GI microflora, alter the immune system, prevent colon cancer, reduce pathogen invasion including pathogens such as Salmonella enteridis and

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E. coli and reduce cholesterol and odour compounds (Cummings and Macfarlane, 2002; Hajati and Rezaei, 2010).

The main problem with the use of bioactives substances is their efficient administration under fully controlled conditions. In order to be effective, they have to be administered to the animals as early as possible. Above that, uncontrolled variables (i.e. water quality, and other environmental factors) should be minimized. To exclude some of these factors that could influence the responses of the animals to these substances, the *in ovo* injection technology of bioactives, directly into the air chamber of chicken embryo, has been defined (Gulewicz and Bednarczyk, Polish patent Nb. 197726). Through *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*. 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 popular point of view that embrionic development of the chicken happens in sterile environment (Amit-Romach et al., 2004) and the first contact with microflora occurs only after hatching; despite, Deeming in 2005 found that microorganisms may be internalized from yolk at 18 day of embryionic 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 very early the developing of immune mechanisms of the innate and adaptive immunity, and this fact may be more important than competitive exclusion in 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.

It has been exposed that a single *in ovo* injection with prebiotics into 12 day old chicken embryo leads to an increase in the number of bifidobacteria at the time of the hatch, although this fact ensures the long-term maintenance of a high level of bifidobacteria in the intestinal tract (Villaluenga et al., 2004; Pilarski et al., 2005; Bednarczyk et al., 2016; Pruszynka-Oszmalek et al., 2015). The same *in ovo* prebiotic injection approach realized on a large scale

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(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, 2011). The individual injection with defined dose and content of bioactives has also additional advantages: first of all, the chicken embryo is not contaminated and might be populated with the beneficial bacteria and secondly, provided a fully developed immune system and can profit from these bioactives. Moreover, prebiotic administration *in ovo* could have a positive effect on chicken hatchability. However, the dose of prebiotic has an important role (Bednarczyk et al., 2015). Even in previous study, Bednarczyk et al., (2011) found a hatchability percentage significantly higher in control group (87.3%) in comparison to the *in ovo* treated group with 1,9 mg of RFO. The reason for the decline in hatchability percentage of the injected embryos is unknown: different factors could affect the hatchability of *in ovo* injected embryos like site of injection, features and doses of injected substances and technology of incubation.

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Chapter 6

6.Structural and biochemical aspects of poultry muscle

6.1 Muscle tissue

Muscle tissue constitutes the largest part of edible poultry meat and for this reason it is very important for further processing. Usually, breast and legs of chicken and turkeys are classify, respectively, as white and dark meat due to different muscles color. Moreover, muscles show different shape and structure according to their different functions performed by live animal. Birds and animals in general, exert three main different activities that request three different types of muscles: skeletal (e.g. used in locomotion), cardiac (e.g. used for pumping blood) and smooth muscles (e.g. used by digestive system) (Barbut, 2002).

6.1.1 Skeletal muscle

Skeletal muscle is also classified as voluntary-type muscle, because its activity can be controlled completely in accordance to the will of the animal. Animals have a large number of different skeletal muscles. They can be relativity large like the *biceps femoris* or very small such as the ones responsible for closing the eyelids. Skeletal muscles look striated when painted and observed under a light microscope and for this reason they are known as striated muscles (Figure 6.1). This aspect is the consequence of the muscular fibres organization, which with their component, form the microstructure of the muscle (Aughey and Frye, 2001).
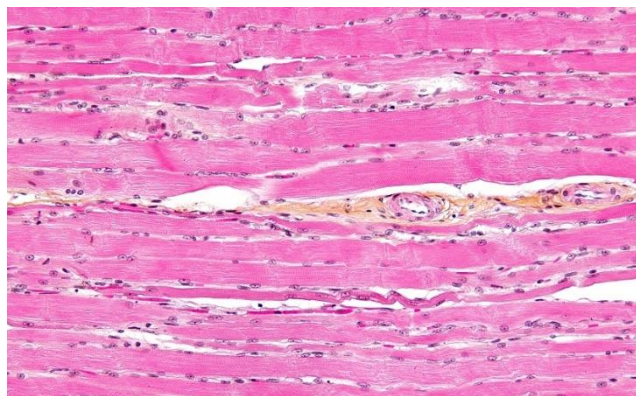

Figure 6.1 *- Striated muscle tissue*

[https://en.wikipedia.org/wiki/Striated_muscle_tissue (downloaded November 2015)]

The basic structural unit of a muscle has been defined as the muscle fiber (Figure 6.2), which constituted of several myofibrils (contractile units). Each muscle fiber was surrounded by a connective tissue called the *endomysium*, muscle fibers were then grouped into fascicles and surrounded by another layer of connective tissue called the *perimysium*, then the whole muscle was made up of group of fascicles and was surrounded by *epimysium* that connected the muscle to bones. Collagen was the major constituent of these connective tissues. Muscle development and subsequent meat quality were influenced by these connective tissues. Skeletal muscles growth was achieved by increasing the size of preexisting muscle fibers (hypertrophy). Fiber number, size, and type varied with function and anatomical location of the muscle. Meat quality was also affected by these factors. A muscle that contained high proportion of oxidative fibers tended to have red color due to a greater amount of myoglobin (e.g. thigh muscles) as compared to glycolytic fibers, which tended to appear white in color that affected the appearance of muscle/meat (e.g. chicken breast muscle). Glycolytic fibers were larger and had a lower rate of protein turnover. Therefore, the white muscles were larger and more efficient. In poultry, genetic selection for increased breast yield resulted in pale breast meat color in broilers (Le Bihan-Duval et al., 1999), ducks (Baeza et al., 1997), and turkeys (Sante et al., 1991), which could result in poor meat quality.

Collagen is the most abundant protein in the body and in connective tissues. The structure of collagen supported its function of providing strength to muscle and other tissues. Glycine constituted about one-third of all the amino acids found in collagen, while proline, which has been classified as an imino acid, and its analog hydroxyproline also constituted about one-third of all amino acids in collagen (Ramachandrana et al., 1973). Lysine has been considered to be another constituent of collagen where both proline and lysine were covalently modified to hydroxyproline and hydroxylysine, respectively. A collagen molecule (tropocollagen) was shown to be composed of three left-handed polypeptide helices coiled around each other to form a right-handed supercoil where glycine was found at every third residue. Strength of the collagen fibrils was due to the covalent bonds formed between and within tropocollagen triple helices, where collagen was cross-linked by lysine side chains that contributed to the strength of the collagen in meat that had an important role in development of meat tenderness (Alnaqeeb et al., 1984).

The connective tissue performs a fundamental role. It provides to the muscular structure to fix the components of the muscle and allows the transmission of movement to generates from sarcomeres (the little units that form muscle). Between various muscle fibres you can also find several blood vessels and groups of nerves of different dimensions (usually are

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microscopic, but sometimes you can find a bigger nerve visible at naked eye) designated to control their contractions (Aughey and Frye, 2001).
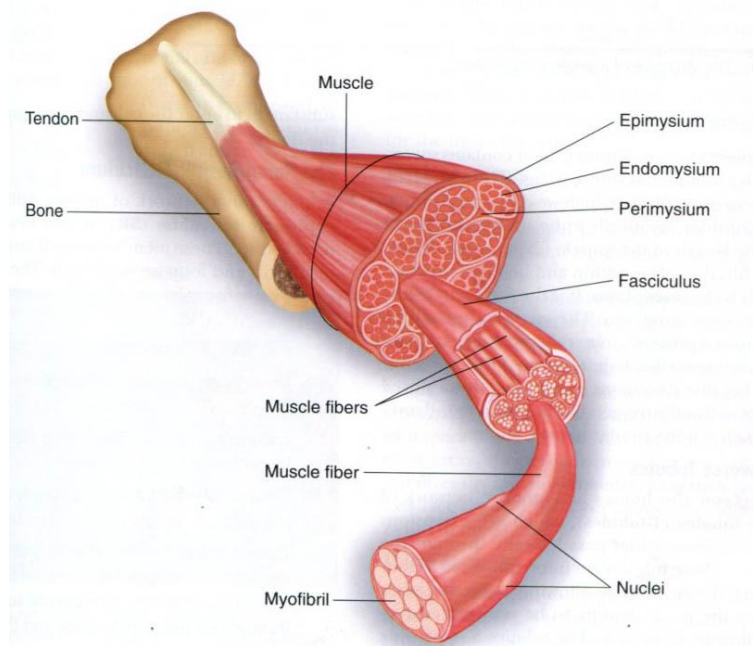

Figure 6.2 *- Structure of skeletal muscle*

http://www.slideshare.net/MohammedAlsailani/muscular-systempowerpoint-7508867

(downloaded November 2015)

Muscular fibres are formed of several myofibrils composed of numerous myofilaments. The myofibrils show a striated pattern because of the repetitive structure obtained by the overlap of thin and thick filaments. In particular, A-band is the dark area created by the superimposing of thin and thick filaments. The A-band includes the H-zone, a less dark area consisted of only thick filaments. The lightest area called I-band is composed of only thin filaments and it is divided by the Z-line. The thick filaments that shift to the Z-line, the sarcomer, become shorter, causing the muscle contraction, responsible for the movements (Barbut, 2002; Swartz et al., 2009).

6.1.2 Conversion of muscle to meat

The process of converting muscle to meat has started with killing the bird. Upon exsanguination the blood/oxygen supply was removed, the muscle tried to maintain its functions even after oxygen depletion through anaerobic glycolysis of its glycogen reserves to produce ATP but with the absence of blood supply to remove the waste, heat and lactic acid accumulated in the muscle and decreased pH. Due to ATP depletion, the muscle remained contracted as a result of actin and myosin binding that resulted in muscle stiffness.

This marked the onset of rigor mortis and conversion of muscle to meat where muscleproteins start to denature due to high temperature and low pH. Temperature and pH were the main postmortem factors influencing meat quality through affecting the onset and progression of rigor mortis and subsequent resolution (Wulf and Page, 2000; Cavitt and Sams, 2003; Jensen et al., 2004; White et al., 2006). During resolution, proteolysis of Z-disk proteins took place, and myofibrillar proteins were degraded into myofibrillar fragments by proteolytic enzymes that affect meat tenderness. In chickens, the process of converting muscle to meet has been found to start immediately after slaughter and be resolved within 2-4 hours. The extent of meat tenderization postmortem could be altered by the conditions under which the meat was processed. Factors have included temperature and duration of chilling, deboning time, postchill aging/holding duration, and marination.

6.2   Chemical composition of the skeletal muscle

In general, and for the poultry species, muscle chemical composition changes in function of several factors, for example specie, breed, gender, age, feeding and commercial cut. In order to assess the composition of muscles proximate analyses can be used to evaluate the quantity of moisture, protein, lipid, ash and carbohydrate. Whereas the mean values, the large part of the muscles contain about: 1% of ash that comprises mainly mineral like potassium, phosphorus, sodium, chloride, magnesium, calcium, and iron; 1% of carbohydrate predominantly glycogen *ante mortem*, and lactic acid *post mortem*; 5% of lipid; 21% of nitrogenous compounds (mostly proteins); and the rest 72% as moisture. These values are compared to the composition of fat and bone as shown in Table 6.1 and Figure 6.3. (Kauffman, 2012; Keeton and Eddy, 2004).

Table 6.1 - Summary Proximate analysis a of muscle fat and bone (Kauffman, 2012)

	Muscle	**Fat**	**Bone**
Moisture (%)	72	9	25
Nitrogenous compound (%) *(primarily protein)*	21	1	10
Lipid (%)	5	90	20
Ash (%)	1	<	45
Carbohydrate (%)	1	>	<

Meat shows an inverse relation with the percentage of fat and other meat components except carbohydrates. Practically, fat percentage increases with the decreasing amounts of moisture, protein and ash in muscle tissue. However, the amount of carbohydrates does not change significantly.

The 99.0% of animal's body composition is constituted by eleven primary chemical elements: 65.0% of oxygen, 18.0% of carbon, 10.0% of hydrogen, 3.0% of nitrogen, 1.5% of calcium, 1.0% of phosphorus, 0.35% of potassium, 0.25% of sulfur, 0.15% of sodium, 0.15% of chlorine and 0.05% of magnesium. The remaining part is constituted by 25 microelements: cobalt, copper, iodine, iron, manganese, molybdenum, selenium and zinc are required for normal metabolic function (essential elements), whereas barium, bromine, cadmium, chromium, fluorine and strontium are considered nonessential (Keeton and Eddy, 2004).
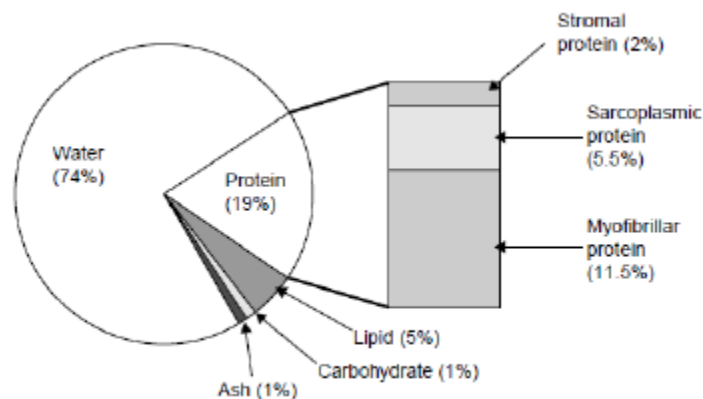

Figure 6.3 - *Composition of skeletal muscle tissue (Keeton and Eddy. 2004)*

6.2.1 Water

Muscle tissue conteins 75% of water. The largest part of water is located inner the muscular structure and the muscle cells. In particular, water is located into muscle cells, myofibrils, between myofibrils and between myofibrils-sarcolemma (muscular cells membrane), between muscle cells and between muscle bundles. Water molecules could be attracted to charged species like proteins because their dipolar property. In muscle cells, water is sometimes very closely bonded to the proteins (Huff-Lonergan and Lonergan, 2005).

In muscle tree main water categories are definite: bound, entrapped and free water. Bound water is the water that is directly linked to the other molecules (i.e. proteins). This is the reason why it has low mobility, for example it is usually static and rarely goes away from its compartments. In fact, this water does not easily freeze with low temperatures or

evaporates due to conventional heating. The bound water fraction located in the muscle cells represents a small part of the total water, about 0.5 g of water per gram of protein, less than the 10% of the total water in muscle. The bound water content is not affected at all by the post-rigor activity (Keeton and Osburn, 2010).

Entrapped or immobilized water is the water fraction that is linked to the other molecules either by steric effects and/or by attraction to the bound water. This type of water is not directly bounded to the proteins even if it is located between the structure of the muscle (Huff-Lonergan and Lonergan, 2005). Entrapped water in live muscle does not flow freely from its compartment, but it may be evaporated easily by drying or heating process, and could be converted to ice through freezing process. Entrapped water is very sensible to the rigor process and all the reactions that lead the conversion of muscle to meat. This water could flow like purge as a result of the alteration of muscle structure and pH following.

Free water is the water that can freely flow from one compartment to another. It is linked to muscle structure by weak surface forces. This water fraction is not contained on the pre-rigor muscle, but it is consequents due the conditions change that permit the shift of the entrapped water from its compartment (Huff-Lonergan and Lonergan, 2005; Keeton and Osburn, 2010).

6.2.2 **Muscle proteins**

It is known that proteins are the essential building units of the muscle structure. About the 20% of the lean muscle weight are proteins, the remaining part is represented by about 75% water and about 5% fat. According to different levels of salt solubility muscle proteins could be classify in three different main groups (Table 6.2; Goll et al., 2008).

Sarcoplasmic proteins are soluble in water or in low salt solutions (<50 mM) and are distributed entirely into the cells through the cellular fluid called sarcoplasm. They form about the 30% (w/w) of the total muscle tissue. All glycolytic enzymes and metabolic pathways enzymes are comprised in this protein class. They contain also myoglobin and the oxygen carrying molecule.

Myofibrillar proteins are also called contractile or cytoskeletal proteins. They are about 60% (w/w) of total muscle tissue. This group of proteins is soluble in high salt solution (about 0.6 M). These proteins build up the myofibril of skeletal muscle that consists principally in myosin (thick filaments) and actin (thin filaments) (Smith, 2010). This group of proteins, sited entirely into the cell, could be divided in three subgroups: muscle contraction protein (22%

actin and 43% myosin), enzyme and proteins that lead the muscle contraction, and cytoskeletal proteins that make up the cytoskeletal and are responsible for muscle cells integrity and rigidity (8% titin, 5% tropomyosin, 5% troponin, 3% nebulin, 2% C protein, 2% a-actinin, 2% M protein and <1% desmin (Barbut, 2002; Smith, 2010).

Stromal proteins are not soluble in water or in salt solutions. They mainly comprise the connective tissue (collagen and elastin) but also some membrane proteins may be included. Stromal proteins are extracellular molecules, which are about 10% of total muscle's proteins. They constitute the connective tissue proper *(perimysium, epimysium, endomysium),* and supportive connective tissue (ligaments, tendons, and cartilage) (Keeton and Eddy, 2004).

In general, collagen represents the most diffuse protein in animal's body, because it is the most important protein that constitutes the connective tissue. Its quantity depending mostly of muscle's physical activity and varies in function of different type of muscles. For example higher collagen concentration is content in muscles of leg, which do a higher activity and have the function to support body weight. The relatively high connective tissue rate makes leg meat less tender than more inactive muscles (Barbut, 2002).

Table 6.2 - Muscle proteins are classified in three different main groups, according to the different levels of salt solubility (19% of total muscle protein amount) [Barbut, 2002]

Group	Protein	Amount (%)
Sarcoplasmic		**(5.5)**
	mioglobin	0.2
	hemoglobin	0.6
	cytochromes	0.2
	glycolytic enzymes	2.2
	creatine kinase	0.5
Myofibrillar		**(11.2)**
	myosin	5.5
	actin	2.5
	tropomyosin	0.6
	troponin	0.6
	protein C	0.3
	α-actinin	0.3
	β-actinin	0.3
Stromal		**(2.0)**
	collagen	1.0
	elastin	0.05
	mitochondrial	0.95

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6.2.3 **Lipids**

Muscle tissue contains from 1.5% to 13% of fat. It presents mainly neutral lipids (triglycerides or triacylglycerols) and phospholipids. Other kind of lipids is the sterol esters (i.e. cholesterol) and cerebrosides. In general, lipid performs different functions: as a reservoir of energy for the cell, as a functional and structural constituent of the cellular membrane, as isolation or preservation for vital organs, and as hormones and vitamins (A, D, E, K) solvent. Fats are 2.25 times more caloric then carbohydrates or proteins (energydense nutrient). (Keeton and Eddy, 2004; Wood et al., 2007).

Triacylglycerols consist of a glycerol (i.e., three-carbon alcohol) base-structure bonded (ester bond) with three long-chain fatty acids that in muscle tissue they usually contain more than 10 fewer carbon (Figure 6.4). Triacylglycerol can be classified as simple (glycerol bonded with three identical fatty acids) or mixed (glycerol bonded with two or three different fatty acids). The large part of fatty acids in animal triacylglycerols is palmitic (16:0), stearic (18:0), and oleic acid (18:1n-9), in general consisting of 20–25%, 10–30%, and 30–55% of the total meat lipids. Animal triacylglycerols contain in minor amount also myristic (14:0), palmitoleic (16:1n-7), linoleic (18:2n-6), and α-linoleic acid (18:3n-3). Their concentrations depend of the different kind of species and diet (reviewed by Rimini, 2014). The large part of animal's fatty acid shows a even number of carbon atoms. Fatty acids may be saturated (without double bonds carbon-carbon), monounsaturated (with only one double bond carbon-carbon), and polyunsaturated (with two or more double bonds carbon-carbon).

Fat owns properties depending of the different fatty acids chemical characteristics (i.e. carbon chain length, number of double bonds, melting point, fluidity, hardness, susceptibility to lipid oxidation). The length of carbons chain and degree of saturation determine fats melting point. The most saturated fats, with higher melting points, are the internal fats that protect the organs, while the less saturated are the external subcutaneous fats. Saturated fatty acids with twelve fewer carbons are solids at body temperature. The main fatty acid in poultry is palmitic (C16, 26%), whereas in lamb, cattle and pigs it is oleic acid (C18:1, 20–47%) (Wood et al., 2007).

The fat saturation degree from most saturated (hard fat) to least saturated (oily fat) is listed by species as follows: lamb > cattle > pigs > poultry > fish. In general, polyunsaturated fatty acids are easily oxidized from oxygen, pursued by monounsaturated, while the saturated fatty acids are the less sensible to oxidation of lipid. (Keeton and Eddy, 2004).

Phospholipids (0.5-1% of muscle lipid) are located in the cell membranes where carry out the structural and functional activities. This fraction is more sensible to oxidation of lipid than neutral fatty acids fraction (triacylglycerols). Phosphoslipids have almost the same structure of triacylglycerols, but they present a phosphoric acid group interpolated between the glycerol ester and the third fatty acid.

The lipid content of the skeletal muscle varies in function of species, age and diet. During animal's life fat is accumulated in the subsequent order: around vital organs, under the skin (subcutaneous fat), between muscles (intermuscular fat) and in the end between muscle bundles (intramuscular fat or 'marbling') (Wood et al., 2007).

6.2.4 Carbohydrates

When the animal is alive, glycogen is the main carbohydrate of the skeletal muscle tissue (from 0.5% to 1.5%). This carbohydrate continent in the live muscle is important because it affects colour, texture, firmness, water-holding capacity, emulsifying capacity and shelf-life of the meat after rigor process. Glycogen can be a big molecule stored in the muscle cell like a glucose reserve. It is present in granules that could have a small molecular weight (proglycogen; 400,000 Da) and the "classic" glycogen or (macroglycogen; 10,000,000 Da). Glycogen in live tissue is a source of energy that allows muscle contraction. Animal's tissue carbohydrates are also located in the extracellular matrix of connective tissues (glycosaminoglycans and proteoglycans) and in plasma and blood (glycoproteins). Some hormones, glycolytic intermediates, nucleotides, nucleosides and the glycolipids are classified as carbohydrate (Claflin et al., 2008).

6.2.5 Minerals

Minerals are typically expressed as percent ash. Ashes contain several kinds of minerals: oxides, sulfates, phosphates, nitrates, chlorides and other halides. In meat tissues ashes are about 1% and derive from the total mineral content of myoglobin, haemoglobin, enzymes, bone fragments, mechanically separated tissue, advanced meat recovery systems and in cases of adding ingredients during the processing like sodium chloride, potassium chloride, alkaline phosphates, lactate salts, spices, seasonings, batters, breading (Table 6.3).

Table 6.3 - *Mineral composition of chicken, beef and ostrich (Sales and Hayes, 1996).*

Minerals (mg/100g)	Chicken	Beef	Ostrich
Sodium	77	61	43
Potassium	229	350	269
Calcium	12	7	6
Magnesium	25	20	22
Phosphorous	173	180	213
Iron	0.9	2.1	2.3
Copper	0.05	0.14	0.10
Zinc	1.5	4.3	2.0
Manganese	0.02	0.04	0.06

Muscle tissue presents low content of calcium (3-6 mg/g), but high contents of potassium (250-400 mg/g), phosphorus (167-216 mg/g), sodium (55-94 mg/g), magnesium (22-29 mg/g), zinc (1-5 mg/g), iron (1-3 mg/g) and copper (0.5-0.13 mg/g). Meat contains haem iron (about 50% of the total iron) that is easily absorbed as a nutrient. Calcium, magnesium, sodium and potassium permit the muscle contraction in live animal, while magnesium and calcium allow the contraction of muscle fibre after slaughter. Sulfur (2.5 mg/g) is present in sulfur-containing amino acids, while chlorine (0.65 mg/g) is mostly present in salt form and located in soft tissues and intracellular fluids. Iron, copper, zinc, iodine, manganese, molybdenum, cobalt and selenium are diet essential microelements, while barium, bromine, cadmium, chromium and fluorine are microelements within meat tissues with specific functions. Aluminum, arsenic, boron, lead, lithium, nickel, rubidium, silicon, silver, strontium, titanium and vanadium are also present, but until now their function is not well-defined or maybe they could be environmental contaminants (Keeton and Eddy, 2004).

6.3   Muscle contraction

As previously mentioned, sarcomere is the smallest repetitive unit that forms the muscular fibre. It is an area comprised between two longitudinally contiguous Z-lines with one A-band and two half I-bands (Figure 6.4). Sarcomeres are linked end-to-end in order to create the myofibril. Sarcomere length could be ranged from 4 to 1 µm or less and it has a diameter of about 1 µm. It is normally enclosed with T-tubule and sarcoplasmic reticulum, the same intracellular membrane elements of the mitochondria. In the overlap region there is a space between the thick and thin filaments of about 25 nm depending of the sarcomere length. Sarcomere is a dynamic unit that can change its diameter with the addition or the subtraction of filament. It could also be divided and developed adding myofibrils to increase the cell

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diameter. Sarcomeres can also be used in order to increase the overall cell length (Swartz et al., 2009).
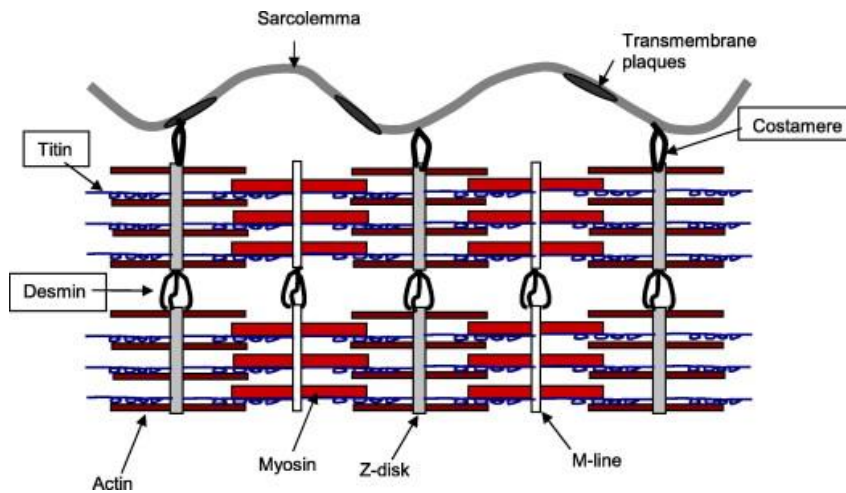

Figure 6.4 - Microstructure of skeletal muscle tissue: sarcomere (Kemp et al., 2010)

Muscle contraction causes muscle movement and it consists of an extremely difficult succession of events. In general, several sarcomeres shift in unison causing a tension that results in a pressure in a definite part of the body. This process practically converts the chemical energy that is stored in the adenosine triphosphate (ATP) molecules as a highenergy bond, into physical movement (Swartz et al., 2009).

Below, it will be briefly discussed the most important proteins implicated in muscle contraction, with their particular structure.

Myosin. The muscle part known as thick filaments is formed by myosin, which represents about 45% of the myofibrillar proteins. This protein presents an elevated molecular weight (around 450000 Da) and an extended rod-shape (Figure 6.5). It has two heavy (myosin's heads) and two light chains, which is possible to divide subjecting myosin toward a particular proteolytic enzyme activity. Heavy chains subsist of myosin's heads that are able to use chemical energy stored as ATP-bond molecule, splitting ATP into adenosine diphosphate and phosphate. They use this energy to modify their orientation (i.e., bend), to form cross bridges with the actin molecules and consequently generating movement (Smith, 2010).

Actin. The thin filaments are formed by actin, a protein with lower molecular weight (42000 Da) when compared to myosin. It presents two chains of G-actin, in each one is formed from several F-actin molecules. The arrangement of the actin's chain is favoured at a definite salt concentration. Double helix of actin molecules that forms the thin filament, is made by two actin's chains rolled together (Smith, 2010).
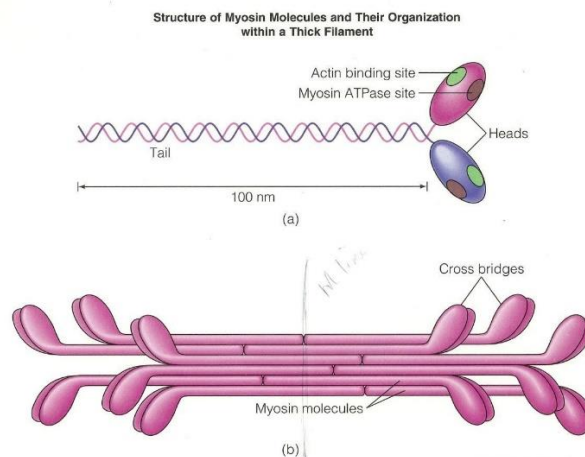
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Figure 6.5 content:

Structure of Myosin Molecules and Their Organization
within a Thick Filament

Actin binding site
Myosin ATPase site
Heads
Tail
100 nm
(a)

Cross bridges
Myosin molecules
(b)

Figure 6.5 *- Molecule of myosin (Smith, 2010).*

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Tropomyosin. It is about 5% of the total myofibrillar proteins; it is located around the actin double helix structure and shows a rod-like shape. Every seven molecules of actin you can find one molecule of tropomyosin molecule. It is located at the side of the actin molecule and is situated in the inner void space between the two actin filaments (helical structure).

Troponin. This globular protein is, as tropomyosin, about 5% of the myofibrillar proteins. It is also situated in the groove inside the double helix of actin, located within the tropomyosin strands. Molecules of troponin are organized according to a recurring pattern alongside the actin filament. Three different types of troponin can be found: troponin-C (binds Ca), troponin-I (inhibits ATP) and troponin-T (binds tropomyosin) (Swartz et al., 2009).

In order to explain the phenomenon of the muscle contraction more than one explanation has been proposed. The most suitable explanation is known like "slidingfilament theory". This theory shows that thick filaments (myosin) move in the direction of the Z-lines between thin filaments (actin). As previous cited, the releasing of energy that allows the operation of this process is provided by the molecular splitting of ATP in ADP, which is performed from myosin heads in their specific site. Using this energy myosin heads turn or entwine on actin filaments moving to the Z-line. The start signal that allows the beginning of muscular contraction comes from the brain and through the nervous system reaches the muscles. The depolarized membrane and the intern electrical potential variation (from 80 mV to 20 mV) allow the transmission of the signal via nerves. In the rest time, in order to maintain constant the potential difference among inside and outside of the cell, pumps located

in the membrane of the nerve transfer positive sodium ions from inside to outside (Barbut, 2002).

After the message transmission (known as action potential), electrical potential returns very quickly at initial condition, it takes about one thousandth of a second to restore the original base situation. The signal is communicated from the nerve to the muscle, when it achieves the end of the nerve, by chemical means. The last part of the nerve releases acetylcholine and induces the depolarization of the cell's membrane of the muscle. This chemical meaning is deactivated very rapidly by a specific enzyme named acetycholinesterase to avoid an incessant messaging to the muscle. The chemical signal cause the electrical depolarization of the membrane of the muscle cell and moves to the myofibrils through a specific way of T-tubules located in the sarcoplasmic reticulum (Swartz et al., 2009). Below it is showed schematically the mechanism of muscle contraction (Barbut, 2002):

1. calcium is released from the terminal cisternae of the sarcoplasmic reticulum to the sarcoplasm;
2. troponin-C rapidly bonds free calcium molecules;
3. this allows the loop operation that permits the tropomyosin to move from the actin binding sites;
4. cross bridges between actin and myosin molecules are formed;
5. ross bridges are submitted to a repetitive process of creation and breaking, as consequences thick filaments shift in direction of Z-line and, thus cause the shortening of the sarcomere.

The chemical message coming from the nerve stops for the duration of the relaxation phase:

1. re-polarization of the sarcolemma and the T-tubules prepared them to receive the subsequently chemical signal;
2. calcium situated inside the sarcoplasmic reticulum is pumped back into the terminal cisternae by calcium pump;
3. breaking of acto-myosin bridges;
4. tropomyosin molecules come back again to the binding sites of actin;
5. filaments move passively and they came back to the initial position so sarcomeres come again to their relaxing status.

The sarcoplasmic concentration of free calcium leads to muscle contraction. It is less than 10-8 mole/litre, for the relaxing period, but this concentration could increase until around 10-5 mole/litre with the releasing of free calcium. This is the reason why the troponin-C bond calcium causes the movement from myosin binding sites away to actin molecules of

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tropomyosin-troponin system. For all the duration of the relaxation time, free calcium is sequestered again, and its concentration returns around 10-8 mole/litre (Swartz et al., 2009).

6.4 Fibre type

Fibre of skeletal muscle is constituted of multinucleate, membrane-bound cells with a diameter that ranges from 10 to 100 μm and lengths that could range from less than 1 cm to more than 30 cm. Fibre type could be clearly different according to species and muscle types, depending on the function.

Moreover, several aspects contribute to fibre type variation: sex, age, breed, hormones, type of muscle and physical activity. These differences are affected by their molecular, metabolic, structural, and contractile properties. The diversity of the individual muscle fibres results in a different type of skeletal muscle (Choi and Kim, 2009; Lee et al., 2010).

Commercial poultry meat shows a clear division in white and dark meat. White meat consists almost entirely in the breast muscle from chicken and turkey, while dark meat consist principally in leg and back meats. Thus poultry meat is classified according to the colour. It is well known that this classification on chicken and turkey meat depends also in the different muscle amounts of red and white fibres. In general the large part of muscles is composed from a mixture of red and white fibres; only a small number of muscles contain all white or all red fibres. In fact, on chicken and turkey the meat that presents a high proportion of red fibres is darker and more red than the white meat (Barbut, 2002).

Red and white muscle fibres present some significant metabolic and functional differences. In general, the red muscle fibres present more red appearance due to the higher content of myoglobin. Red fibres make slower contractions than white fibres, but they can work for longer periods of time (Table 6.4; i.e., slower but sustained activity). Red fibres are able to work for longer time when compared with the white ones and for this reason they present bigger mitochondria and are presented in a more elevated number. Besides they also present a superior percentage of total lipids, which represents the energetic source. Muscles used to sustain the skeleton in an upright position present a high percentage of red fibres. These muscles present a defined metabolism that allows them to be more resistant and less fatigable. Red fibres can be operated for a long period of time, but in order to work in the proper way, they need an excellent provision of oxygen and a large number of enzymes from the oxidative metabolism (Taylor, 2004; Choi and Kim, 2009). The content of myoglobin and the activity of the oxidative enzyme are lower in white fibres than in red. Fibres usually work

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utilizing the glycolytic metabolism, it can be operated with or without the presence of oxygen (i.e., aerobic and anaerobic metabolism).

Muscles with high number of white fibres does not need a fast transport of nutrient therefore they present lower capillary density. These muscles are known to perform more rapidly contraction for a very short time (e.g., the quickly shake of the chicken wings) and they are more susceptible to the tiredness. It is interesting to note that various migratory wild type birds (i.e., ducks and geese) present a quite dark/red breast muscle due to a higher percentage of red fibres that allow the muscle to work for long periods of time. Another midway fibre type can also be found and presents an intermediate characteristic (Barbut, 2002; Lee et al., 2010).

Table 6.4 - Muscular fibre classification

	Fiber type		
Characteristics	**Oxidative** (βR)	**Intermediate** (βR)	**Glycolitic** (βR)
Colour	Red	Red	White
Myoglobin content	High	High	Low
Capillary density	High	Intermediate	Low
Fibre diameter	Low	Low/Intermediate	High
Number of mitochondria	High	Intermediate	
Speed of contraction	Low	High	High
Contractile action	Tonic	Phasic	Phasic

The existence of different muscle colours is very important for the market of meat product because it can influence consumer acceptability therefore also the market price. For example, in North America white meat is more expensive than dark meat. On the contrary, in some parts of Asia (Far East), dark poultry meat presents higher prices (Barbut, 2002).

6.5 Meat Quality Defects

Meat production is projected to double by 2020 due to increased per capita global consumption of meat and population growth. Genetic selection based on important economic traits such as growth rate, body size, edible meat yield, and feed conversion has resulted in gross changes in commercial meat poultry (Havenstein et al., 1994a,b; Anthony, 1998; Pollock, 1999). The age to reach market weight, the amount of feed necessary to produce a kilogram of meat, and the age at which slaughter occurs has been reduced continuously during the last 50 years (Gous, 1986). However, during the last decade, intense selection pressure has been applied to improve breast meat yield and muscle mass in response to a shift

in the market from whole birds to further processed products and an increasing demand for white meat by consumers (Ewart, 1993). Consequently, the growth of the *Pectoralis major* muscle of broilers and turkeys has increased at a rate that has exceeded body weight growth (Lilburn, 1994). Unfortunately, along with this progress has been a concomitant increase in muscle abnormalities and problems. Genetic selection has resulted in profound changes in muscle fibers and vascular structure of poultry skeletal muscle (Wilson et al, 1990; Dransfield and Sosnicki, 1999; Hoving-Bolink et al., 2000; Remignon et al., 2000). These alterations have lead to an increase incidence of muscular problems such as leg weakness and edema, focal myopathy, deep pectoral myopathy, and muscular dystrophy in broilers and turkeys (Grunder et al, 1984; Siller, 1985; Sosnicki et al, 1988).

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 Elminowska-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 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. 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).

6.5.1 **PSE-like breast meat defect**

Pale, soft and exudative (PSE) meat is a quality defect in the meat industry, which accounts for huge losses particularly in the pork and poultry meat. The incidence of PSE meat has been well-documented in swine, where meat had a very light gray color and was soft in texture and lacked the ability to hold water (Wismer-Pedersen, 1959; Cassens, 2000). It was first described by Ludvigsen for pork in 1953 (Briskey, 1964). PSE meat development was earlier related to increased rate of early post-mortem glycolysis, indicated by elevated muscle temperature and rapid pH decline, which results in sarcoplasmic and myofibrillar protein denaturation and meat with pale color, soft texture and high water loss (Briskey, 1964). More recently, it's been shown that extended glycolysis and low ultimate pH could also result in PSE meat (Sellier and Monin, 1994). More recently the PSE defect was also reported in

turkey (Sosnicki et al., 1998; Owens et al., 2000), chicken (Figure 6.6) (Swatland, 2008; Petracci et al., 2009), and ostriche (Van Schalkwyk et al., 2000) meat. However, it was more difficult to distinguish and identify these characteristics in poultry meat compared to pork. This condition has been referred to as PSE since characteristics were similar to PSE in pork which is misleading since both conditions were not exactly the same. Poultry researchers have preferred to refer to the condition in poultry as "PSE-like" or "Pale poultry muscle syndrome" (Petracci et al., 2009; Smith and Northcutt, 2009). The PSE and PSE-like conditions were detrimental to the industry profit ability since it affected important meat quality attributes involved in the production of value-added products and further processed meat. Affected muscle has been reported to lose their rheological properties and become unable to hold water. For example, "*mortadella*" prepared with PSE-like chicken meat has reduced water holding capacity, altered texture, and diminished emulsion stability, and required additives to restore the functional properties of normal meat (Kissel et al., 2009).

Also, poultry processors have been concerned with the appearance of PSE-like meat in fresh tray packs as the pale color affected color uniformity within the package and thus consumer acceptance. The occurrence of PSE-like in poultry meat has been believed to be the result of accelerated postmortem glycolysis (rapid pH decline) while the carcass was still warm (Pietrzak et al., 1997). In poultry, normal pH values at 15 min postmortem (pH_{15}) were around 6.2 to 6.5 (Berri et al., 2005) whereas normal ultimate pH (pH_u) values were around 5.8 (Van Laack et al., 2000). If the pH_{15} value was low (below 6.0) when the muscle was still warm, the proteins were subject to denaturation, which led to a decreased water-holding capacity and a lighter color of the meat.

The reasons for PSE-like condition have remained unclear, but up to 30% of broiler breast meat and up to 40% of turkey breast meat have shown this defect in commercial processing plants (Barbut, 1997a,b; Zhang and Barbut, 2005a,b). Furthermore, it has been reported that the occurrence of PSE-like meat in birds may be affected by alteration to the intracellular calcium homeostasis caused by a mutation in the ryanodine receptor gene, which was different from the ryanodine receptor gene in swine, and also depended upon several aspects of pre-slaughter and post-slaughter management practices (Chiang et al., 2004; Oda et al., 2009). It was thought that the application of ''snow chilling'' with carbon dioxide intensified meat quality abnormalities (Wynveen et al., 1999). Also, other factors have been thought to contribute to this problem, such as heat stress during the finisher period or during the pre-slaughter period (Petracci et al., 2009), and stress and struggling prior to slaughter (Ma and Addis, 1973).

Differentiating PSE-like meat from normal meat has beens based on instrumental or visual assessment of color lightness (*L**). However, the cut-off value for classifying meat as PSE-like has differed among researchers. Petracci et al. (2004) considered a *L** value of 56 as the cut-off, while Barbut (1993, 1996, and 1998) suggested to classify turkey breast meat as PSE-like when *L** values were greater than 52 at 24 h post-mortem. Fraqueza et al. (2006) classified breast meat as PSE-like when the *L** was greater than 50 and pHu was less than 5.8, while Woelfel et al. (2002) used *L** values greater than 54 in broilers as their standards.

Using *L** as an indicator of PSE-like condition has not been considered accurate and could be misleading because several factors have influenced poultry meat color. Feed ingredients used in poultry have been reported to change breast meat color (e.g. wheat-based vs corn-based diets). In addition, it has been shown that genetic selection for increased growth and breast meat yield resulted in a marked increase in muscle fiber size (Remignon et al., 1995; Guernec et al., 2003) with a shift towards a greater proportion of white fiber (glycolytic) and reduced dark fiber (oxidative), which produced meat that appeared pale but still had a high pH_u. Color measurement was also affected by muscle thickness (Bianchi and Fletcher, 2002) and color measurement position on the fillet (Goshaw et al., 2000). Therefore, color, pH_u and water holding capacity should all be considered when classifying poultry as PSE-like meat.

Figure 6.6 - Pale, soft and exudative (PSE)-like broiler breast meat (Petracci and Cavani, 2012)

6.5.2 Dark firm and dry meats

Meat with DFD condition is dark in color, has a firm texture and dry appearance, which are mainly related to its high pH and higher protein functionality resulting in higher water holding ability and in return a firm texture and dry surface characteristic for this meat (Barbut et al., 2005; Owens and Sams, 2000). The high WHC of DFD meat might increase its susceptibility to microbial contamination and therefore result in shorter shelf life for this meat

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(Allen et al., 1998). In addition, higher WHC of DFD meat results in lower light scattering from the surface and therefore a substantially darker color for DFD meat (Barbut et al., 2005; Swatland, 1994). Breast meat with DFD characteristics was investigated for both broiler chickens (Barbut, 1997a, 2005; Qiao et al., 2001; Woelfel et al., 2002) and turkeys (Zhang and Barbut, 2005a,b). From a morphological point of view the muscle fibers in DFD meat are arranged in a much denser and more compact manner compared to normal meat, which had a fairly loose microstructure with no abnormalities (Barbut et al., 2005).

It is believed that DFD meat is related to long-term stress before slaughter that causes depletion in muscle glycogen resulting in higher post-mortem muscle pH because of the prevention of glycolysis by elimination of its substrate (Owens and Sams, 2000). Many factors including transport exhaustion (Lesiow et al., 2007; Warris et al., 1999), feed withdrawal (Kotula and Wang, 1994), climatic stress, in particular cold stress (Nicol and Saville-Weeks, 1993; Webster et al., 1993), resting prior to slaughter (lairage time) (Warris et al., 1999), and aggressive behavior could contribute to the depletion of muscle glycogen and in return limit the amount of lactate formed post-mortem. The incidence of DFD breast meat in poultry has been studied by Lesiow et al. (2007) and Petracci et al. (2004). According to Petracci et al. (2004), breast meat color was significantly darker in winter compared to summer. Lesiow et al. (2007) further confirmed that transportation during winter season caused a significant increase in the incidence of DFD broiler breast meat. Zhuang and Savage (2010) did not find difference in average flavor intensity scores of breast meat between different groups categorized based on raw meat color (light, $L^*>60$; medium, $55<L^*<59$; dark, $L^*<55$). In addition, these authors did not find any difference in texture profiles between the cooked medium and dark fillets.

The characteristics of DFD breast meat are already established, however, the causes for this defect in the poultry industry are not clear. It has been shown that cold winters could increase the incidence of DFD breast meat in broiler chickens. However, more studies are required to establish the exact causes of this defect and the biochemistry behind DFD development in poultry breast meat. It is important to understand the challenges to lower the incidence of DFD in the poultry industry especially in the winter season and find solutions to use this meat properly in further processed products, i.e. by manipulating the pH.

6.5.3 Deep pectoral myopathy in broiler chickens

Deep pectoral myopathy (DPM, Figure 6.7), is a degenerative muscle disease of poultry commonly referred to as green muscle disease. Green muscle disease is a hidden problem in

today's large broiler chickens that is not detected until these birds are deboned at the processing plant. It is characterized by necrosis and atrophy of the deep pectoral muscle, which is commonly referred to as the breast tender (Bilgili and Hess, 2008). Green muscle disease is not a new disease, but it is becoming increasingly more common in meat-type broiler chickens selected for high breast meat yield. The disease was first described by Dickinson et al. (1968) in adult turkeys. It was later noted by Page and Fletcher (1975) in broiler breeder hens and finally reported in young broiler chickens by Richardson et al. (1980). As the number of broilers marketed at a heavy body weight for the further processed industry continues to grow, so do the reported occurrences of DPM. According to some researchers (Bilgili et al., 2000), the condition appears to be more common in males than females. However, there is some disagreement on this matter (Lien et al., 2012). The problem is also seen in free-range broilers. According to Siller (1985), DPM occurs exclusively in birds that have been specially selected for breast muscle development. It is generally recognized that DPM 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 (Harper et al., 1983; Siller, 1985). The resultant necrotic muscle has a characteristic hemorrhagic appearance, with a swollen reddish-brown lesion (early developing stage) that later becomes green and shrunken and then pale green (old stage), depending upon the time of induction of the vigorous wing exercise (Bilgili and Hess, 2002). Evidence that this condition only appears because of intensive selection is supported by the absence of DPM in wild turkeys, even when these birds are experimentally forced to produce DPM by wing flapping (Siller, 1985; Calnek et al., 1997; Bilgili et al., 2000). Moreover, because commercially reared broilers and turkeys are relatively inactive during the growing period, the pectoralis minor muscle is not exercised, and this determines a further reduction of the elasticity of the muscle compartment that does not allow the accommodation of the swollen muscle after wing flapping. 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 1 or both pectoralis minor muscles, respectively. No

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public health significance is associated to DPM, but it is aesthetically undesirable. The fillet should be removed, whereas the rest of the carcass is still fit for human consumption (Jordan and Pattison, 1998). However, the required trimming operations determine 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 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).
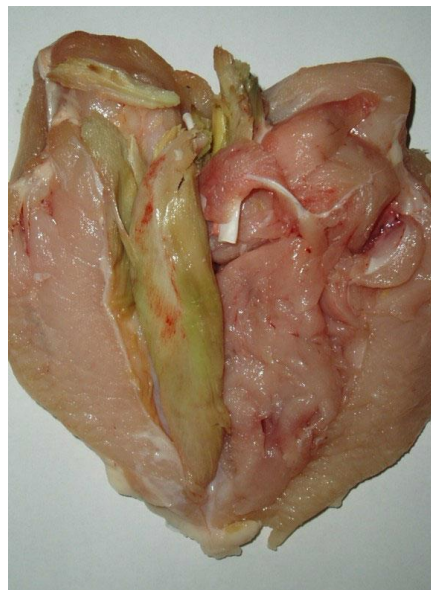

Figure 6.7 - *Deep pectoral myopathy (Kijowski and Konstanczak, 2009)*

6.5.4 White striping in broiler breast muscles

White striping was a condition described in broiler chickens as characterized by the occurrence of white striations parallel to the direction of muscle fibers on both breast fillets and thighs of broilers (Figure 6.8). White striping was considered to be an emerging issue by the poultry meat industry that could be associated with enhanced growth rate and heavier body weight in birds (Bauermeister et al., 2009; Kuttappan et al., 2012a,b), especially in the age of 6 to 8 wk (Bauermeister et al., 2009), and higher fat content in broiler breast fillets (Kuttappan et al., 2012a). At present, there is few information concerning the occurrence of white striping defect under commercial conditions. Petracci et al. (2013a) reported that the incidence of breast white striping in broiler chickens evaluated under commercial processing

was around 12.0% of which 3.1% had severe striping and 8.9 had moderate incidence. However, recent studies carried out under experimental conditions have estimated that the incidence of white-striped breast fillets is over 50% (Kuttappan et al., 2012a,b, 2013a). Based on the incidence of the white stripin, fillets can be classified as normal (NORM, any distinct white lines), moderate (MOD, white lines < 1mm), severe (SEV, white lines > 1 mm) (Figure 6.9) (Kuttappan et al., 2012c).

It was possible that intense selection for rapid growth rate in birds could have accidentally been accompanied by the selection for inadequate capillary/fascial growth or muscle fiber defects leading to myopathic changes referred to as growth-induced myopathy (Mahon, 1999). The precise etiology of white striping has not been defined yet (Kuttappan et al., 2013b), however several speculations have been reported. In turkeys, Wilson et al. (1990) reported that rapid growth rate may have led to limited ability of muscle support systems leading to a condition called focal myopathy, which affected the major pectoral muscle.

Ischemia could also result from rapid growth rate and lead to muscular damage in turkeys (Sosnicki et al., 1991). It was also possible that reduced oxygen supply to breast muscle resulted from lower capillary density in fast-growing chickens (Hoving-Bolink et al., 2000).

Higher growth rate could also lead to defective cation regulation in muscles leading to an increased level of sodium, potassium, magnesium, and calcium in muscle tissue (Sandercock et al., 2009). An increased level of calcium in muscle tissue could initiate several tissue changes including the activation of intracellular proteases or lipases resulting in myopathic changes (Mitchell and Sandercock, 2004; Millay et al., 2009). Kuttappan et al. (2013b) reported that breast fillets showing severe white striping had reduced protein content and myopathic lesions, while Petracci et al. (2013a) observed poor cohesion beneath the striation area.

White striping is not confined on impairing visual appearance only, but could be also associated with differences in chemical composition (Kuttappan et al., 2012a) and reduced technological properties of the meat (Petracci et al., 2013b). Recent results by Petracci et al., 2014 show that both severe and moderate white-striped fillets exhibit higher fat content, lower protein level and decreased quality of protein as proven by higher collagen content. Petracci et al. (2013a) observed that meat with severe white striping resulted in significantly lower shear values (indicating a more tender meat) compared with moderate and normal groups; the same author found also a higher collagen content in muscle affected by white striping (Petracci et al., 2014).

Figure 6.8 *- Breast muscle of chickens affected by white striping*

Figure 6.9 *- Classification of breast meat samples showing white striping defect*
(normal, moderate, and severe). [Petracci et al., 2014]

Chapter 7

7.Meat quality traits

7.1   Chicken Meat Quality

Meat quality can be described as the overall characteristics of meat including its physical, chemical, biochemical, microbial, technological, sensory, nutritional and cooking properties. Some properties are important to consumers including appearance (color), texture, juiciness, tenderness, odor and flavor, which influence their judgement of the meat quality prior or after purchasing the meat, whereas properties such as water holding capacity, shear force, drip loss, cook loss, pH, shelf life, protein solubility, and fat binding capacity are essential for processors of value added meat products (Allen et al., 1998). However, the poultry grading system used worldwide is based on visual attributes such as presence or absence of carcass defects, bruises, missing parts, and skin tears without paying attention to the functional properties of meat, which are extremely important for the further processing industry (Barbut, 1996). However, the two most important quality attributes for poultry meat were appearance and texture since they influenced initial consumer selection of the product as well as final satisfaction (Fletcher, 2002). Appearance quality attributes have included skin color, meat color, and appearance defects such as bruises and hemorrhages. Any deviation from a normal appearance has resulted in meat product rejection, subsequently leading to consumer complaints. Despite the importance of these quality attributes, the poultry grading system used was still based on aesthetic attributes such as conformation, presence or absence of carcass defects, bruises, missing parts, and skin tears, without taking into consideration the functional properties of meat (Barbut, 1996), which have been important for the further processing industry that was mainly interested in the functional properties of meat. Many factors have influenced poultry meat quality, including sex, strain, age, environmental factors, exercise, diet, and processing practices (i.e. chilling, deboning time, marination, and electrical stunning) (Lyon and Wilson, 1986; Braxton et al., 1996; Poole et al., 1999; Northcutt et al., 2001). Meat quality is usually assessed by measuring its pH, color and water holding capacity (WHC), since these are three main attributes for fresh and further processed products.

7.2   Meat color

Meat color is an important quality factor and has a great influence on consumer acceptability and purchase decisions, because consumers relate meat color to its freshness and overall quality. Customers ascribe an intense red colour to raw meat, while they attribute brown-gray or tan colour to cooked meat. Pink colour is characteristic of cured meat products. Poultry is the only species with meat that shows marked differences in color due to muscle biochemistry and histology. These meats are classified as either white (breast meat with pale pink color) or dark (thigh and leg meat with dark red color) meat. White and red fibers have different characteristics, which will be discussed later, but the main noticeable difference to consumers is their color, which is a result of myoglobin pigment concentration. Changes in breast muscle color is more noticeable because of its natural light color and because breast comprises a higher proportion of the carcass (Anadon, 2002). Color variation within a package of skinless fillets could cause rejection of the entire package and a great loss to the industry, because consumers are more sensitive to color variations than to absolute color (Fletcher, 2002).

Meat color varies based on the concentration of myoglobin and hemoglobin (major pigments imparting meat color), pigment chemical state, or the way light is reflected off the meat (Froning, 1995). Hemoglobin is found in red blood cells and its concentration in meat depends on the efficiency of bleeding during slaughter (Swatland, 1994). Myoglobin is a soluble protein formed from a single polypeptide chain, which surrounds an oxygen-carrying heme group composed of an atom of iron and a porphyrin ring. The primary function of myoglobin is to transport oxygen within the muscle fiber (Swatland, 1994). Concentration of myoglobin can be affected by factors such as species, age, sex and genotype (Barbut, 2002), location of the muscle, and muscle activity (Barbut, 2002). Raw meat presents three forms of myoglobin: (1) purple-red deoxymyoglobin (fresh meat and absence of air), (2) bright red oxymyoglobin (presence of oxygen), and (3) brown metmyoglobin (when myoglobin is oxidized). The brown-grey or tan colour of cooked meats is caused from the oxidation and heat-denaturing of the globin hemichrome pigment (Suman and Joseph, 2013). The pigment denatured globin haemochromes can be reduced and becomes pink. This reaction can occur in the anaerobic core of large roasts or meats packaged under vacuum, independently of the cooking level. It is important to notice that denatured globin haemochromes is not the same known as mononitrosylhaemochrome, which is another pink pigment typical of cured meats. It is the consequence of the reaction between pigments of the meat and nitrate or nitrite salts,

or when the meat is smoked with nitrogen dioxide. Oxidation and/or microbial spoilage may promote the development of several green pigments: metsulfmyoglobin and cholemyoglobin in raw meats, and nitrimetmyoglobin in cured meats (Cornforth and Jayasingh, 2004).

Fiber type and myoglobin content have strong effects on meat color, since the basic difference in meat color is due to relative amounts of white and red fibers. Post-mortem temperature and pH play crucial roles on the extent of protein denaturation and physical appearance of meat (Lawrie, 1998). Light scattering from a muscle surface is directly proportional to the extent of protein denaturation, where at a pH $\geq$ 6.0 that protein denaturation is minimal, water molecules are tightly bound, causing more light to be absorbed by the muscle, and the meat appears darker in color. Whereas, at pH $\leq$ 6.0 protein denaturation is higher, light scattering increases, and muscle becomes opaque. Changes in light scattering affect meat lightness (L*), but has a minimal effect on meat redness (a*) and yellowness (b*) that is opposite to that caused by heme pigment concentration (Barbut, 1997a; Swatland, 1994).

Color of meat can be evaluated using different systems, the most popular of which is the CIE LAB, defined by the CIE (1978). Another frequently used method is Hunter L, a, b solids scale. In the CIE LAB, the L* value is an expression of the lightness of the surface ranging from 0-100 (black to white), a* value indicates red, ranging from negative to positive (green to red), and b* value also ranging from negative to positive, which stands for blue to yellow (Barbut, 2002). The time at which L* value is measured could affect reading values of L*, since it is reported that meat lightness increases significantly over time post-mortem (McCurdy et al., 1996).

Meat color have been related to other meat quality parameters and functional properties of meat. L* measurements can be used as an indicator of poultry breast meat quality for further processed products as well as poultry meat defects including PSE (pale, soft, exudative) and DFD (dark, firm, dry) conditions. Dark broiler breast fillets are reported to have significantly lower lightness values (L*), higher redness values (a*), and lower yellowness values (b*) than light broiler breast fillets (Barbut et al., 2005; Bianchi et al., 2007). A wide range in lightness (L*) of breast meat has been reported by different researchers ranging from; 35.3 to 55.6 (Barbut, 1997a), 42.0 to 71.0 (Woelfel et al., 2002), 45.0 to 67.0 (Wilkins et al., 2000), 47.7 to 66.5 (average of 56.7) (Anadon, 2002), 40.0 to 66.0 (Petracci et al. 2004), and 41.0 to 56.0 (Barbut, 1998; Lesiow et al., 2007). This wide variation in L* values are reflective of the wide distribution of muscle pH values at 24 h post-

mortem because these traits are inversely correlated. Variation in L* value depends on many factors (e.g. genetics, age, sex, flock, nutrition, season of the year).

7.2.1 Myoglobin

As mentioned before the most important meat pigment is myoglobin. It is a composite molecule formed by two different portions: protein globin and an haem prosthetic group. Eight helical segments, named from A to H make the globin polypeptide, which form the empty space containing the haem part (Figure 7.1). The haem group has a hydrophobic property and it is arranged in order to orient the vinyl groups in the direction of the hydrophobic core of the box formed by the protein portion around the prosthetic group. The main roles of the globin portion is to confer water solubility and protection from oxidation to the haem prosthetic group. The reason why myoglobin is able to absorb visible light (and reflect a range of red colours) is because of the resonant nature of the conjugated double bonds of haem (Castigliego et al., 2012).

Haem ferrous iron (Fe^{2+}) may form six coordinate bonds, one for each electron in its external orbital. It is fixed to the haem porphyrin ring through four bonds with pyrrole groups and to the globin through one bond with histidine F8 (also known as histidine 93 or proximal histidine). Moreover, another amminoacid residue stabilizes (without bond) the haem group into the haem box the histidine E7 (also known as histidine 64 or distal histidine). The sixth position is vacant and could be used for binding oxygen or other small ligands such as carbon monoxide (CO) The formation of metmyoglobin is due by the oxidation of ferrous iron (Fe^{2+}) to the ferric form (Fe^{3+}), which is physiologically inactive because haem ferric form is unable to bond oxygen. Myoglobin molecule is only polypeptide composed of 153 amino acid residues. Its molecular weights vary according to different species and it is about 17 kDa (Castigliego et al., 2012).

Figure 7.1 - Molecule of myoglobin (Castigliego et al., 2012).

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7.2.2 **Common fresh meat pigments**

Myoglobin presents three different forms deoxy-, oxy-, and metmyoglobin, that are soluble in water. Oxymyoglobin confers to the fresh cut meat its typical colour (bright red).

When raw cut meat is exposed to air, it goes toward the process of oxygenation called also 'blooming'. Oxygen takes about 30 min to oxidize myoglobin and obtain oxymyoglobin, that presents ferrous state (Fe^{2+}) haem iron. Metmyoglobin is obtained from the oxidation of the myoglobin haem iron. Luckily, when dioxygen is liberated from oxymyoglobin, it does not oxidize directly the haem iron. In fact if it would be the operation, oxymyoglobin would not function like storage molecule of oxygen. Nevertheless, oxygen represents an oxidizing agent in meat products, this oxidation is the cause of formation of metmyoglobin and one superoxide (one electron reduction product of dioxygen) (Figure 7.2). In presence of oxygen (20%) the process of metmyoglobin formation in meat is slow (over days). This process could be much faster at concentrations of oxygen equal or less than 1%. Other factors that promote the metmyoglobin formation are: high temperatures, acid conditions (pH < 5.5), presence of oxidizing agents (i.e. sodium nitrite, potassium ferricyanide, sodium chloride). A successful inhibiting process of the metmyoglobin formation in meat is the vacuum packaging (Cornforth and Jayasingh, 2004; Castigliego et al., 2012).
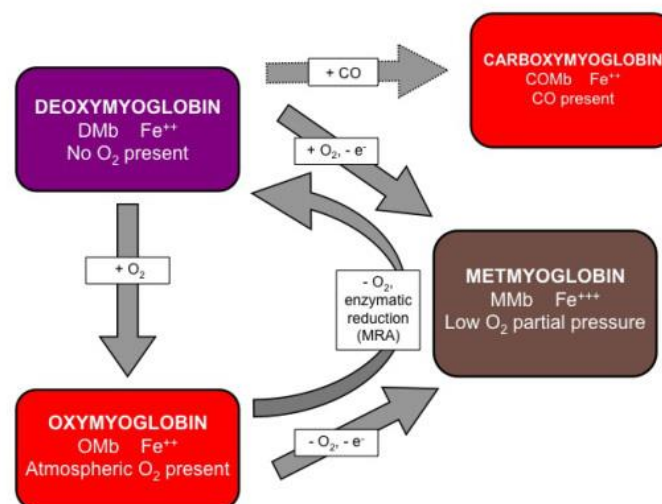

Figure 7.2 *- Forms of myoglobin, adapted from Mancini & Hunt, 2005*

[https://meatisneat.wordpress.com/2009/09/23/color-of-fresh-meat/ (downloaded 1st February 2016)]

7.3 Meat pH

Meat pH by far is the most important parameter contributing to meat quality and protein functionality. Muscle pH is associated with numerous other meat quality attributes such as color, WHC, tenderness, juiciness and microbial stability (shelf-life) due to its effect on protein structure and hydration properties. The ultimate pH is the value measured at *rigor mortis*. The pH of a solution represents the concentration of H+ that when reacts with a H_2O produces a hydroxonium ion (H_3O^+). pH is expressed as negative decimal base logarithm (log10) of the H+ ions in the solution. In water solutions pH varies from 0 (acid) to 14 (basic). Only water without any salts dissolved shows a pH value of 7.0 (Honikel, 2004). The formation of hydrogen ions in solution occurs when acids for example lactic acid (typical on meat; CH3–CHOH–COOH) dissociates as below (Barbut 2002; Honikel, 2004):

$$CH3–CHOH–COOH \quad CH3–CHOH–COO− + H$$

The initial rate of pH decline and ultimate pH of meat, reached at 5-6 h post-mortem, are the two principal causes of variation in the quality of chicken meat. pH changes during the process of transformation of muscle to meat, more precisely pH strongly decreases from alive muscle (about 7.0 – 7.2) to the final value measured in the carcass (about 5.3 – 5.8) known as ultimate pH (pHu). This pH downfall is determined by the development of lactic acid (about 0.1 mol/l) derived from anaerobic glycogenolytic pathway that uses glycogen to obtain glucose and consequently, energy. When the same amount of lactic acid is dissociated in a water solution the pH detected is around 2 units, instead of the pH 5.3–5.8 measured on meat. This difference (more than 3 units) is the consequence of the buffering capacity of some constituents in meat (amino acids side chains, peptides as carnosine and anserine, phosphate ions, etc.).

The detection of the meat pHu is done in numerous times after slaughtering of animals according with species, kind of muscle and level of stress during the pre-slaughter period. In general, values of pHu are: 5.5–5.8 in pork muscles after 6–8 h *post mortem*; 5.5–5.6 in beef muscles after 18–36 h *post mortem*; about 6.0 in chicken after 2–4 h *post mortem* (Barbut 2002; Honikel, 2004).

A strong relationship between pHu and lightness (L* value) of the breast meat in chicken (Allen et al., 1997, Qiao et al., 2001; Anadon, 2002) and turkey (Owens et al, 2000) is reported. Furthermore, pHu and L* are strongly linked and correlated with drip loss of the

raw meat (Le Bihan-Duval et al., 2001) and water-holding capacity (Le Bihan-Duval et al., 2001) and cooking yield (Debut et al., 2003). As pH of meat drops to the isoelectric point (where positive and negative charges are equal on proteins), solubility and water binding capacity are minimal, because firstly there is no net charge on the proteins to bind to water molecules and secondly there is less space for water within myofibrils due to increased affinity within myofibrils. Isoelectric point of muscle myofibrillar proteins is close to a pH of 5.5 (Swatland, 1994). However, Srihari et al. (1981) have reported different isoelectric points for various components of myofibrillar proteins, with actin having pH from 5.5 to 5.8; myosin heavy chain from 6.3-7.3 and myosin light chain from 4.8 to 5.6. Understanding the relationship between pHu and L* value could be useful for the quality differentiation of poultry meat, making it possible to use this meat adequately by the industry.

The speed of pH alteration *post mortem* is an important trait that affects meat quality. High final or "ultimate" pH produces dark, firm and dry (DFD) meat with high WHC, but a poor storage quality due to high moisture content and a faster rate of off-odor production and accelerated microbiological growth (Allen et al., 1998; Le Bihan-Duval, 2004). In this case pH downfall stops prematurely when pH achieves values around of 6.0 due to the end of glycogen reserves. In fact less glycogen content on meat results in lower content of lactic acid. In this case the high pH values promotes the obtainment of darker meat, with lower drip loss and less tender meat (Shen et al., 2009). On the contrary, a fast drop in pH *post-mortem* and a low final pH produces meat with an improved shelf life but a pale color, soft texture and reduced WHC (PSE; pale soft, exudative). When pH value falls very fast, and the meat is still warm, meat proteins undergo to denaturation process.

7.4   Water holding capacity (WHC)

As mentioned previously in the chapter 6, water represents the largest portion of muscle tissue (about 75%) and it is organized in layers located around polar molecules and among stratums of cellular materials. Several forces control meat water movements (Pearce et al., 2011).

WHC is one of the most important functional properties of meat and an important quality attribute for both processors and consumers. Water-holding capacity or juiciness is definite as the aptitude of meat to hold water (naturally present in meat or added) during the application of forces like heat and pressure. The ability of meat to hold water helps with tenderness, juiciness, firmness and appearance of the meat, leading to an improvement in quality and economic value. WHC of meat can be categorized as water binding potential

(WBP), expressible moisture and free drip, each having different applications. For instance, WBP is defined as the ability of the muscle proteins to retain excess water and under the influence of external forces; therefore it represents the maximum amount of water that muscle proteins can retain under the established condition (Swatland, 1994). Expressible moisture represents the amount of water that can be expelled out of the meat by use of force, and free drip is the amount of water lost from the meat without any force other than gravity (Swatland, 1994), which is important for retail display and consumer acceptability of tray packed meat.

Most of the water (88 to 95%) inside the muscle is held within intracellular spaces between actin and myosin filaments, and only a small portion (5 to 12%) is located between the myofibrils (Offer and Knight, 1988); the portion of water that affects more the meat water-holding capacity (WHC) is located among those miofibrillar proteins (actin and myosin) where it is detained in intermolecular spaces by capillary force. Several factors such as pH, sarcomere length, ionic strength and osmotic pressure affect WHC (Offer and Knight, 1988) because they all influence the distance between myosin and actin/tropomyosin. This space can range from 320 Å to 570 Å and is preserved by electrostatic forces, which are active even for relatively long distances (Brewer, 2004; Pearce et al., 2011). Development of rigor mortis also influence WHC. After animal death, lactic acid is produced and pH declines causing a reduction in water binding ability of the meat due to protein denaturation, loss of protein solubility and therefore reduction of reactive groups available for water binding on muscle proteins (Offer and Knight, 1988). When pH decreases to values close to the isoelectric point of proteins (4.9-5.3), water binding ability of the proteins is impaired, thick and thin filaments move closer together, myofibrils shrink, and the volume of sarcoplasm increases. Eventually, muscle fibers deplete all their ATP, their membranes no longer confine the cell water, and fluid is lost from the muscle fiber that may contribute to the exudate lost from the meat (Swatland, 1994).

Proteins (for example actin and myosin) are molecules formed by amino acids jointed among themselves by peptide bonds in order to form an amino acidic chain, that represents the primary structure (sequential order of amino acids). The polypeptide chain is structured in order to form a three-dimensional molecule, which represents the second and the third structure. Finally proteins can show a quaternary structure that explains the geometric organization between different polypeptide chains usually bonded with each other through no covalent bounds. Amino acids present several side chains that are externally located respect the main protein filament. They can be charged in different ways (neutral, positively or negatively) according to the type of amino acid and environmental pH. As previously

mentioned, during the transformation of muscle to meat, pH decreases as a result of the increase of the muscle lactic acid amount. This dramatic decreasing of pH value causes the reduction of the protein reactive charged groups, which represents the ones able to bond free water. This variation of pH determines a relevant decrease of WHC values it mainly depends of three factors:

a) **Net charge effect**. Definite as the total quantity of amino acidic charged groups able to bond water molecules. Meat pH, as a consequence of lactic acid accumulation, decreases until it reaches the isoelectric point (at this pH protein presents the same number of negatively and positively charged groups). In this way, for muscle proteins the pH is around 5.5. In this conditions, only a few groups of the side chain are able to interact with water (net charge effect). When pH is around 7.0 (live muscle pH), muscle WHC presents higher value than after rigor-mortis with pH around 5.6. This shows the possibility to obtain a better net charge at a higher pH and consequently proteins can held more molecules of water (Barbut, 2002; Huff-Lonergan and Lonergan, 2005).

b) **Steric effect**. Definite as the repulsion observable fact noticeable between different side chains charged with a analogous charge. It is well known that charged groups with the same charge repulse themselves. This represents a positive phenomenon, especially for the meat processor, because more molecules of water can be held if bigger spaces between fibre filaments are formed. It can be possible at pH lower or higher according to the isoelectric point, where an elevated quantity of negatively or positively charged groups are present, resulting in more repulsion. WHC of *post mortem* meat is abridged, for the reason that the pH is near to isoelectric point. *Post mortem* meat WHC can be increased with the variation of meat pH using alkaline (e.g. phosphates) or acid ingredients (Huff-Lonergan and Lonergan, 2005).

c) **Ions exchange**. Definite as the phenomenon that occurs when *rigor mortis* has been completed; during the process of aging, when ions are relocated after degradation of cell structure performed by enzymes located in myofibrillar proteins. Some divalent cations as Mg^{2+} or Ca^{2+} are replaced with monovalent cations like Na^+ and K^+, resulting in the creation of free side groups charged of protein, which increases the meat WHC. Calcium (Ca^{2+}) is a divalent cation that is released during *post mortem* process. It has the ability to bind and consequently neutralize two negatively charged side groups. When calcium is

Influence of different prebiotics and mode of their administration on growth, carcass traits and meat quality in broiler chickens

Cinzia Abiuso (2015)

substituted by monovalent ions, proteins increase the number of free binding site to bind water (Pearce et al., 2011; Barbut, 2002).

Every chemical, physical or enzymatic process that increases the space between protein filaments improve the quantity of water to be held and thus increasing the WHC. This is possible because the large part of water in muscle is located in the space between thick (myosin) and thin (actin) filaments that form myofibrils. This is the reason why salt (NaCl or KCl) and phosphates are used to improve water holding capacity in meat product. Usually salt and phosphate in water solution are included into raw meat by injection, marination or tumbling, resulting in a higher juiciness and cooking yield of the product.

In order to estimate WHC in raw meat and in meat products the following methods are usually used: (1) applying pressure (mild to severe; by compression or centrifugation); (2) monitoring sample performance during regular processing such as cooking or storage; (3) watching meat product microstructure; (4) applying special technique like nuclear magnetic resonance (NMR) to check water molecules state and position; (5) using optical sensors (Barbut, 2002).

7.5   Tenderness

Poultry meat tenderness was one of the most important meat quality attributes related to consumer acceptance. Tenderness development was a function of myofibrillar proteins denaturation, connective tissue content, and juiciness. Deboning time, age, and strain were some of the major factors that affected poultry meat tenderness (Northcutt et al., 2001). Lyon et al. (1992) reported that as time before deboning increased from 0 to 24 h postmortem, consumer acceptability of the meat texture increased, with fillets deboned at 0 and 2 h postmortem considered tough by a consumer panel, and samples deboned at 6 and 24 h postmortem considered slightly tender to moderately tender. Liu et al. (2004) reported decreased shear force of chicken breast as deboning time increased from 2 to 24 h postmortem. Similar results were also reported by Cavitt et al. (2005). Mehaffey et al. (2006) reported that fillets deboned 2 or 4 h postmortem from broilers raised to 7 wk were significantly more tough than those raised to 6 wk, indicating that age affected tenderness when deboning was performed shortly after harvest. Northcutt et al. (2001) reported that breast fillets harvested at less than 2 h postchill aging were more tender when taken from broilers slaughtered at 42 or 44 d of age compared to those harvested from birds 49 or 51 d of age, irrespective of any sex effect. On the other hand, Young et al. (2001) reported that females had greater fillet yields than males.

Connective tissue content has been reported to increase with age, collagen was the most abundant protein in the body and made up the majority of the connective tissue proteins (Hultin, 1985; Bechtel, 1986). In young broilers (6 to 8 wk), connective tissue did not affect tenderness since mature cross-links had not yet formed between tropocollagen molecules, which were the structural unit of collagen fibril. On the other hand, contraction of myofibrillar protein, which depended upon time and rate of rigor mortis development after the bird was killed, was related to processing rather than intrinsic factors (Fletcher, 2002).

Tenderness together with juiciness and flavour determine the palability of meat (Troy and Kerry, 2010). This attribute is strongly related to the overall acceptability and preference sensitivity of consumers. Tenderness is defined as the easiness which is possible to crash the meat during chomping. The opposite of tenderness is known as toughness, in other words the opposition of meat to be munching and chewing (Miller, 2004). Meat tenderness improving subsists in a multifarious process affected by several factors: structural design of muscle, integrity of muscle fibres, endogenous proteases activity, and influences of extracellular matrix (McCormick, 2009; Maiorano et al., 2015).

Meat is a complex biological tissue, which presents many structural and metabolic characteristics that are responsible for its toughness after cooking, when it is ready to be eaten. Some of those factors, listed in Table 7.1, are called "intrinsic determinants of tenderness". Several intrinsic determinants usually affect the tenderness of meat, and rarely depends of only one of them (Purchas, 2004).

The main components of meat are: lean tissue, fat and connective tissue. As previously mentioned, it is possible to find fat or adipose cells located intramuscularly, known as marbling. Marbling is responsible for the meat flavour, juiciness and tenderness. Meat tenderness is affected by intramuscular fat content, even if this connection is not always clear. In fact, there are four different theories in disagreement discussing the relationship among meat fat contents and tenderness (Miller, 2004; Troy and Kerry, 2010):

a) **Bulk density theory**: because fat is not dense as heat-denatured meat proteins, meat that presents higher quantity of fat or soft tissue isles tough. In other words, meat tenderness is superior when it presents elevated percentage of soft constituents (such as fat);

b) **Theory of the lubrication effect**: while meat is masticated, lipids store in adipose cells, that constitute marbling, are liberated and become available to lubricate the muscle fibres. In this way, muscle fibres that show elevated quantity of lipid may be slide with less difficulty transversely each other, as consequence meat is

perceived as more tender because of the less opposition at the chewing (Miller, 2004);

c) **Insurance theory**: practically fat alleviates the effects of strict heat-induced toughening of meat submitted to a cooking process. Cooking process, if very severe, can strongly denature meat protein and cause loses of part of the aptitude to hold water (cooking loss). The more concentrated the muscle fibre proteins, the less tender the meat. This is why it is important to limit the quantity of liquid lost during cooking in order to not concentrate muscle fibre proteins. Lipids alleviate the protein denaturation because they work like insulators, because heat is not effortlessly transferred through fat. In other words, meat that contains elevated percentage of fat will decrease heat transmission during cooking. In this way, heat is not applied quickly and strictly, an meat proteins can denature in a mild way, causing less cooking loss, producing a more tender meat (Miller, 2004);

d) **Strain theory**: marbling is located in connective tissues (perimysium); if adipose tissue augments, perimysial result destabilized and does not affect negatively the meat tenderness. Connective tissues are consequently stressed and meat is less tough. All those four theories are not easy to investigate and they depend from each other (Miller, 2004).

Meat before considerable ageing can present different initial tenderness according with different muscles, individuals and species. Tenderness is also affected by features like muscle shortening, which is not usually easy to establish (Devine, 2004).

Table 7.1 - Some intrinsic determinants of meat tenderness (Purchas, 2004).

Intrinsic determinant	Relationship with tenderness	Relative importance, and situations in which it might be particularly important
1. Concentration of connective tissue. This tissue consists primarily of the fibrous protein collagen, but will also include some elastin and other substances.	Other things being equal, meat containing more connective tissue will be less tender, but this will depend on the nature of the connective tissue and the cooking conditions.	Of medium importance. More important for comparisons between different muscles, between meat samples from older animals, and when cooking conditions have been mild (i.e. when final internal temperatures are less than about 60 °C so that most collagen is not dissolved).
2. The extent of cross-linking between peptide chains within collagen molecules in meat.	Other things being equal, meat containing collagen with fewer cross-links will be more tender because such collagen will dissolve to form gelatin faster and at lower temperatures.	This is an important source of variation in tenderness if samples vary widely in the level of crosslinking, as might be expected if they are from animals varying widely in age (crosslinking

		increases with increasing age). It is also more important for fast cooking methods such as frying.
3. The ultimate pH (pHu) of the meat, as determined primarily by the amount of lactic acid present, which in turn is a function of the glycogen levels at the time of slaughter.	Other things being equal, an increase in pHu from about 5.5 (the normal pHu for meat from a well-fed and unstressed animal) to 6.1 will initially lead to tougher meat. With further increases from about 6.2 to 7.0, tenderness increases.	An important determinant of tenderness in some situations where the variability of pHu is high. In many situations it is of low importance because there is little variation in pHu between animals.
4. The extent to which muscle is contracted when it sets in rigor mortis, as assessed by the average sarcomere length.	Other things being equal, a greater degree of contraction (shorter sarcomere lengths) will be associated with tougher meat. This relationship is not linear and muscle shortened by more than about 40% of its resting length will actually be more tender due to structural damage.	Very important as a determinant of tenderness under cold-shortening conditions when low temperatures pre-rigor induce muscle contraction. Thaw-shortening of meat frozen prior to the onset of *rigor mortis* can also lead to very tough meat. If shortening is prevented in some way, this is not an important determinant.
5. The extent to which certain proteins in meat are broken down post mortem through the action of proteolytic enzymes such as the calpains and cathepsins.	Other things being equal, a greater degree of protein breakdown is associated with more tender meat, but the extent of this effect will depend on the specific proteins that are cleaved.	An important determinant of the extent to which tenderness improves with ageing of meat at temperatures above freezing. It also accounts for some genetic differences in tenderness through varying levels of proteolytic enzymes (e.g. the calpains) and/or their inhibitors (e.g.calpastatin).
6. The concentration of intramuscular fat (marbling) in muscle. Levels vary from less than 2% in many lean meat products through about 3% when the marbling first becomes clearly visible, up to levels of over 30% in very heavily marbled products.	Other things being equal, more highly marbled meat will be somewhat more tender. The reasons for this are unclear, but probably include the fact that the meat will tend to be more juicy, that muscle fibres and connective tissue are diluted by fat, and a reduced likelihood of pre-rigor shortening.	This determinant seldom accounts for more than 10% of the variation in tenderness, but is likely to be more important when there is a wide variation in marbling level.

If meat is severely affected by muscle shortening, it does not age effectively and as a consequence remains still tough. The degree of cold shortening influences the tenderization process because it impedes the large part of the ageing chemical changes (calpain system, proteolysis). Sometimes cold-shortened meat still presents some changes in tenderness after a while due to the premature beginning of the rigor in several muscle fibres that allows a protection effect against cold-shortening.

Other factors can add more variability, for example the different temperature crossways muscles. In some cases muscle's sarcomere length depends also from the way that the carcass is hung. For example, it enhances considerably if carcass is suspended from pelvis rather than from Achilles tendon. In this way meat is ready to be consumed greatly earlier compared with traditional carcasses hanging. This results in a difference in tenderness (longer sarcomere

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allows more tender meat), but this divergence can also be compensated with a long ageing period (Devine, 2004; Juárez et al., 2012).

When pre-rigor meat is took away from carcass skeletal connections, during operation of hot or warm, boning rigor shortening can occur, resulting in less tender meat. This drawback occurs only if temperature falls under 10°C. Thus if temperatures around 10–20°C are maintained during *rigor mortis*, is possible to obtain hot-boned meat with similar ageing characteristics of meat leaved attached on skeletal.

To promote aging process and to obtain more tender meat in a short time, it is possible to use electrical stimulation. This practice allows the earlier start of rigor for some fibres compared with no stimulated muscle resulting in a faster aging that occurs at high temperatures before cooling down (Devine, 2004; Juárez et al., 2012).

Meat and meat batters rheological properties and tenderness depend from several changes performed by different proteins. Muscle texture is largely affected by the amount of collagen and the number of its cross-linking, tissues morphological configuration of meat, muscle biochemical condition (pre and post rigor) and mechanical breakdown of the meat structure (Juárez et al., 2012).

The tenderness of the muscle structure and the formation of collagen gels are affected by the changes that occur after the animal death (*post mortem* changes or aging) and the time –temperature management. When heat is applied on meat the below facts occur: (1) at 40–50°C meat tenderness decreases for the reason that actomyosin denatures; (2) at 60–75°C meat tenderness further decreases because shrinkage of intramuscular collagens; (3) at 70–90°C meat presents further actomyosin shrinkage and dehydration and collagen gelatinization; (4) at superior temperatures the general effect of heating results in the softening of the intramuscular collagen fraction and in the hardening of myofibrillar proteins fraction (Figure 7.3; Palka, 2004; Juárez et al., 2012).

Meat tenderness varies according to fibre size, sarcomere shortening grade (during rigor and during heating at 70°C) fibres type, numbers of collagen cross-linking. For example, endomysium during cooking shrinks and presses out the intramuscular water and as a consequence, it can produce a less tender meat with tightening structure. When meat undergoes through a long mild cooking process (4–8 h for 50–60°C) its tenderness improves, even if juiciness and flavour are negatively affected. Meat with elevated pH and low drip loss shows less cooking losses and it is less tough after heating (Palka, 2004; Juárez et al., 2012).
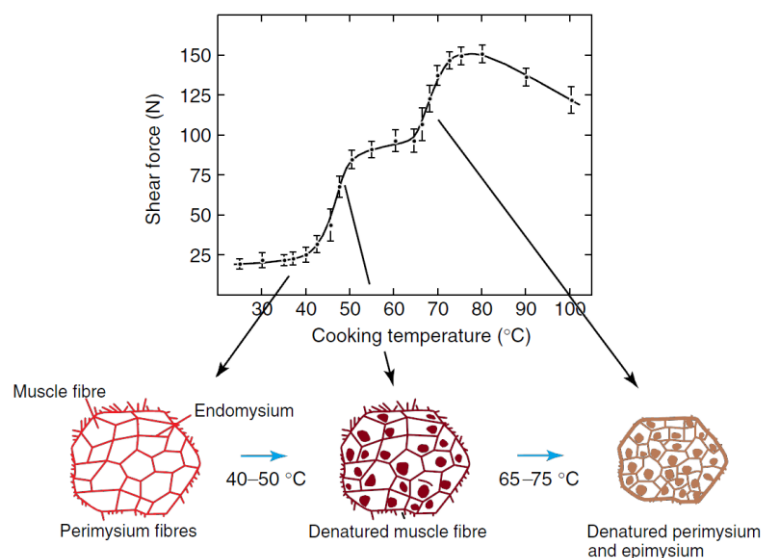
Figure 7.3 *- Meat structure and tenderness changes with increasing cooking temperature (during one hour)*
(Devine, 2004)

As previously mentioned during meat pH decreasing, lateral electric charge of amino acids modified until achieves the isoelectric point (some amounts of negative and positive charges). As a consequence myofibrillar structures filled with water shrink because negative and positive charges were attracted by themselves. This phenomenon causes the reduction of the space capable of holding the water (Pearce et al., 2011; Juárez et al., 2012).

This water goes to the sarcoplasm, where it is not strongly held (only by the action of the cellular walls). Meat enzyme or mechanical action during process operations break cellular membranes, that make weaker the 'bound' water – intact cell membranes resulting in water loss when forces like gravity, pressure and vacuum are used. In a few words, pH, shrinkage and breakdown of membranes reduce meat water-holding capacity and as a consequence also the tenderness decreases. During the aging period enzymatic action reduces the toughness of meat, breaking the cross-links between filaments, that are formed during *post mortem* period (meat presents the higher hardness values at the beginning of *rigor mortis*) (Juárez et al., 2012).

Final tenderness of meat is extremely affected by the degree of alteration and deteriorating of myofibrillar fibre, explaining the increase in tenderness in aged meat compared to non-aged meat. The rupture at the junction between the I-band and Z-disk, in addition to the degradation of cytoskeletal proteins, the acto-myosin complex, and other myofibrillar proteins like titin, desmin, troponin-T, or nebulin, strongly influence the meat tenderization (Koohmaraie and Geesink, 2006). Lysosomal cathepsins, calpain system and multicatalytic proteinase complex are the three endogenous proteolytic enzymatic systems that are responsible for the enzymatic meat tenderization as a consequence of the myofibrillar

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degradation (Koohmaraie and Geesink, 2006). Cathepsins are situated in lysosomes. This group of enzymes (endo- and exopeptidases) was the first enzymatic system to be linked with *post mortem* mechanisms of meat tenderization, but this primary attention was later abandoned on basis of numerous observations (Kemp et al., 2010). Even though this observation, cathepsins show a proteolytic activity on nebulin, myosin, actin, and tropomyosin analyzed in vitro. However, the degradation of samples is still different when compared with the ones observed in *post mortem* muscles. Consequently, calpain and caspase systems seem to be responsible for proteolysis of the majority of myofibrillar proteins for the duration of the *post mortem* period (Koohmaraie and Geesink, 2006).

The calpain system is leaded from minimum three calcium-activated proteases: μ-calpain, m-calpain, and calpain 3. In this system is also important the role of calpastatin, the inhibitor of μ- and m-calpain. μ- and m-calpain are activated respectively by a micro- and millimolar concentration of calcium. m-calpain does not present a significant effect on meat tenderization because it is more stable than μ-calpain in *post mortem* muscles, where there is not enough calcium to activate m-calpain. Moreover, different studies on mice indicate an elevated effect of μ-calpain on protein degradation and meat tenderization, but almost nothing on calpain 3 (Kemp et al., 2010). μ-calpain and calpastatin interaction seems to be the most important cause of *post mortem* proteolysis and as a consequence, meat tenderization.

Although the most common assumption of the significance influence of calpain system on meat tenderness, latest approaches propose that other processes and systems can contribute, for example caspases protease (Ouali et al., 2006). With the slaughter and exsanguinations processes, cells stay without oxygen and nutrients, during which caspases starts their catalytic pathways. Until now the role of caspases is still not well known and it is still under study. Some in vitro studies about degradation of myofibril proteins by caspases, sustain the hypothesis of an important interaction among calpain and caspase protease systems (Kemp et al., 2010).

7.6   Flavour of poultry meat

Meat products' flavour, as for other foods, is one of the most important factors that determines its acceptance between consumers. Flavour is defined as a combination of two main senses: taste (perceived by tongue receptors) and aroma (perceived from olfactory receptors of the nose) (Farmer, 1999). The perception of flavour and taste is still not completely comprehended because it represents an extremely complex system that is

influenced by numerous elements (e.g., flavour compounds composition and temperature of food).

Taste is detected by tongue sensors able to notice four main sensations called salty, sweet, acid and bitter. Other feelings like "umami" (Japanese word that means very tastiness), astringency, metallic and painful ("hot" and "cooling" foods) are also noticed. Several studies have reported how tasty compounds contribute to meat flavour. The large part of the works are focused on red meat, but comparable results are estimated also for poultry meat. Meat Flavour is affected principally by genetic type, meat component, diet, post processing process, cooking, additives and use of irradiating and pressure (Jayasena et al, 2013).

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Chapter 8

8. Connective tissue

8.1 Composition of connective tissue

Connective tissue consists of proteins, complex polysaccharides and water as different mixtures depending on the type of tissue. In intramuscular connective tissue (IMCT) the main protein is collagen and another important protein is elastin. Polysaccharides, which often are bound to protein, form together with water the ground substance (of IMCT). The IMCT in meat is in three hierarchical levels (Figure 6.2): epimysium is the layer surrounding the whole muscle, perimysium surrounds bundles of muscle fibres, and individual muscle fibres are surrounded by the endomysium (Bailey and Light, 1989; Alberts et al., 2002).

8.1.1 Proteins

The major protein in connective tissues is collagen and different types of collagen comprise up to 25-30 % of total protein in mammal bodies (Bailey and Light, 1989). Collagen forms rather inelastic fibrous structures, but also filamentous and network-like structures in order to hold the structure of organs, to provide a framework for transforming the force developed in the contractile filaments and to give the environment for cell proliferation and growth (Bailey and Light, 1989; Kjaer, 2004). To date, 29 different types of collagen have been identified (Myllyharju and Kivirikko, 2004; Veit et al., 2006).

8.1.1.1 Collagen

Collagen is the most abundant mammalian and avian protein and it is a connective tissue constituent that is present in all tissues. The family of collagen types can be divided into fibre collagens (I, II, III, V and XI), network forming collagens (IV, VIII and X), with special attention to type IV collagen in basement membranes, fibre associated collagens with interrupted triple helix (IX, XII, XIV, XIX and XXI), filamentous collagen (VI), anchoring fibres forming collagen (VII), collagens with transmembrane domai (XIII and XVII) and the collagens that have been only partly characterised (Bailey and Light, 1989; Prockop and Kivirikko, 1995). Most abundant types of collagen in IMCT are, however, the types I, III, IV, and V (Light and Champion, 1984). Molecules of all collagen types are similar in basic

structure which is triple helix composed of three polypeptide chains (α-chains). This folding is not as common in protein as α helix or β sheet folding. Thus, in this way collagen is special. The molecules of the most abundant fibre forming collagen, type I (approximately 300 nm long), align next to each other forming striated fibrils. These striated fibrils form the collagen fibres (Alberts et al., 2002; Bailey and Light, 1989; Orgel et al., 2001).

The difference between collagen types comes from the amino acid composition of the α-chains and either the similarity or difference of the three α-chains in one molecule (Bailey and Light, 1989; Prockop and Kivirikko, 1995; Alberts et al., 2002). The amino acid compositions of the most abundant types of collagens in meat are shown in Table 8.1. Hydroxyproline, the special amino acid for collagen, is not frequently found in other proteins in animal tissues and so the amount of it has been used for decades as a tool to determine the collagen content of tissues (Neuman and Logan, 1950; Bergman and Loxley, 1963; Alberts et al., 2002). Quantity, solubility and cross-linking of collagen has been widely studied in relation to meat tenderness as will be discussed later (for example Hill, 1966; Fang et al., 1999).

Table 8.1 - *Amino acid composition of the a-chains of the main collagen types in meat (Bailey and Light, 1989)*

Aminoacid	Type I		Type III	Type IV		Type V	
	α1(I)	α2(I)	α 1(III)	α 1(IV)	α 2(IV)	α 1(V)	α 2(V)
Hyp	102	87	127	146	127	107	108
Asp	43	46	43	48	52	55	51
Thr	17	19	13	18	28	27	22
Ser	39	36	39	34	26	34	26
Glu	74	69	72	77	62	90	99
Pro	139	123	109	79	69	92	119
Gly	352	350	354	318	313	318	320
Ala	121	113	97	31	49	59	46
Val	21	34	14	30	24	30	19
Cys	-	-	2	-	-	-	-
Met	7	5	8	15	12	10	7
Ile	7	15	13	34	42	18	20
Leu	21	33	22	54	59	37	40
Tyr	2	4	3	5	6	-	1
Phe	13	12	8	24	37	12	12
Hyl	5	9	5	61	42	25	36
His	2	11	6	6	8	10	7
Lys	31	23	30	5	6	18	19
Arg	51	53	47	20	45	57	48

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8.1.1.2   *Elastin*

Elastic fibres give the resilience, which is necessary in muscles, but also for example in skin, blood vessels and lungs to recoil after transient stretch. Elastic fibres contain 90 % elastin, another important connective tissue protein that forms a core. The core is surrounded by microfibrils that are composed of distinct glycoproteins. Elastin is highly hydrophobic and insoluble and it is formed from its soluble precursor tropoelastin via cross-linking (Alberts et al., 2002; Mithieux and Weiss, 2005). Elastin is also very stable: its estimated half life is about 70 years (Petersen et al., 2002). Elastic fibres can undergo several cycles of stretching and recovery and the maximum extension of elastic fibres before rupture varies between 100-220 % of the original length. The structure of elastic matrices varies among tissues depending on the function of the tissues (Fung et al., 1993; Mithieux and Weiss, 2005).

Because of the hydrophobic and insoluble nature of elastin, it is also highly resistant to proteolytic attack. However, an elastin degrading enzyme, elastase, has been established. Elastase is secreted from macrophages and granulocytes. The inactive form, pre-elastase, is activated by a trypsin-like enzyme (Bailey and Light, 1989). To the author's knowledge the exact mechanism of elastin degradation is not completely known.

8.1.2   Carbohydrate containing components of connective tissue

8.1.2.1   *Proteoglycans*

Proteoglycans are connective tissue macromolecules that provide a hydrated space around cells. The physical behaviour of proteoglycans, however, is mostly determined by the common properties of the glycosaminoglycans (GAGs) that are covalently bound to the core protein (Ruoslahti, 1988). The core proteins of proteoglycans are an extremely diverse group as only few small families of core proteins have been recognised but no common structure for the proteins has been identified. Scott (1988) concluded that only a very simple classification could be done on core proteins: i) small, ii) large and iii) very large.

The GAGs are unbranched polysaccharide chains that consist of repeating disaccharide units. One of the disaccharide units is always an amino sugar. A molecule to be titled as proteoglycan has to have at least one GAG attached to the core protein, but the number of GAGs bound to the core protein can vary between 1 and over 100 (Ruoslahti, 1988; Scott, 1988; Alberts et al., 2002). The GAGs can be divided into four types: 1) hyaluronan, 2) chondroitin sulphate and dermatan sulphate, 3) heparan sulphate and 4) keratan sulphate.

Most of the extracellular proteins, such as collagens, fibronectin, and growth factors have binding sites for GAGs. The GAGs also bind large amounts of water relative to their weight; therefore also the matrix with GAGs can bind large amounts of water (Ruoslahti, 1988). However, for whole muscle, most of the water is located in the myofibrils between actin and myosin filaments and thus, the properties of myofibrillar proteins determine the water holding capacity of whole meat (Lawrie, 1998 pp. 219-220).

Weber et al. (1996) showed that the binding of core protein of decorin, a small proteoglycan with chondroitin/dermatan sulphate GAGs, to collagen molecule promotes the formation of correct type of collagen fibril. Velleman et al. (1996) reported that in chickens with genetic muscle weakness an increased amount of decorin at 20 d of embryonic development was followed by an increase in the content of mature collagen cross-link, hydroxylysylpyridinoline (HP), in breast muscles at six weeks post hatch. Based on these, McCormick (1999) suggested that the expression of decorin would regulate the content of HP via the regulation of alignment of collagen molecules in during the formation of collagen fibril.

8.1.2.2   *Glycoproteins*

Glycoproteins differ from proteoglycans most clearly by structure and polysaccharide chain content. Glycoproteins contain typically 1-60% carbohydrate by weight whileproteoglycans can contain up to 95% carbohydrate by weight. In addition, the oligosaccharide chains of glycoproteins are relatively short and branched, whereas the GAG chains of proteoglycans are long and unbranched (Alberts et al., 2002).

Fibrillin, a glycoprotein, has been found in muscles in perimysium and in endomysium, but also in skin, vasculature, kidney and lung. It has been located in the elastic fibres (Sakai et al., 1986). Fibrillin is one of the many glycoproteins that surround the elastin core in the elastic fibres. It has been demonstrated that fibrillin together with collagen VI contribute to the cell adhesion *in vitro* (Kielty et al., 1992).

Fibronectin, a large glycoprotein, is a dimer that is composed of two large subunits. Each subunit has functionally distinct domains which are separated with regions of polypeptide chain. In connective tissue, fibronectin is in insoluble fibrillar form and it acts as a link between extracellular matrix (ECM) and actin filaments inside the cells (Hynes and Destree, 1978; Bailey and Light, 1989; Alberts et al., 2002). Nakamura et al. (2007) showed that in bovine *M. masseter*, fibronectin was found in perimysium and in endomysium. They

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also reported that the amount of fibronectin increased with increasing exercise (the time of mastication of the feed) of the masseter muscle.

Laminin (laminin-1) is a large, asymmetric cross-shaped glycoprotein that consists of three polypeptide chains. It is very flexible and acts as a binding protein in ECM. Several isoforms of the polypeptide chains make a formation of a large family of laminins possible (Alberts et al., 2002). Laminins are the major component of basement membranes of skeletal muscles and critical for muscle fibre differentiation. Foster et al. (1987) have demonstrated that laminin enhances the myoblast proliferation *in vitro*. Laminins bind to for example integrins. The two laminin variants, 2(α2β1γ1) and 4(α2β2γ1), also called merosin, are the most abundant laminin variants in developing and adult muscles. These laminins are needed for muscle fibre survival (Belkin and Stepp, 2000). However, the association between laminins and meat quality has not been intensively studied.

8.2   Collagen formation and degradation

8.2.1 Biosynthesis of collagen in fibroblasts

Genes and each mRNA only code for one polypeptide chain of collagen. The typical structure, triple helix of collagen (Figure 8.1) is formed in the endoplasmic reticulum (ER) of a fibroblast after a series of enzymatic modifications of the single polypeptide chains (pro-α-chains) (Bailey and Light, 1989). Hydroxylation of proline and lysine are carried out by enzymes prolyl-4-hydroxylase and lysyl hydroxylase located in the ER. These hydroxylations are essential for the stable triple helix formation and also for the bifunctional cross-link formation between collagen molecules after fibre formation (Figure 8.2). Glycosylation of some hydroxylysine residues is carried out by galactosyltransferase and galctosylhydroxylysineglucosyltransferase (Bailey and Light, 1989; Prockop and Kivirikko, 1995). Also molecular chaperones such as protein disulphide isomerase, glucose regulatory protein 94 and heat shock protein 47 assist collagen molecule formation (Lamande and Bateman, 1999).

After triple helix formation the procollagen molecules are secreted to the extracellular space via Golgi apparatus. In the extracellular space enzymes pC-propeptidase and pNpropeptidase remove the procollagen propeptides from both ends of procollagen molecules before the spontaneous alignment of the collagen molecules to collagen fibrils (types I, II and III). Non-fibrous collagen types are not fully processed by the peptidases in the extracellular space as are the fibrous collagens (Bailey and Light, 1989).
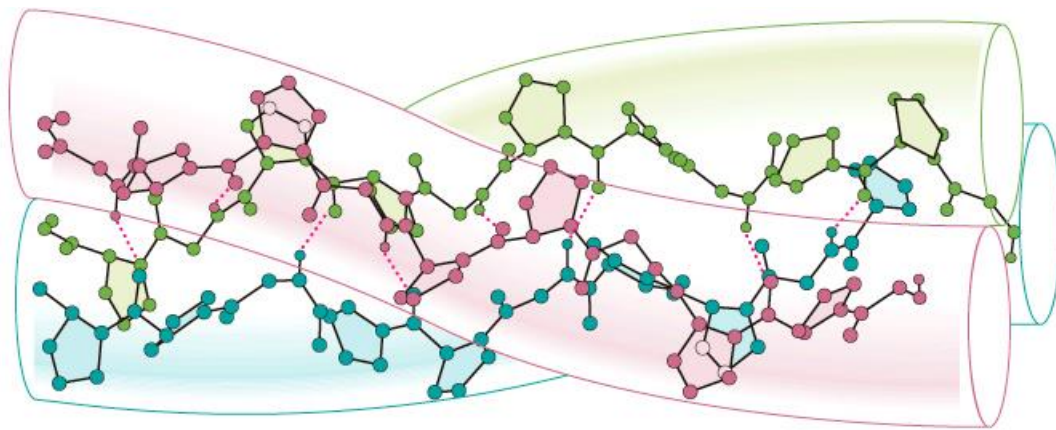
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***Figure 8.1** - Collagen triple helix*

[http://aris.gusc.lv/NutritionBioChem/bio_chemistry/en/32_proteins.html (downloaded 7th december 2015)]
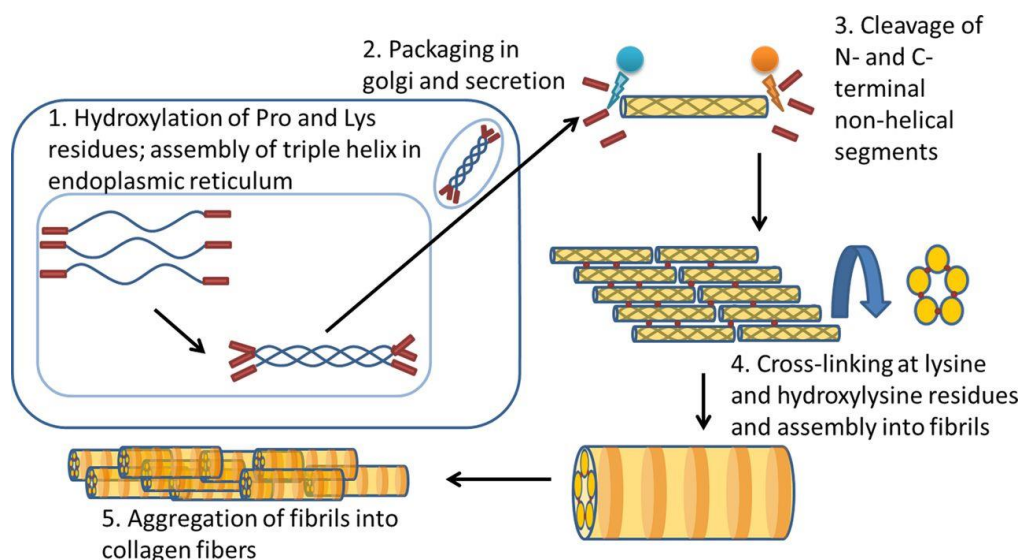

***Figure 8.2** - Collagen synthesis begins with the translation of collagen pro-α chains in the rough endoplasmic reticulum. After these chains form a triple helix, the molecule is secreted from the cell, where it undergoes further processing and aggregation into fibrils and fibers.*

[http://ajplung.physiology.org/content/304/11/L709 (downloaded 7th december 2015)]

8.2.2 Degradation of collagen

Most of the proteinases cannot cleave collagen triple helix but fibroblasts have been shown to synthesise collagen degrading enzymes which act under physiological conditions. Collagen degradation occurs even before the molecules are secreted from the cells. The amount of collagen degraded before secretion has been estimated as 15 % of the collagen produced. This has been suggested to be a way of eliminating abnormal pro-α- chains

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(Laurent, 1987; Bailey and Light, 1989). In non-mineralised matrices the collagen degradation has four pathways: matrix metalloproteinase-dependent, plasmin-dependent, polymorphonuclear leukocyte serine proteinase-dependent and phagocytic pathways (Birkedal-Hansen et al., 1993). Degradation of collagen is a series of actions of enzymes on the normally resistant triple helix and on the non-helical terminal regions (Bailey and Light, 1989).

In vertebrates there are at least 20 different types of matrix metalloproteinases (MMPs) (Nagase and Woessner, 1999). According to Kjaer (2004) the collagenases (MMP-1 and MMP-8) initiate the degradation of collagen types I and III, whereas the gelatinases (MMP-2 and MMP-9) break the collagen type IV and other compounds in the connective tissue. However, for example MMP-2 is also able to degrade type I collagen, so the degradation of connective tissue as a whole seems a very complex process (Kjaer, 2004). The inhibitors of MMPs are called tissue inhibitors of matrix metalloproteinases (TIMPs) (Woessner, 1991; Nagase and Woessner, 1999; Kjaer, 2004). Two of the four identified TIMPs (TIMP-1 and TIMP-2) are able to inhibit the activities of all MMPs, especially MMP-2 and MMP-9, respectively. The TIMPs are usually activated together with the MMPs, most likely to control the degradation of collagen (Kjaer, 2004).

Balcerzak et al. (2001) reported activities of MMP-2 and MMP-9 and most likely the inactive forms of MMP-1 and MMP-3 in bovine MAS muscles and SM muscles. In addition, they reported mRNA encoding MMP-1, -2, -9, -16, TIMP-1, -2 and -3, meaning that these enzymes are potentially associated with the degradation of IMCT. Generally, collagen turnover rate is high during growth but reduces to a plateau by maturity (Bailey and Light, 1989). Turnover time of collagen is very short for example in periodontal ligament (only few days) but longer in skin and tendon (Laurent, 1987; Bailey and Light, 1989).

It has been established that collagen plays a key role in determining the background toughness of meat from different domestic animals including birds (Maiorano et al., 2012). Lepetit (2007) analyzed various studies in wich collagen crosslinks in muscle tissue were measured. He suggested that measurement of crosslinks (pyridinoline) is a reasonable predictor of tenderness. In addition, McCornick (1999) suggested that mature crosslinks and collagen concentration have an additive effect on the toughening of meat.

8.3   Cross-links in collagen

8.3.1 **Formation of cross-links**

When the collagen molecules have arranged to form the fibrils, cross-link formation between the collagen molecules starts. Lysyl oxidase, an extracellular enzyme, converts the lysine residues in N- and C-terminals of each a-chain to aldehyde groups. This enables the formation of several types of lysine derived cross-links in fibrillar collagens (Figure 8.3) (Eyre et al., 1984; Bailey and Light, 1989; Reiser et al., 1992). In general, tissues bearing heavy loads contain predominantly cross-links formed via the hydroxyallysine route (Eyre et al., 1984). The pyrrole and pyridinoline cross-links occur in about equal amounts as a result of maturation of bone collagen. The maturation product of cartilage collagen is mainly hydroxylysyl pyridinoline (Eyre et al., 2008).
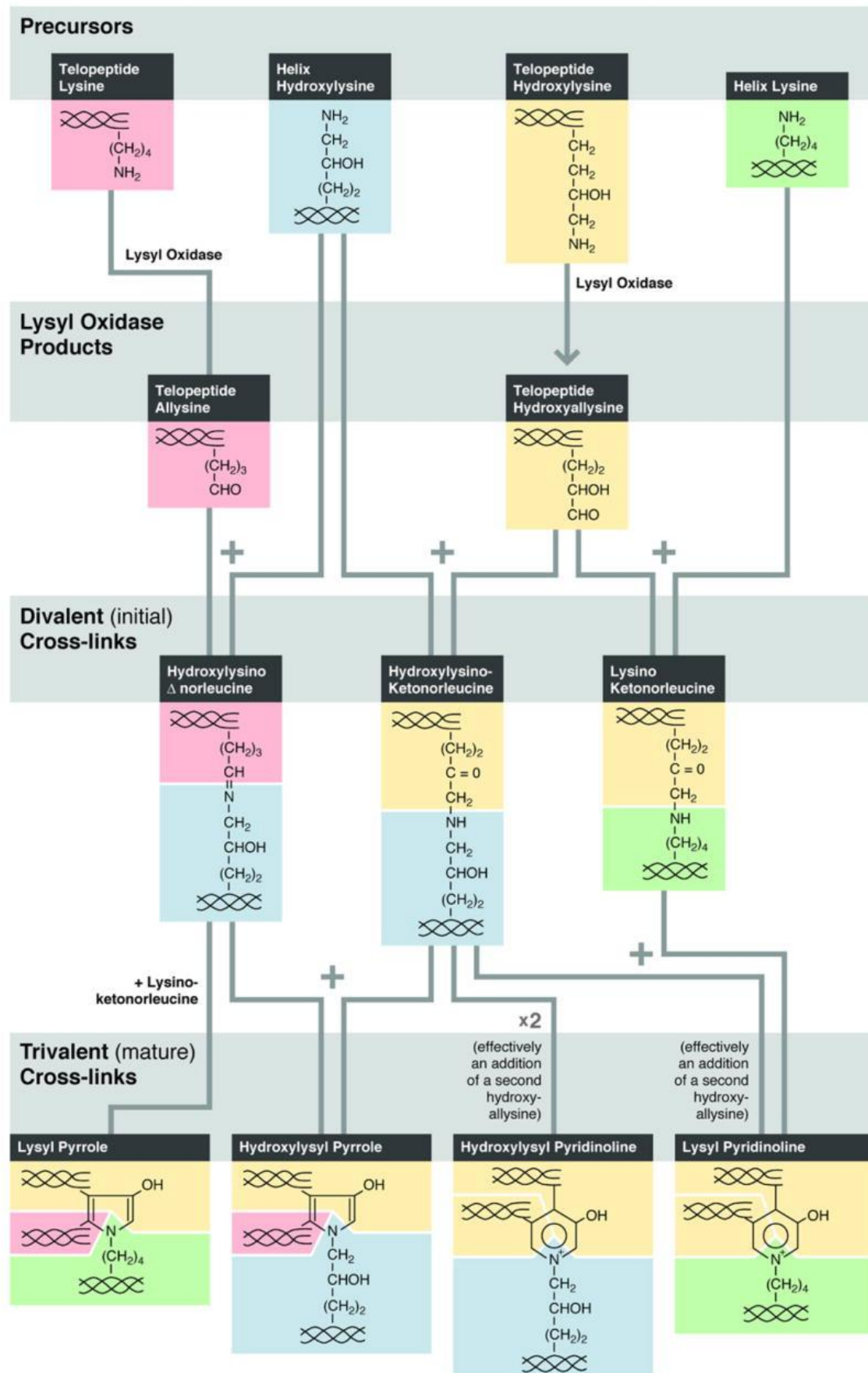
Figure 8.3 - *Chemical pathway of cross-linking (from Eyre et al., 2008)*

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PART 2: RESEARCH WORKS

This research work was a part of an European research project (THRIVE RITE) conducted by the "University of Science and Technology" in Bydgoszcz (Poland) in collaboration with our Department of Agricultural, Environmental and Food Science in Campobasso (University of Molise, Italy). This research was financed by Seventh Framework Programme (FP7/2007-2013, Project number: 315198).

Chapter 9

Research n°1

9. Influence of different prebiotics and mode of their administration on broiler chicken performance

9.1 Aim

In recent years, the ban of antibiotics contributed to increase prevalence of enteric diseases in the farms, causing epidemiological and economic damage in the poultry industry. In the post-antibiotics era, there is an increased interest in finding antibiotic alternatives for production of poultry. Probiotics and prebiotics are proposed as a solution to the intestinal problems of poultry. Prebiotics are commonly used in poultry production to stimulate growth and development of a healthy microflora in chickens. There are different ways to deliver bioactives into avian gastrointestinal tract; but, to achieve desired efficacy, prebiotics must be administered to an animal as early in life as possible.

Conventionally, in-feed or in-water supplementation has been used at first hours/days post hatching. However, this approach relies on amount of feed and/or water intake, the quality of water (chlorinated), and other experimental factors (Waldroup et al., 2003; Ciesiolka et al., 2005; Schneits, 2005; Biggs et al., 2007; Midilli et al., 2008; Huyghebaert et al., 2011). As a consequence, consumed dose of prebiotics varies in the first hours/days after hatching. Also, during early post hatching period, infection of chicks by detrimental bacteria

is also possible. Therefore, *in ovo* approach for injection of prebiotics directly to the incubating egg has been developed. It allows for a precise delivery of the bioactive substance to all embryos at early stage of development, which unifies the effects of prebiotics across the flock and assures proper development of gut microflora in all chicks. It was determined that day 12 of incubation is the optimal time for prebiotic injection into the air cell of the incubating egg (Villaluenga et al., 2004). At this time, embryo is totally immersed in amniotic fluid. Allantochorion is completely developed and highly vascularized, allowing for transfer of the bioactive solution from air cell to embryonic gastrointestinal tract. This method has been successfully used for prebiotic (Pilarski et al. 2005; Bednarczyk et al., 2011) or symbiotic (Maiorano et al., 2012; Slawinska et al., 2014a; Slawinska et al., 2014b; Madej and Bednarczyk, 2016; Madej et al., 2015; Pruszynska-Oszmalek et al., 2015) *in ovo* delivery. As a consequence, *in ovo* delivery of prebiotics not only have improved performance traits, such as the growth rate, feed intake, nutrient digestibility (Bednarczyk et al., 2011) and meat quality (Maiorano et al., 2012), but also significantly increased total activity of pancreatic enzymes (amylase, lipase, and trypsin) (Pruszynska-Oszmalek et al., 2015) and influence immune system development and function (Slawinska et al., 2014b; Madej and Bednarczyk, 2016; Madej et al., 2015; Płowiec et al., 2015).

Evaluation of prebiotics for *in ovo* injection comprises of few steps. First, oligosaccharide has to prove complete solubility in physiological salt. Only fully solved prebiotics can be precisely injected *in ovo* and pass the egg membrane into the bloodstream and guts of the embryo. Secondly, prebiotic has to be delivered *in ovo* in a specific dose that assures high hatchability of the eggs and microflora development already at hatching. Third, prebiotics should confer beneficial properties to the host in performance and fitness traits. So far, the "golden standard" for *in ovo* injection was RFO (raffinose family oligosaccharides) extracted from lupin, which assures long-term maintenance of a high level of intestinal bifidobacteria at hatching (Villaluenga et al., 2004; Pilarski et al., 2005) and optimal performance of broiler chickens (Bednarczyk et al., 2011). However, there are many other biologically active oligosaccharides available, that could be validated for *in ovo* injection.

The aim of this study was to evaluate applicability of different prebiotics to *in ovo* injection using a two-step evaluation (Trials 1 and 2). The aim of the first trial was to select the doses best suited for *in ovo* administration and to estimate their effects on the hatchability and the bacteriological status of the hatched chickens. The objective of the second trial was to define the optimal route of prebiotic administration by evaluating the influence of prebiotics

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on broilers' performance using different routes of delivery (*in ovo* vs. in-water vs. *in ovo* and in-water combined).

9.2   **Material and methods**

9.2.1 **Prebiotics**

Three prebiotics were selected, based on their solubility in physiological saline: DN (DiNovo®, Bioatlantis Ltd., Ireland) an extract of beta-glucans obtained from algae; BI (Bi[2]tos, Clasado Ltd., Malta) a non-digestive trans-galactooligosaccharides (GOS) from milk lactose digested with *Bifidobacterium bifidum* NCIMB 41171; and RFO (in-house) extracted from lupin (*Lupinus luteus*) seeds (Gulewicz et al., 2000). DN is an extract from *Laminaria* spp. containing laminarin and fucoidan. BI is composed of 45% lactose, 9.9% disaccharides [Gal (β 1–3)- Glc; Gal (β 1–3)- Gal; Gal (β 1–6)- Gal; Gal (α 1–6)- Gal], 23.1% trisaccharides [Gal (β 1–6)-Gal (β 1–4)- Glc; Gal (β 1–3)- Gal (β 1–4)- Glc], 11.55% tetrasaccharides [Gal (β 1–6)- Gal (β 1–6)- Gal (β 1–4)- Glc], and 10.45% pentasaccharides [Gal (β 1–6)- Gal (β 1–6)- Gal (β 1–6)- Gal (β 1–4)- Glc] (Tzortzis *et al.*, 2005). RFO solution contains 6.1% sucrose, 9.4% raffinose, 65.2% stachyose, 18.0% verbascose and 1.3% other saccharides (Bednarczyk et al., 2011).

9.2.2 **Extraction method for the isolation and purification of α-galactosides from lupin seeds**

α-Galactosides, called also raffinose family oligosaccharides (RFOs), belong to low molecular weight, nonreducing sugars, soluble in water (Arentoft and Sorensen, 1992). They are α-(1-6)-galactosides linked to carbonc-6 of the glucose moiety of sucrose. Raffinose family oligosaccharides are widely distributed in the plant kingdom. Large amounts of RFOs occur in the generative parts of higher plants where they perform protective physiological functions (Dey, 1985; Kuo et al., 1988; Larsson et al., 1993; Bachmann et al., 1994; Horbowicz and Obendorf, 1994).

However, from the nutritional point of view, RFOs are considered arduous factors because they are not hydrolyzed by mucosal enzymes in the small intestine of monogastric animals but pass into the lower gut where they are fermented with the deliberation of gas (Cristofaro et al., 1974; Saini and Gladstones, 1986; Prince et al., 1988). On the other hand, their ingestion in the form of pure compounds in diet increases the bifidobacteria population in the colon, which in turn contributes to human health in many ways (Minami et al., 1983; Tomomatsu, 1994).

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These low-molecular weight oligosaccharides are of interest to many nutritionists as health food ingredients. Oligosaccharides are presently one of the most popular functional foods in Japan and they are added to such products as soft drinks, cookies, cereals, and candies (Tomomatsu, 1994). The RFOs can be obtained by extraction from plants, mainly from legume seeds, via enzymatic synthesis in vitro in the presence of glycosyltransferases, or via synthesis by the saccharide metabolic pathway in vivo (Muzquiz et al., 1999; Ichikawa et al., 1994).

In the following paragraphs were described a rapid method to obtain high purity RFO powder from lupin seeds.

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.

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.

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.

_____

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 (Figure 9.1). 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 and then placed in the drier (figure 9.2).

Figure 9.1 - Glass funnel with diatomaceous earth and charcoal (1:1 w/w)

Figure 9.2 - RFO extract

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.

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.

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).

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

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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.

Trial 1. Dose optimization of DN and BI for in ovo injection

In Trial 1, two commercial prebiotics (BI and DN) were *in ovo* injected into the chicken embryo to optimize the doses per embryo for a Trial 2. RFOs had been optimized in studies of Bednarczyk et al., 2011. Hatching eggs (60 g average weight) were obtained from the same 32-weeks-old breeder flock (Ross 308). Eggs were incubated in a commercial broiler hatchery (Drobex-Agro Sp. z o.o., Poland) in a Petersime incubator (vision version, Petersime NV, Zulte, Belgium) (Figure 9.3 a,b).

Figure 9.3 - a) Drobex-Agro (Solec Kujawski, Poland); b) Petersime incubator

On day 12 of incubation, prior to the injection, the eggs were candled to select only the ones containing viable embryos. Eggs were randomly divided into three experimental groups: two groups were treated with different doses of the two prebiotics administered *in ovo*, and the control group.

The trial was conducted in three replicates, consisting of 100 (in the first replicate) and 197-200 (in the second and third replicates) eggs per prebiotic-treated and control groups. Injections solution consisted of 0.2 mL of physiological saline and different testing doses of prebiotic: 0.18, 0.88, 3.5 and 7.0 mg/embryo. Control group was injected with 0.2 mL of physiological saline. Solutions of prebiotics were injected *in ovo* using automatic system (Figure 9.4).

Figure 9.4 *- In ovo injection with automatized system*

First, hole was made in the air cell of the egg; 0.2 mL solution was deposited in air cell and the egg was sealed. After that, incubation proceeded using the standard hatching procedure. At hatching (Figure 9.5), number of healthy chicks was scored for each replicate experiment. The values were expressed as a percentage of the total number of injected eggs (a total of 3995 eggs).

Figure 9.5 *- Hatched chicken*

Fresh meconium and feces samples were collected from chicks at each batch of hatching (three batches total). For each batch, samples from 20 randomly selected chicks were pooled. Each samples was analyzed in three technical replicates.

Counts of *Bifidobacterium* spp. and *Lactobacillus* spp. were determined based on the EN 15785 and EN 15787 protocol, respectively. Pooled material was first weighed and buffered. Peptone water (Argenta Mikrobiologia Sp. z o.o., Poznan, Polska) was added to each sample as a diluent at 1:9 sample-to-buffer (g/mL) ratio. Subsequently, ten-fold dilutions were prepared using 9 mL of buffered peptone water plus 1 mL of starting material. This operation was repeated until reaching 10^{-9} dilution.

To count *Bifidobacterium*, 0.1 mL suspension of inocula were aspirated from the prepared serial dilutions, each inoculated to a MRS substrate with reduced pH of 5.7 (Argenta Mikrobiologia Sp. z o.o., Poznan, Polska) and distributed in drop-wise manner over surface of the culture plate. Culture plates were placed in an anaerobic atmosphere (Argenta Mikrobiologia Sp. z o.o., Poznan, Polska) and incubated at 37°C for 36-48hours. After incubation the colonies were counted at the appropriate dilutions and confirmatory tests were carried out. For this purpose, five out of the most characteristic colonies were picked up from each plate and subjected to Gram staining. *Bifidobacterium* spp. colonies were defined based on the morphological assessment of the test-derived candidate colonies.

To count *Lactobacillus* ssp., sample dilutions were prepared as described above. Culture plates were incubated in anaerobic atmosphere at 37°C for 36 - 72h. After incubation the colonies were counted at the appropriate dilutions and confirmatory tests were carried out. For this purpose, five out of the most characteristic colonies were picked up from each plate and subjected to Gram staining and catalase production test. All catalase - negative bacteria, morphologically corresponding to gram -positive non sporous bacilli were considered as belonging to *Lactobacillus* spp. family.

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Trial 2. Comparison between in ovo, in-water and in ovo + in-water routes of prebiotics delivery

In Trial 2, three prebiotics (DN, BI and RFO) were used for comparison between different routes of delivery: (T1) *in ovo* injection, (T2) *in ovo* injection combined with in-water delivery, and (T3) in-water delivery. Control group (C) was injected *in ovo* with physiological saline only and did not receive any prebiotic in-water. Hatching eggs were collected from the same breeder flock and incubated in the same commercial broiler hatchery as in Trial 1. *In ovo* injection (groups T1 and T2) was carried out in the same manner as in Trial 1. At day 12 of incubation 1500 eggs containing viable embryos were randomly allotted into four experimental groups (375 eggs/group).

Eggs were injected *in ovo* with 0.2 mL solution containing: 3.5 mg/embryo BI, 0.88 mg/embryo DN and 1.9 mg/egg RFO. Afterward injection, each hole was sealed with hot glue and the egg incubation was continued until hatching. After hatching chicks were sexed and 600 males (42.0 g average weight) were randomly assigned to ten experimental groups (60 males/group): T1 (DN, BI and RFO), T2 (DN, BI and RFO), T3 (DN, BI and RFO) and C. Birds were grown to 42 day of age in collective cages (n = 6 replicate cages, 10 birds in each cage) (Figure 9.6). Chicks from T1 and C groups were raised without any additional supplementation with prebiotic. T2 and T3 groups were supplemented in-water with respective prebiotic (DN, BI or RFO) for first seven days of life. Those animals received 12 ml of the prebiotic dissolved in water per pen (20 mg of prebiotic/ml).

Birds were reared according to the Polish Local Ethical Commission (No 22/2012. 21.06.2012) and in accordance with the animal welfare recommendations of European Union directive 86/609/EEC, in an experimental poultry house Drobex-Agro (Solec Kujawski, Poland) that provided good husbandry conditions (e.g., stocking density, litter, ventilation).

Figure 9.6 *- Birds in collective cages*

Animals were fed *ad libitum* the standard commercial feed mixtures (Table 9.1): starter (day 1-21), grower (day 22-35), finisher (day 35-42). Along the rearing period, chickens were weighed and counted within each cage. Body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR= BW/FI on pen basis) and mortality were calculated.

Table 9.1 - Composition and nutrient content of diets

	Starter (1-21 d)	Grower (22-35 d)	Finisher (36-42 d)
Ingredient (g/Kg)			
Wheat	26.73	29.19	30.66
Maize	30.00	30.00	30.00
Soybean meal	32.50	28.20	25.33
Canola seeds	5.00	6.00	7.00
Soybean oil	2.10	1.33	1.80
Lard	-	2.00	2.50
NaCl	0.30	0.30	0.28
Mel stern	1.09	0.95	0.85
Phosphate 1-Calcium	1.15	0.94	0.63
DL-methionine	0.25	0.18	0.13
L-lysine	0.32	0.32	0.27
L-threonine	0.06	0.09	0.05
Vitamin - mineral premix[1]	0.50	0.50	0.50
Calculated composition			
ME (kcal/kg)	2980	3100	3200
CP (% DM)	22.00	20.50	19.50
Lysine (% DM)	1.35	1.25	1.15
Methionine (% DM)	0.57	0.49	0.43
Methionine + Cystine (% DM)	0.95	0.85	0.78
Ca (% DM)	0.90	0.80	0.70
P (% DM)	0.40	0.35	0.28

DM = dry matter.

[1] Provided per kilogram of diets: vitamin A 12,500 IU, vitamin D3 - 4,500 IU, vitamin E 45 mg, vitamin K3 3 mg, vitamin B1 3 mg, vitamin B2 6 mg, vitamin B6 4 mg, pantothenic acid 14 mg, nicotinic acid 50 mg, folic acid 1.75 mg, choline 1.6 g, vitamin B12 0.02 mg, biotin 0.2 mg, Fe 50 mg, Mn 120 mg, Zn 100 mg, Cu 15 mg, J 1.2 mg, Se 0.3 mg, fitase 500 FTU

Slaughter surveys and analyses

At 42 d of age, broilers (12 for each group), identified by numbered permanent wing bands, were individually weighed, hot carcass weight was recorded, and carcass yield percentage was calculated.

The right pectoral muscle (PM) was removed from all carcasses (n=120) and its percentage was calculated based on hot carcass weight. The pH and color (L*, a*, b*) of PM were measured at 45 min (pH_{45}) and 24h (pH_{24}) *post mortem* (Figure 9.7, 9.8). Samples of the left pectoral muscle of all carcasses (n=120), 12 birds for each experimental group, were

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taken and frozen in liquid nitrogen (-196°C) for histological analyses. The right pectoral muscle was packaged and stored frozen (-40°C) until intramuscular collagen and cholesterol content analyses.
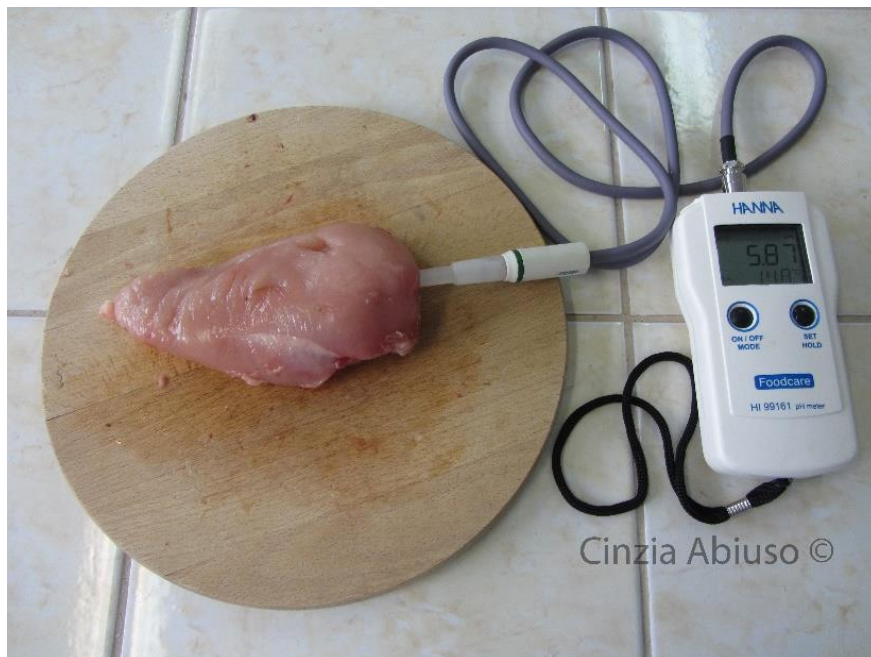

Figure 9.7 - pH measurement

Figure 9.8 - Meat color measurement

Water holding capacity

It was measured by method of Grau & Hamm (1953). This method consist by pressing a 300 mg fresh muscle sample onto filter paper No. 1 Whatman filter paper under contant pression for 5 minutes between balance weights (figure 9.9). The pressure developed is about of 50 kg/cm^2. The meat area on filter, before dryied on open air, was measured with a Image J software.

The formula applied for the measurement of WHC is the following one:

$$\textbf{WHC (\%) = [liquid/meat area (cm}^2\textbf{) / filter area (23,6cm}^2\textbf{)] * 100}$$
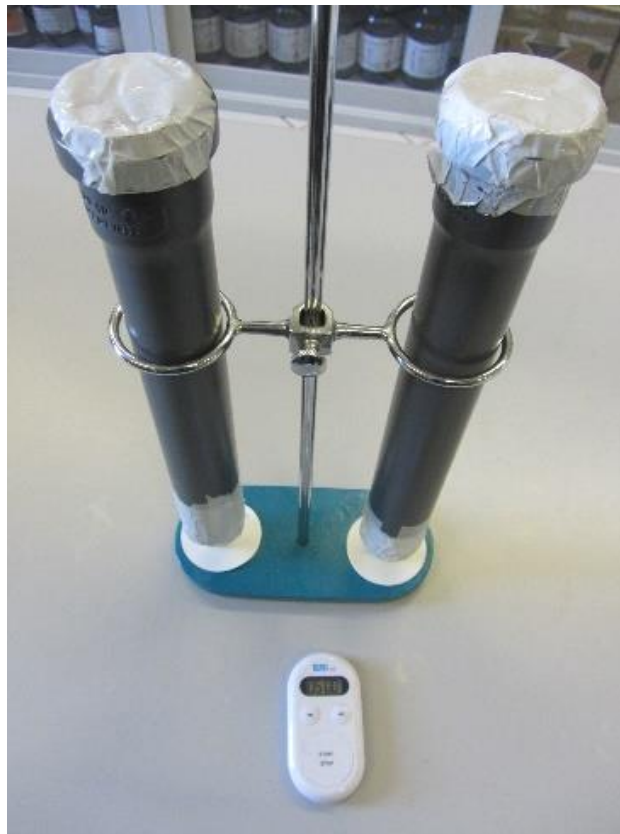
Figure 9.9 *- Measurement of WHC*

Histological analyses

The samples of *pectoralis superficialis* muscle were collected from chickens for histological analysis 45 min *postmortem*. Directly after the collection, the samples were frozen in liquid nitrogen at -196°C. Frozen samples were cut into 10 µm cross-sections in a Thermo Scientific cryostat (Figure 9.10) and later placed on glass slide and stained using staining reactions.

Figure 9.10 - Thermo Scientific cryostat

It was used H+E staining (Hematoxylin and Eosin) - the most commonly used in histology and routine histopathology - according to the method of Dubowitz and Brooke (1973) to measure the diameters and the number of muscle fibers in the pectoral muscle (Figure 9.11).

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Figure 9.11 - H+E staining

The intramuscular fatty tissue content in *Pectoralis superficialis* was determined by Oil red O staining method (Dubowitz and Brooke, 1973). Using oil red O, neutral lipids (mainly triglycerides) are stained with an orange-red tint (Lillie and Ashburn, 1943). Afterward the staining, the analysis were done on the Computer Microscopic Image Analysis System.

Using the InterVideo WINDVR program (Kworld Computer Co. Ltd., New Taipei, Taiwan), 6 images of microscopic pictures were taken per each sample, using magnification 12.5 x 10. Afterwards, all muscle fibers were counted and their diameters (µm) were measured on the area of 1,0 mm^2 cross-section of the pectoral muscle of the chickens. For the determination of intramuscular fatty tissue, 12 successive microscopic images, from an area of 2 mm^2 cross-section, were saved and analysed in order to define the percentage of fatty tissue in the muscles. MultiScan v.14.02 Image Analysis System (Computer Scanning System Ltd., Warszawa, Poland) was used to estimate the muscle fiber diameter and fatty tissue content.

Measurement of muscle cholesterol

The muscle cholesterol content was determined using the method by Maraschiello et al., (1996). 100mg of breast muscle was saponified with 2ml of methanolic KOH (0.5N) and heated in water bath at 80°C for 1 hour. All analyses were carried out in duplicate. After cooling, 2 ml of distilled water saturated with NaCl was added. The tubes were vortexed followed by addition of 3 ml ether/hexane (1:1, *v/v*) and centrifuged for 10 min at 3000g. The upper phase was recovered and the hexane/ether extraction step was repeated twice. 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.

Collagen analysis

Approximately 50 g of *Pectoralis superficialis* muscle (wet weight) was thawed at room temperature, trimmed of fat and epimysium, lyophilized for 24h, and stored frozen (-20°C) until collagen analysis. The lyophilized muscle tissue was weighed (100 mg), and hydrolyzed in Duran tubes (Schott AG, Mainz, Germany) in 5 ml of 6N HCl at 110°C for 18 to 20 h (Etherington and Sims, 1981) for determination of hydroxyproline (Woessner, 1961) and crosslinking. The hydrolyzate was filtered (Whatman #1) and diluted with water plus. An aliquot of the hydrolyzate was removed for hydroxyproline determination and the remaining part was subjected to HLP (Hydroxylysilpyridinoline) crosslink analysis.

Intramuscular collagen

The 4-hydroxyproline (intramuscular collagen concentration) was quantified using the colorimetric procedure of Woessner et al. (1961). The hydroxyproline was oxidized with chloramine T (sodium p-toluenesulfonchloramide) that was then destroyed by adding perchloric acid. Finally, a solution of p-dimethylaminobenzaldehyde solution (Ehrlich solution) was added and the tube was placed in a 60°C water bath for 20 minutes. The absorbance of the solution was then determined spectrophotometrically (UV 8500, Techomp, Japan) at 557 nm (Figure 9.12). The hydroxyproline concentration was determined directly from the standard curve of L-hydroxyproline. Intramuscular collagen concentration was

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calculated, assuming that collagen weighed 7.25 times the measured hydroxyproline weight (Eastoe and Leach, 1958) and expressed as µg hydroxyproline/mg of lyophilized tissue.

Figure 9.12 *- Spectrophotometer, UV-8500, Techomp*

Crosslink concentration analysis

Hydroxylysylpyridinoline (HLP) concentration, is the principal non-reducible crosslink of muscle collagen and highly correlated with the thermal stability of collagen (McCormick, 1999). Its concentration was determined using the procedure described by Eyre et al. (1984). Hydrolyzate HLP was concentrated and separated from the bulk of the other amino acids by elution from a CF1 cellulose column using the procedure described by Skinner (1982). The obtained eluate, added of pyridoxamine as an internal standard, was concentrated (Speed Vac® Plus SC110A, Savant Instruments, Farmingdale, NY), resuspended in 1% (v/v) n-heptafluorobutyric acid (HFBA) and filtrated (Nylon syringe filter 0.45µm, Whatman).

Quantitation of the HLP crosslink was performed by reversed phase high performance liquid chromatography (RP-HPLC) using the procedure described by Eyre et al. (1984). A Kontron HPLC (Kontron Instruments, Milan, Italy) model 535, equipped with a Luna C18 column (250 x 4.6 mm x 5 µm; Phenomenex, Torrance, CA), was used. The HLP was expressed as moles of HLP per mole of collagen and also as microgram of HLP per milligram of lyophilized tissue.

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Statistical analyses

Growth performance, FI, FCR and mortality data were evaluated by ANOVA (SPSS/PC1Statistics 18.0, SPSS Inc., Chicago, IL, USA, 2010). Differences among groups were determined by contrasts (prebiotic contrast 1: $3 \times \mu_C - \mu_{DN} - \mu_{BI} - \mu_{RFO} = 0$; contrast 2: $2x\ \mu_{DN} - \mu_{BI} - \mu_{RFO} = 0$; contrast 3: $\mu_{BI} - \mu_{RFO} = 0$. Mode of prebiotics' administration contrast 1: $3 \times \mu_C - \mu_{T1} - \mu_{T2} - \mu_{T3} = 0$; contrast 2: $2x\ \mu_{T1} - \mu_{T2} - \mu_{T3} = 0$; contrast 3: $\mu_{T2} - \mu_{T3} = 0$; μ = overall mean). Hatchability, number of *Bifidobacteriaceae* and *Lactobacillaceae* were analyzed by one-way ANOVA using the same statistical package. Differences among the means were determined with Scheffe's test. The cage was considered the experimental unit.

9.3   Results and Discussion

Trial 1. Dose optimization of DN and BI for in ovo injection

In this study, we have optimized conditions of prebiotics *in ovo* delivery and validated this method for broiler chickens production by comparison to in-water supplementation. Prebiotics were sourced from different materials, i.e. marine algae (DN), cow milk (BI) or plant seeds (RFO), and differing in biological composition and bioactive properties.

DN is an extract of *Laminaria* spp., a seaweed containing laminarin. A major active compound of a *Laminaria* seaweed extract used in this study is a low-molecular-weight polysaccharide containing beta-glucans, effective pro-immunological modulator working through the gut (Vetvicka and Oliveira, 2014). Preparations of a soluble polysaccharide laminarin (β-(1,3)-(1,6)- β-glucan) supplemented in-feed, have showed a positive effect on productive traits in monogastrics. In broilers, an oxidative stability of a meat has been improved (Ahmed et al., 2014) and in piglets, reduction of inflammatory factors and improved morphology of an intestine have been reported (Heim et al., 2015). Beta-glucans have been shown to exhibit prebiotic properties by increasing numbers of intestinal *Bifidobacterium* and *Lactobacillus* spp. (Jaskari et al., 1998). More recently, *Laminaria* spp.-derived laminarin has been shown to increase intestinal *Lactobacilli* numbers in weaned pigs and also reduce coliforms (Murphy et al., 2013). Fucoidan has also been shown to have prebiotic effects in the porcine monogastric model (Lynch et al., 2010a; Sweeney et al., 2011).

BI is belongs to GOS prebiotics, that are produced from lactose by enzymatic digestion with glycoside hydrolases (Torres et al., 2010). GOS used in this study (BI) was synthesized using enzymes from *Bifidobacterium bifidum* 41171, which is a common intestinal human bacteria. GOS generated with those enzymes have stronger affinity to stimulate growth of bifidobacteria and as such expressed better prebiotic activities based on *in vitro* studies in pigs and in humans (Tzortzis et al., 2005; Depeint et al., 2008; Tzortzis, 2009). GOS have been known to increase bacterial populations in poultry when supplemented in-feed (Jung et al., 2008). This prebiotic was also reported to mitigate harmful effects of heat stress in chickens and in mice by stabilizing intestinal integrity in jejunum and alleviating associated inflammatory responses (Akbari et al., 2015; Varasteh et al., 2015).

RFO are synthesized from sucrose in plants, where they are used for storage and transportation as well as they were reported to play a role in draught tolerance (Sprenger and Keller, 2000; Hincha et al., 2003). As a prebiotic, RFO have been used in chickens in

combination with *in ovo* method of delivery, also in combination with probiotic bacteria. One of the recently studied properties of RFO is protective mechanism against oxidative stress and related liver damage in mice (Zhang et al., 2013). Studies in which a model of the chicken embryo is used to compare the biological activity of various prebiotics require determination of the optimal dose for *in ovo* injection (Schneitz, 2005).

Hatchability results are presented in Table 9.2. Hatchability scored for tested doses of 0.18, 0.88 and 3.5 mg of prebiotics/embryo was similar ($P > 0.05$) among control, DN and BI groups. For both prebiotics, the highest tested dose (7.0 mg/embryo) decreased hatchability, resulting in 71.4% hatched chicks for BI (P > 0.05) and 56.5% for DN ($P < 0.05$). However, optimal doses of prebiotics were defined as the highest one which did not reduce hatchability (as compared to a control group) and were determined as 0.88 mg/embryo for DN and 3.5 mg/embryo for BI. Many papers report decrease in overall hatchability as a result of *in ovo* injection of different solutions, for instance glucose (Salmanzadeh, 2012), royal jelly extracts (Moghaddam et al., 2014) or avian influenza vaccines (Cai et al., 2011). The growing embryo is susceptible to homeostatic disturbances and such factors as site of injection, embryo age, solution sterility and immune response to the bioactive factor. Our study shows that the optimal dose of prebiotic solution delivered at day 12 of incubation assures proper growth conditions for the growing embryo

Figures 9.13 and 9.14 illustrate impact of prebiotics DN and BI delivered *in ovo* on the number of *Bifidobacterium* and *Lactobacillus*. Effect of DN on bifidobacteria count was not statistically significant compared to the control group ($P > 0.05$). Bifidobacteria count increased in all BI groups ($P < 0.05$) (Figure 9.13). In case of lactobacilli, differences ($P < 0.05$) were scored between control group and DN for lower doses (0.18 and 0.88 mg/embryo). BI increased (P < 0.05) the number of *Lactobacillus* at all doses compared to the control group (Figure 9.14).

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Table 9.2 - *Dose effect of prebiotics delivered in ovo on chicks hatchability*

	C[1]	DN[2]				SEM	Significance	C[1]	BI[3]				SEM	Significance
Prebiotic dose (mg/embryo)	0.0	0.18	0.88	3.50	7.00	-	-	0.0	0.18	0.88	3.50	7.00	-	-
Eggs injected (n)	500	500	499	500	499	0.24	-	500	500	500	497	500	0.60	-
Hatchability (%)	88.7[a]	90.4[a]	89.2[a]	84.5[a]	56.5[b]	6.42	**	88.7[a]	91.0[a]	92.6[a]	89.7[a]	71.4[a]	3.89	ns

[1] C = Control, *in ovo* injection of physiological saline; [2] DN = DiNovo® ; [3] BI = Bi^{2}tos;.

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

Means in a row with different letters are significantly different at: [a,b] *P* < 0.05.
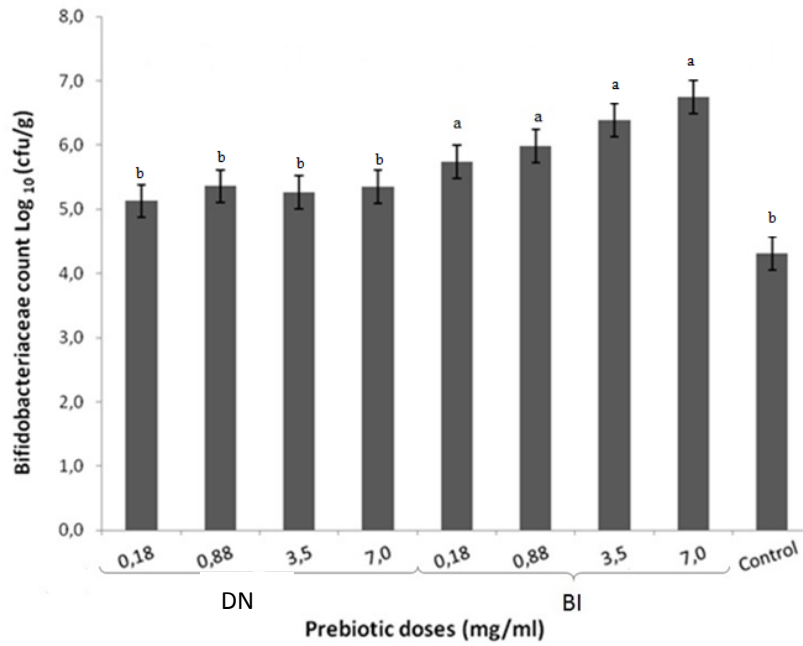
Figure 9.13 - *Impact of prebiotics delivered in ovo on Bifidobacteriaceae count in feces of one-day-old chicks. DN = DiNovo® L; BI = Bi2tos; Control = in ovo injection of physiological saline. Prebiotic effect: P < 0.01. Means with different letters are significantly different at: a,b P<0.05.*
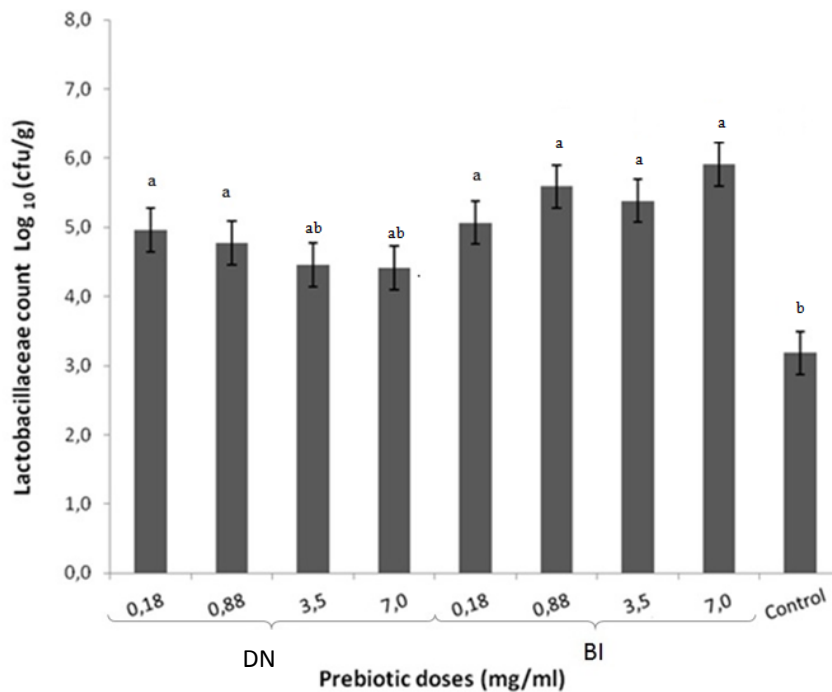
Figure 9.14 - *Impact of prebiotics delivered in ovo on Lactobacillae count in feces of one-day-old chicks. DN = DiNovo® L; BI = Bi2tos; Control = in ovo injection of physiological saline. Prebiotic effect: P < 0.01. Means with different letters are significantly different at: a,b P<0.05.*

As aforementioned, in Trial 1, both prebiotics injected *in ovo* (DN and BI) stimulated number of lactobacilli and bifidobacteria in chicken feces. This observation confirmed our earlier findings, that a single *in ovo* prebiotic (oligosaccharides) injection into 12 days-old chicken embryo leads to an increased number of bifidobacteria at the moment of hatching; it also ensures the long-term maintenance of a high level of intestinal bifidobacteria (Villaluenga et al., 2004; Pilarski *et al.*, 2005). The mechanism of this process is of considerable interest, particularly in the light of the commonly held opinion that embryonic development of the chicken happens in a sterile environment (Amit-Romach *et al.*, 2004). However, in contrary to the existing paradigm, some authors have indicated that the gastrointestinal tract of the embryo is not sterile. In fact, Deeming (2005) found that microorganisms might be internalized from yolk at the 18th day of embryonic development. Pedrosa (2009) discovered that the embryo's intestinal tract is far from being sterile and the pioneer microbial community demonstrates signs of evolution in the last 4-5 days before the hatch.

In summary, results of Trial 1 showed that the number of beneficial bacteria increased with the doses of the both (BI and DN) applied prebiotics. As a consequence, and taking into account both criteria (the number of bacteria and the hatching results) we decided to choose the dose of 0.88 mg/embryo for DN and 3.5 mg/embryo for BI for the Trial 2. RFO dose had been optimized earlier, therefore it was not included in this trial.

Trial 2. Comparison between in ovo, in-water and in ovo + in-water routes of prebiotics delivery

Effects of DN, BI and RFO prebiotics and three different routes of delivery (T1, T2 and T3) on BWG, FI and FCR of broiler chickens are presented in Table 9.3. Mortality of the chickens during this trial was low (ranged from 2.2 to 5.0%) and not dependent on type of prebiotics or route of delivery.

All prebiotics (DN, BI and RFO) significantly improved BWG within the first three weeks of life, irrespective of route of delivery (T1, T2 or T3), as compared to the control group ($P < 0.05$). During this period, chickens from RFO group were heavier than those of BI group ($P < 0.05$). Considering route of delivery, BWG was similar among treated groups ($P > 0.05$) but higher compared to control group ($P < 0.05$).

During the whole rearing period (1 - 6 weeks), BWG was higher in RFO group in comparison to the control group ($P < 0.05$), whereas DN and BI groups did not differ from control ($P > 0.05$). On the other hand, no effect of administration mode was found for BWG in this period ($P > 0.05$).

Type of prebiotics injected *in ovo* did not have any significant effect on FI during first 3 weeks of life; while, birds of T3 group (in-water) showed higher FI compared to the other experimental groups ($P < 0.05$). Considering the whole rearing period (1-6 weeks), prebiotics-treated groups were characterized by higher FI compared to the control group ($P < 0.05$), with the highest values for DN and BI. Similarly, route of prebiotic administration had effect on FI showing higher value for all the prebiotic-treated groups (T1, T2 and T3) compared to control group ($P < 0.05$). However, there were no differences between T1, T2 or T3.

Chickens from the control group showed overall better FCR compared to BI group (1.76 vs. 1.94, respectively; P < 0.05), with intermediate values for DN and RFO ($P > 0.05$). Moreover, RFO group had lower FCR than BI ($P < 0.05$). The combination of *in ovo* and in-water administration of prebiotics (T2) resulted in a significantly higher FCR as compared to the chickens of control group ($P < 0.05$); while T1 and T3 groups had intermediate FCR values ($P > 0.05$).

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Table 9.3 - *Effects of prebiotics delivered in ovo, in-water and in ovo combined with in-water on performance traits of broiler chickens*

	Group[1]						Treatment[2]					
	C	DN	BI	RFO	SEM	Significance	C	T1	T2	T3	SEM	Significance
Body weight gain (g)												
1 to 3 week	784[b]	845[ac]	829[a]	855[c]	3.25	*	784[b]	836[a]	845[a]	849[a]	3.60	**
1 to 6 week	2619[b]	2697[ab]	2667[ab]	2721[a]	8.25	*	2619[a]	2699[a]	2681[a]	2706[a]	12.25	ns
Feed intake (g)												
1 to 3 week	1434[a]	1487[a]	1508[a]	1487[a]	12.16	ns	1440[b]	1451[b]	1464[b]	1567[a]	8.16	**
1 to 6 week	4612[b]	5067[a]	5181[a]	5001[a]	31.20	**	4612[b]	5053[a]	5083[a]	5114[a]	33.20	**
Feed conversion ratio												
1 to 6 week	1.76[b]	1.88[ab]	1.94[a]	1.84[b]	0.01	**	1.76[b]	1.87[ab]	1.90[a]	1.89[ab]	0.01	*

[1] Group: C = Control, *in ovo* injection of physiological saline; DN = DiNovo® ; BI = Bi$_2$tos; RFO = raffinose family oligosaccharides.
[2] Treatment: C = Control, *in ovo* injection of physiological saline; T1 = *in ovo*; T2 = *in ovo* + in-water; T3 = in-water.
Significance: ns = $P > 0.05$; * $P < 0.05$; ** $P < 0.01$.
Means in a row with different letters are significantly different at: [a, b] $P < 0.05$.

Results of Trial 2 regarding BWG provide further support for the hypothesis concerning well-established growth promoting effect of dietary prebiotics, attributed to their ability to strongly bind the pathogenic bacteria and decoy pathogens away from the intestinal lining. Positive effects of the in-feed prebiotic supplementation of broiler chickens on the body weight was observed by Dizaji et al. (2012). However, statistically significant differences were detected no earlier than 42 day of age (finisher period). We find our results in line with values obtained by Shahir et al. (2014) for BW and FI which increased significantly through whole rearing period in broilers supplied with oligosaccharides at 0.1%/ton of feed. Jung et al. (2008) reported significant promotion of *Bifidobacteria* in intestine after supplementing GOS at 3kg per 25kg feed but no change in BW, FI and FC. Similar effect of trans-galacatooligosaccharides on broiler performance is shown by (Biggs et al., 2007). However, in laying hens, supplementation with RFO in blue lupin seed meal at 20% promoted daily egg productivity comprised by decrease in BW and FI (Zdunczyk et al., 2014). The third tested prebiotic, beta-glucan is well established in aquaculture, and treatment with 1-2% w/w feed allowed for significant improvement in FCR and increased BW in marine species (Kuhlwein et al., 2014). In poultry, addition of 0.025% modified beta-glucan did not improve broiler performance apart from increased BWG in starter period of rearing (Jozefiak et al., 2008). Whereas, the inclusion of laminarin and fucoidan beta-glucans as prebiotics improved growth parameters in weaned pigs (O'Doherty et al., 2010). In our study prebiotic treated chickens show trend for increased FCR. It has been suggested that the effect of prebiotics on chicken growth performance, could be related to metabolism modification linked to an increase in the digestive enzymes activity (Pruszynska-Oszmalek et al., 2015), the decrease in bacterial enzymes activity and ammonia production along with the improved feed intake and digestion (Kabir, 2009). Our results indicate a positive stimulation of the broiler BW expressed as soon as in the starter period (1-21 days), which might be explained by early supplementation of chicken embryos with prebiotics using *in ovo* method.

Tendency of the increased FI and FCR in the prebiotics-treated groups could be due to the stimulation of the intestinal microbiota expansion in the chicken guts by the injection of prebiotics during the *in ovo* development. The enhanced colonization of the intestines with the microflora, apart from significant effects on the host's growth performance, gut immunity and development, can increase the energy requirements for the maintenance of the bacterial population inhabiting the chickens' gastrointestinal

tract. It was also demonstrated by Mountzouris et al. (2010) that the inclusion of the growing dosages of probiotics into broilers' feed caused the proportional increase in the FCR, especially in the grower and finisher growth phases. It is generally accepted that the bacterial species inhabiting the gut compete for the nutrients with the host, which results in an higher energy uptake (Furuse and Yokota, 1985). Moreover, intestinal microflora requires increased mucus secretion and epithelial cells turnover in the guts, which is accompanied by an extremely high rate of metabolism and protein synthesis, resulting in 23 to 36% of the whole body energy expenditure (Dibner and Richards, 2005).

Effect of prebiotic and prebiotic administration methods on performance and carcass traits of broiler chickens are presented in Table 9.4. Final body weight was higher in all prebiotic group; in particular, significant differences (P < 0.01) were recorded between RFO and Control group. This finding corroborate the results reported by Pelicano et al. (2005). They reported that pre-slaughter body weight was greater (p < 0.05) in the birds fed probiotics based on a bacterial pool, whereas lower weight was observed in the birds fed single-culture probiotics in the diet. No differences (P > 0.05) were found between experimental groups and Control group in case of carcass weight and carcass yield, it ranged from 1,900.0 to 1,935.1 g, and from 69.66 to 71.67 %, respectively. These results are similar to findings reported by Maiorka et al. (2001), and Pelicano et al. (2005). Dietary treatment with prebiotics did not cause any significant effect on carcass weight and carcass yield also in study conducted by Midilli et al. (2008).

Breast weight and breast yield were higher in prebiotic group however significance differences (P < 0.05) were only observed between DiNovo® and control group; in particular pectoral muscle percentage in DiNovo® was 33.3% and 30.7 in control group. Our results are in contrast to those described by Pelicano et al. (2005) and Ashayerizadeh et al. (2009). They reported that the cut yields were not affected by different prebiotics. Important is to highlight that, it is suggested that such promoters might be used in broiler diets, since they do not interfere, or interfere positively, on the yield of the most commercial edible cuts (Pelicano et al., 2005).

Broiler chickens from T1 and T2 were heavier than C group (P < 0.05). The different modality of administration affected carcass weight. Value of this trait, was significantly higher (P < 0.01) for *in ovo* in comparison to the group that received prebiotics only in the water. The intermediate value of carcass weight was found in the

group T2 that received prebiotic *in ovo* and in water. Carcass yield was higher (P < 0.05) in control group compared with group T3 that received prebiotic only in water. Control group value of carcass yield was tendencially higher (P=0.064) than T2 group. Breast weight was similar among the experimental groups, differently breast yield was markedly higher (P < 0.01) in birds that received prebiotics only in water than in control group (33.7 *versus* 30.7).

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Table 9.4 *- Effect of prebiotic and prebiotic administration methods on performance and carcass traits of broiler chickens*

	Group[1]						Treatment[2]					
	C	DN	BI	RFO	SEM	Significance	C	T1	T2	T3	SEM	Significance
Initial BW (g)	42	42	42	42	-	-	42	42	42	42	-	-
Final BW (g)	2,651.7^B	2,726.1	2,711.5^b	2,778.8^{Aa}	8.04	0.001	2,651.7^b	2,751.8^a	2,740.6^a	2,725.0	8.04	0.008
Carcass weight (g)	1,900.0	1,917.1	1,910.3	1,935.1	9.92	0.713	1,900.0	1,958.0^A	1,936.0	1,872.0^B	9.92	0.004
Carcass yield (g)	71.67	70.32	70.45	69.66	0.32	0.362	71.67^a	71.16	70.65	68.71^b	0.32	0.005
Breast weight (g)	583.3^b	638.4^a	622.8	627.0	5.34	0.024	583.3	632.4	625.3	630.9	3.87	0.040
Breast yield (%)	30.7^b	33.3^a	32.6	32.4	0.24	0.023	30.7^B	32.3	32.3	33.7^A	0.24	0.002

[1] Group: C = Control, *in ovo* injection of physiological saline; DN = DiNovo®; BI = Bi^{2}tos; RFO = raffinose family oligosaccharides.
[2] Treatment: C = Control, *in ovo* injection of physiological saline; T1 = *in ovo*; T2 = *in ovo* + in-water; T3 = in-water.
Means in a row with different letters are significantly different at:$^{a, b}$ $P < 0.05$;$^{A, B}$ $P < 0.01$.

Effect of treatment with different prebiotics and methods of prebiotic administration on pH and color of breast muscle, after 45 minutes and 24 hours *post mortem*, and WHC are presented in Table 9.5.

The bioactives treatment did not affect (P > 0.05) pH_{45}, pH_{24} and color coordinates, except for redness (a*) values at 45 min., which was higher (P < 0.05) in the control group in comparison with DiNovo® and RFO groups and lightness (L*) values at 24 hours, which was higher (P < 0.05) in RFO group in comparison with Bi^2tos group. Considering that the effect of prebiotic was evident only at pH_{45}, this result confirmed that the bioactives influence only marginally the pH. Similar result was obtained by Pelicano et al. (2005), who reported that pH values of the pectoral muscles, measured 5 hour after slaughter, were not affected by the presence of prebiotic and/or prebiotics. Also Quadros et al. (2001), did not find significant differences in pH values, measured 45 min and 24 hours after slaughter, in pork from swine fed diets containing growth promoters. The pH-value or acidity of meat is important in relation to the meat's microbiological and keeping quality. Evaluation of pH meat is a part of experiments aiming to searching a new methods which allow to improve mentioned meat attributes. According to Pearson (1994), biochemical processes, such as pH changes, should occur so that the animal muscle is converted into meat. pH values in the muscle of live animals is approximately 7.4. Furthermore, considering the effect of bioactives on color parameters, obtained results are partially in line with findings presented by Pelicano et al. (2005). Their results showed that the color parameters (L* - lightness, a* - redness, and b* - yellowness) were not affected by the use of different growth promoters at 42 days of age. In presented study, water holding capacity was slightly higher in all prebiotic groups compared with control group (P > 0.05). A similar results was reported by Park and Park (2011), who found a higher WHC values in meat from broilers supplied with oligosaccharides at 0.1%/ton of feed inuloprebiotics (250 g/ton) compared with the control ones.

The way of prebiotic administration influenced the chemical-physical parameters. Breast meat pH values measured 45 minutes after slaughter was higher (P < 0.01) in T3 group (only water) compared with other groups. Differently, after 24 h the meat from T3 group showed the lowest value (5.78) that differed (P < 0.05) when compared with *in ovo* + in water (5.86). Except to redness (a*), color parameters were not significantly affected by modality of prebiotic supplementation. In fact, a* value was higher (P < 0.01) in C and T2 groups compared with T3 ones and, in addition, was higher from C

meat ($P < 0.05$) than that of T1. After 24 hours significant differences ($P < 0.05$) were also observed for values that was higher in C than in T1. The different modality of bioactives administration was studies also by Pelicano et al., (2003). They reported that the concomitant use of probiotics in drinking water and diet reduced significantly the values of L* (lightness) in breast muscle 45 minutes and 5 hours after slaughter, resulting in a less pale meat. According to Petracci et al. (2013a) values of lightness measured in the meat of present study are normal. Aformentioned observation is important especially considering the fact that the color of meat is important because consumers associate it to fresh and high-quality products. Water holding capacity was influenced by prebiotic way of administration: meat form T3 group had higher ($P < 0.05$) WHC compared to C and T2 groups ($P > 0.05$).

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Table 9.5 - *Effect of treatment with different prebiotics and methods of prebiotic administration on pH and color of breast muscle after 45 minutes and 24 hours post mortem*

	Group[1]						Treatment[2]					
	C	DN	BI	RFO	SEM	Significance	C	T1	T2	T3	SEM	Significance
pH_{45}	5.81	5.89	6.02	5.93	0.02	0.042	5.81[B]	5.82[B]	5.86[B]	6.05[A]	0.02	0.001
pH_{24}	5.86	5.81	5.84	5.79	0.01	0.350	5.86	5.82	5.86[a]	5.78[b]	0.01	0.011
Color 45												
L*	46.29	47.11	46.67	47.10	0.38	0.910	46.29	47.37	46.20	46.80	0.38	0.417
a*	4.14[a]	2.97[b]	3.15	3.01[b]	0.10	0.012	4.14[Aa]	3.35[b]	3.64[A]	2.96[B]	0.10	0.001
b*	3.55	3.20	3.31	3.77	0.16	0.533	3.55	3.22	3.71	3.44	0.16	0.445
Color 24												
L*	50.01	50.93	48.70[b]	51.30[a]	0.37	0.029	50.01	50.82	50.15	49.74	0.37	0.496
a*	3.90	3.06	3.35	3.00	0.10	0.077	3.90[a]	3.11[b]	3.40	3.47	0.10	0.036
b*	4.11	3.50	4.47	4.50	0.19	0.155	4.11	4.04	4.15	4.26	0.19	0.952
WHC (%)	16.84	18.59	18.50	18.24	0.27	0.320	16.84[b]	18.12	17.07[b]	18.94[a]	0.27	0.001

[1] Group: C = Control, *in ovo* injection of physiological saline; DN = DiNovo®; BI = Bi^{2}tos; RFO = raffinose family oligosaccharides.
[2] Treatment: C = Control, *in ovo* injection of physiological saline; T1 = *in ovo*; T2 = *in ovo* + in-water; T3 = in-water.
Means in a row with different letters are significantly different at:[a, b] $P < 0.05$;[A, B] $P < 0.01$.

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IMC properties were not significantly affected by both different prebiotics and the way of prebiotic administration (Table 9.6). Significant effect of bioactives on IMC properties have been reported in study conducted by Maiorano et al. (2012), who observed that the IMC concentration was significantly higher in meat of control chickens than for prebiotics treated groups. Taking into consideration aforementioned collagen maturation (mol of HLP/mol of collagen) and HLP concentration (µg HLP), also in research by Maiorano et al. (2012) no significant differences were reported. No difference (P > 0.05) was found between the experimental groups considering the fiber diameter as well; it ranged from 47.37 µm to 54.69 µm. This result is in line with finding presented by Maiorano et al. (2012). Compared to control group, the bioactives treatment slightly increased intramuscular fat content, but differences between groups were not significant (P > 0.05). The breast muscle cholesterol content, ranging from 47.20 to 49.44 mg/100g, was found to be similar (P > 0.05) among experimental groups. Also Maiorano et al. (2012) did not found any effect from the prebiotics. In addition, the cholesterol content found by Maiorano et al. (2012) (ranging from 70.45 to 78.12 mg/100 g) were higher than those reported in present research.

The way of prebiotic administration affected the fiber diameter of PM, birds from control group showed a lower (P < 0.05) value of this trait than those from T3 group (in-water). T1 and T2 had intermediate values (P > 0.05). Nevertheless, must be considered that at higher fiber diameter in prebiotics groups had corresponded a heavier breast muscles in prebiotics groups (Table 9.4). Compared with the control group, prebiotics groups had higher content of intramuscular fat; however, the differences were not statistically significant (P > 0.05). The way of prebiotic administration didn't affect the cholesterol content in the meat.

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Table 9.6 - *Effect of treatment with different prebiotics and methods of prebiotic administration on collagen, hydroxylysylpyridinoline, fiber diameter, fat and cholesterol content.*

	Group[1]						Treatment[2]					
	C	DN	BI	RFO	SEM	Significance	C	T1	T2	T3	SEM	Significance
Collagen (µg/mg)	14.42	14.09	14.50	14.16	0.19	0.828	14.42	14.40	13.97	14.50	0.19	0.492
HLP[3] (mol/mol)	0.075	0.077	0.082	0.010	0.100	0.171	0.075	0.089	0.078	0.095	0.01	0.440
HLP[3] (µg/mg)	1.62	1.54	1.73	1.99	0.09	0.368	1.62	1.77	1.57	1.97	0.09	0.430
Fiber diameter[4] (µm)	47.37	54.02	52.23	54.69	0.85	0.097	47.37[b]	52.76	50.08	53.39[a]	0.85	0.020
Fat[5] (%)	3.22	6.77	4.80	5.31	0.44	0.095	3.22	6.01	5.85	5.28	0.44	0.364
Cholesterol (mg/100g)	47.73	49.44	47.20	47.37	0.59	0.447	47.73	49.11	47.71	46.99	0.59	0.317

[1] Group: C = Control, *in ovo* injection of physiological saline; DN = DiNovo®; BI = Bi^{2}tos; RFO = raffinose family oligosaccharides.

[2] Treatment: C = Control, *in ovo* injection of physiological saline; T1 = *in ovo*; T2 = *in ovo* + in-water; T3 = in-water.

[3] Hydroxylysylpyridinoline (C: n = 7; *in ovo* = 19; *in ovo*+water = 18; in water = 19 C: n = 7; DN = 14; BI = 23; RFO = 19).

[4] (C: n = 8; *in ovo* = 24; *in ovo*+water = 24; in water = 24 C: n = 8; DN = 24; BI = 24; RFO = 24).

[5] (C: n = 4; *in ovo* = 12; *in ovo*+water = 12; in water = 14 C: n = 7; DN = 14; BI = 12; RFO = 12)

Means in a row with different letters are significantly different at:[a, b] $P < 0.05$;[A, B] $P < 0.01$.

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9.4   Conclusions

In summary, the study has established an elegant protocol for stimulation of the intestinal microflora populations in broiler chickens. It was achieved using a single *in ovo* prebiotics delivery during embryonic development. This study has also demonstrated dose optimization method using hatchability and microbiological screening. Also, it was validated this method by comparing it to well-established in-water supplementation with prebiotics. By all means, *in ovo* prebiotics delivery proved to be no different than in-water supplementation or the two methods combined. At the same time, the amount of the prebiotic used was at least ten times lower in case on *in ovo* method (3.5 mg BI/embryo *in ovo* vs. 40 mg BI/chick in-water). As such, *in ovo* method should be further recommended to the poultry industry. The prebiotic that resulted in the best performance traits improvement was RFO. All prebiotics increased FI and FCR compared to control, which is a typical trade-off of the energy and nutrients use by a better developed intestinal microflora. However, has known the gut health is also associated with a better immune status of the animals. Meat quality was slightly affected by both prebiotics treatment and the modality of their administration. Nevertheless, must be considered that at higher fiber diameter in prebiotics groups had corresponded a heavier breast muscles in prebiotics groups. In basis of the results obtained from this study, it is possible to propose that *in ovo* route of prebiotic delivery can replace prolonged and costly in-water supplementation of the broiler chickens with those bioactive compounds.

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Chapter 10

Research n°2

10. *In ovo* validation model to assess the efficacy of commercial prebiotics in enhancing broiler performance and reducing lipid oxidation of meat

10.1 Aim

As has been previously reported, the use of bioactives such as pre/synbiotics has a positive effect on gut microbiome of chickens. Compared to dietary prebiotics inclusion, *in ovo* injection at early stage of development increases the population of beneficial microflora on the day of hatch (Bednarczyk et al., 2016), and leads to a high and stable level of Bifidobacteria throughout the broiler chickens growing period (Villaluenga et al., 2004). The *in ovo* technology has enabled delivery of sustainable bioactives such as pre/synbiotics as early as possible, namely at the 12th day of embryonic development on shaping the microbiome in newly hatched chicks (Pilarski et al., 2005; Bednarczyk et al., 2011; Slawinska et al., 2014; Madej et al., 2015; Płowiec et al., 2015).

In broilers, which are obviously lacking the enzymes galactosidases, a non-digestible galacto-oligosaccharides supplemented to the diet showed functional response in performance and promoted colonization of the gastrointestinal tract by Bifidobacteria (Jung et al., 2008). A bioactive mixture of galactooligosaccharides (GOS) produced through the enzymatic activity of β-galactosidase from *Bifidobacterium bifidum* NCIMB 41171 was earlier proposed as B-GOS prebiotic for human treatment (Clasado Biosciences Ltd., Jersey, United Kingdom). B-GOS possess bifidogenic and immunomodulatory properties which were proven in monogastric model earlier (Tzortzis, 2009), thus a derivative candidate non-digestive trans-galactooligosaccharides in the novel formulation Bi^{2}tos (Clasado BioSciences Ltd., Jersey, United Kingdom) has been proposed for a routine treatment in poultry.

Other studies have shown that dietary provision of a *Laminaria* spp. derived seaweed extract, containing laminarin and fucoidan, promotes improved growth and feed efficiency of

pigs in the absence of in-feed antibiotics (Gahan et al., 2009; McDonnell et al., 2010). Furthermore, previous investigations indicated that the supplementation of a seaweed extract to pigs suppressed enteric *Enterbacteriaceae* populations (Reilly et al., 2008; Lynch et al., 2010b) and increased lactobacilli numbers (Lynch et al., 2010b; McDonnell et al., 2010). This suggests that seaweed extracts may provide a means to improve productivity and gut health in monogastric animals. The previous study (Research n° 1), conducted on broiler chickens, evidenced that Bi^2tos injected *in ovo* (3.5 mg/embryo) increased the number of lactobacilli and bifidobacteria in the chicken excreta, as well as the body weight gain. Differently, feed intake (FI) and feed conversion rate (FCR) were negatively affected by Bi^2tos.

Still, the results of the performance parameters including BW and FCR appear inconsistent among the studies conducted on small cohorts (200-500 animals) after different prebiotic treatments (Hanning et al., 2012; Kim et al., 2011; Mookiah et al., 2014), provided that the breed, sex and environmental conditions are respected. Nevertheless, Ricke (2015) concluded that the type of host immune responses vs. growth performance may not necessarily be positively correlated with each other.

Quality broiler meat has been emphasized in several studies. However, it has been reported that prebiotics can alter lipid metabolism (Letexier et al., 2013) and enhance the PUFA/SFA ratio in chicken meat (Zhou et al., 2009; Velasco et al., 2010) with benefits to human health but a greater risk for the shelf life of the meat. There is no available information regarding the potential effect of Bi^2tos trans-galactooligosaccharides and DiNovo® seaweed beta-glucans, injected *in ovo*, on the shelf life of chicken meat. The current study has aimed to explore the potential of Bi^2tos and DiNovo®, injected *in ovo* with an automatized system, and determine their influence on *in vivo* performance, carcass traits and lipid oxidation of meat in chickens reared under commercial condition.

10.2 Materials and methods

10.2.1 Birds, experimental design and rearing measurements

A total of 350,560 Ross 308 crossbreed eggs were incubated in a commercial hatchery (Drobex-Agro Sp. z o.o., Makowiska, Poland) for the *in ovo* protocol (Figure 10.1). The eggs were randomly divided into 2 prebiotic groups: DN (DiNovo®, Bioatlantis Ltd., Ireland; dietary *Laminaria*-derived laminarin and fucoidan) and BI (Bi^{2}tos, Clasado Ltd., Malta; a non-digestive trans-galactooligosaccharides (GOS) from milk lactose digested with *Bifidobacterium bifidum* NCIMB 41171), and a control (C) group. The composition of the 2 prebiotics are described in details in Bednarczyk et al. (2016). The eggs were automatically injected *in ovo* (Bednarczyk et al., 2011), into the air chamber, with 200 µl of physiological saline solution (Figure 10.1) containing BI at dose of 3.5mg/egg or DN at dose of 0.88 mg/egg, on d-12 of embryonic incubation (Petersime NV, Belgium).

Figure 10.1 *- Automatically in ovo injection*

The optimal doses of prebiotics were selected in a previous study by evaluating the hatchability and the bacteriological status of the hatched chickens (Bednarczyk et al., 2016). The control group was injected with 200 µl of physiological saline. The research was performed on tree commercial farms (Agro Sp. z o.o., Makowiska, Poland), within 20 minutes driving from the laboratory facilities. All treatment groups were represented on the 3 farms.The experimental design is reported in Figure 10.1. Birds (males and females, approximately ratio 1:1) were reared at stocking density (ranging from 21.2 to 21.5 chicks/m^2) (Figure 10.3).
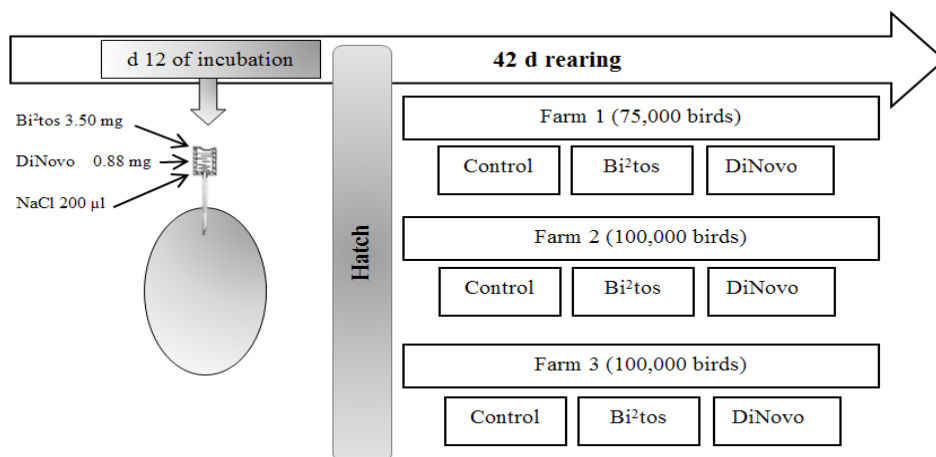
Figure 10.2 - Number of chickens for each chicken house and each treatment

Figure 10.3 - Reared chicken at high density

Temperature was gradually decreased from 33°C on day 0 to 20°C on day 42 and was kept constant thereafter. The lighting program was 23L:1D in the first week and 18L:6D from the second week to the slaughter. The management conditions of broiler chickens were the same in all farms. The broilers were fed ad libitum the standard commercial feed mixtures (Tables 10.1): starter (day 1-10); grower (day 11-35); finisher (day 36-42). Water was

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provided *ad libitum*. The animals were reared according to the regulations and permission of the local Ethical Commission (decision No.22/2012 21.06.2012) and in accordance with the animal welfare recommendations of European Union directive 86/609/EEC.

Table 10.1 - Ingredients and chemical analysis of diets

Item (% unless noted)	Period		
	1 to 10 d	11 to 35 d	36 to 42 d
Ingredients			
Corn (7.75% CP)	61.157	65.999	67.932
Soybean meal (47.75% CP)	33.086	28.158	26.032
Soybean oil	1.754	2.057	2.767
Limestone	1.098	0.982	0.698
NaCl	0.200	0.200	0.234
Dicalcium phosphate	1.605	1.504	1.337
Vitamin-mineral premix 1[1]	1.100	-	-
Vitamin-mineral premix 2[2]	-	1.100	-
Vitamin-mineral premix 3[3]	-	-	1.100
Chemical analysis, %			
DM	88.43	88.52	88.51
CP	21.00	19.00	18.00
Lipid	4.61	4.99	5.72
Crude Fiber	2.69	2.63	2.59
Ash	5.82	5.40	5.02
Calculated analysis			
ME, MJ/kg of diet	12.72	13.00	13.30
Lysine, %	1.32	1.19	1.05
Methionine, %	0.65	0.58	0.52
Methionine+cysteine, %	0.98	0.89	0.82
Threonine, %	0.86	0.78	0.71
Tyrosine, %	0.25	0.22	0.21
Calcium, %	0.90	0.84	0.76
Available P, %	0.71	0.68	0.63
Sodium, %	0.16	0.16	0.15
Salt,%	0.35	0.35	0.35
Potassium, %	0.93	0.83	0.79

[1]Supplied per kilogram of diet: vitamin A, 13,000 IU; vitamin D3, 5,000 IU; vitamin E, 80 mg; vitamin B1, 3 mg; vitamin B2, 9 mg; vitamin B6, 4 mg; vitamin B12, 20 μg; vitamin K, 3 mg; biotin, 0.15 mg; Ca pantothenate, 15 mg; nicotinic acid, 60 mg; folic acid, 2 mg; choline chloride, 0.50 mg; lysine, 2,812 mg; methionine, 3,405 mg; threonine, 745 mg; Ca iodate, 1 mg; Se, 0.35 mg; Fe, 40 mg; Mo, 0.50 mg; Mn, 100 mg; Cu, 15 mg; Zn, 100 mg.

[2]Supplied per kilogram of diet: vitamin A, 10,000 IU; vitamin D3, 5,000 IU; vitamin E, 50 mg; vitamin B1, 2 mg; vitamin B2, 8 mg; vitamin B6, 3 mg; vitamin B12, 15 μg; vitamin K, 3 mg; biotin, 0.12 mg; Ca pantothenate, 12 mg; nicotinic acid, 50 mg; folic acid, 2 mg; choline chloride, 0.40 mg; lysine, 2,831 mg; methionine, 3,018 mg; threonine, 726 mg; Ca iodate, 1 mg; Se, 0.35 mg; Fe, 40 mg; Mo, 0.50 mg; Mn, 100 mg; Cu, 15 mg; Zn, 100 mg.

[3]Supplied per kilogram of diet: vitamin A, 10,000 IU; vitamin D3, 5,000 IU; vitamin E, 50 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitamin B6, 3 mg; vitamin B12, 15 μg; vitamin K, 3 mg; biotin, 0.12 mg; Ca pantothenate, 10 mg; nicotinic acid, 50 mg; folic acid, 1.5 mg; choline chloride, 0.35 mg; lysine, 1,779 mg; methionine, 2,514 mg; threonine, 361 mg; Ca iodate, 1 mg; Se, 0.35 mg; Fe, 40 mg; Mo, 0.50 mg; Mn, 100 mg; Cu, 15 mg; Zn, 100 mg.

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All performance data were collected automatically from the slaughterhouse livestock recording system SRPP-MK (*System Rejestracji Procesow Produkcyjnych*, Production Process Registration System, in-house developed by Drobex-Agro Sp.z o.o. Solec Kujawski, Poland): livestock BW, average BW and final stocking density (kg of live weight/m^2 at slaughter) were calculated. Mortality at first week of age and for the overall experimental period was calculated. In addition, mortality was expressed as a percentage calculated using the formula reported in the European Union (EU) Council Directive 2007/43/EC (1% added to 0.06% multiplied by the age of the birds at slaughter). FI and FCR were calculated for each chicken house replicate (n = 3). Moreover, European Broiler Index (EBI) was calculated for each chicken house replicate, according to the following formula: EBI = liveability (%) x live weight (kg) x 100/age (d) x FCR.

10.2.2 Slaughter Surveys

At 42 d of age, 15 randomly chosen birds (7 males and 8 females) per each treatment were individually weighed (after a fasting period of 12 h) and transported within 1.0 h (including careful catching and loading) to a commercial poultry slaughterhouse (PD Drobex, Solec Kujawski, Poland). After careful unloading and hanging in randomized order, the birds were electrically stunned and slaughtered, hot carcass weight (without head) was recorded, and carcass yield was calculated. In addition, the breast muscle was removed from carcasses of 45 broilers and its percentage was calculated based on hot carcass weight.

10.2.3 Measurement of oxidative stability

For oxidative stability evaluation, breast muscle samples were analyzed as raw meat after storage at 4 °C for 0, 2, 4 and 6 days. Lipid oxidation was determined by the TBA reactive substances (TBARS) method as described by Vyncke (1970, 1975) and with modifications according to Sørensen and Jørgensen (1996). Briefly, 5.0 g of minced meat was homogenized in 15 ml 7.5% trichloroacetic acid with 0.10% propyl gallate and 0.10% EDTA using an Ultra-Turrax T 25 BASIC (Ika-Werke, Staufen, Germany) and filtered; 1.0 ml of the filtrate was mixed with 1.0 ml of TBA 0.020 M and incubated at 100 °C in a water bath for 40 min. Absorbance was measured at 532 nm and 600 nm at room temperature. Lipid oxidation was measured in duplicate for each muscle, and the average absorbance reading was used for each sample. The TBARS value was expressed as mg of malondialdehyde (MDA) per kilogram of sample using a standard curve prepared from 1,1,3,3-tetraethoxy-propane.

*10.2.4*Statistical Analyses

Statistical analyses of the data were performed using SPSS (SPSS, 2010). Data on performance and mortality were analyzed by one-way ANOVA where prebiotic was the main factor. Scheffé's test was applied to compare the mean values among the experimental groups. Slaughter performance data were evaluated by ANOVA, in a 3×2 factorial design. The model included prebiotic treatment and sex. Scheffé's test was used for comparing mean values. The interactions between the main factors were not significant and are not reported in the results. Lipid oxidation (TBARS values) was analyzed as a repeated-measures, the model included the days of storage and prebiotic treatments, sex effect was not included in the model as it was not significant. The interactions between the main factors were not significant and are not reported in the results. For performance and mortality, the individual chicken house was considered an experimental unit; for slaughter performance and lipid oxidation, the individual bird was considered an experimental unit. All statistical tests were performed for a significance level of $P < 0.05$.

10.3 Results and discussion

The automatized *in ovo* technology provided an accurate system for delivering bioactives directly to the egg and allowed for large scale field investigation (Bednarczyk et al., 2011).

Tables 10.2 and 10.3 summarize the housing conditions and productive traits, respectively. At the end of the experiment, the final number of chickens/chicken house (ranging from 22,995 to 23,639) was not different (P > 0.05) among the 3 experimental groups. In general, the mortality recorded at 1st week of life and at the end of the experiment (ranging from 3.56 to 4.32 %) did not differ significantly among the experimental groups. The daily cumulative mortality rate expressed as a percentage and calculated using the formula in the EU directive, was 3.52% (at 42 d) and it was almost similar to the mortality reported for C group, but lower compared to that of BI and DN groups. However, it is necessary to point out that despite the high stocking density used in this farm study, the mortality in all experimental groups was reasonable and lower compared to the results obtained in a smaller-scale experimental study (mortality: 5.7% for a stocking density of 47 kg/m^2 and an average BW of 2.67 kg at 39 d of age; Buijs et al., 2009), but similar to the mortality reported in a field study conducted in Poland (stocking density of 46.8 kg/m^2 and an average BW of 2.41 kg at 42 d of age; Utnik-Banaś et al., 2014). In a large field survey, Dawkins et al. (2004) found that for a

range of stocking densities, from 30 to 46 kg/m^2 (0.073 to 0.047 m^2/bird), broiler health and welfare were to a great extent determined by the quality of the environment provided by producers. However, the EU Council Directive 2007/43/EC has fixed a maximum stocking density for broilers at 33 kg live weight/m^2 with the possibility to increase this limit to 39 and 42 kg live weight/m^2, if some technical requirements (e.g. feeding, heating, ventilation, disinfection and mortality) are fulfilled. In any case, farm-level decisions regarding stocking densities for broilers are still driven by cost-effectiveness and today European farms are often operating at high stocking density (e.g. 45 to 48 kg/m^2 in Belgium, Verspecht et al., 2011).

The recorded values for total BW/chicken house, stocking density (kg/m^2), average BW, FI, FCR and EBI were similar (P > 0.05) among the experimental groups (Table 10.3). However, treatment with BI and DN were associated with small increases in average BW (+ 2.6 % and + 0.9%, respectively) compared to the C group; even if these increases seem minor, considering the high number of the reared chickens, the economic impact could be interesting. In addition, treatment with BI was associated with a small (*P* > 0.05) improvement in FCR compared to the C and DN groups. A recent study by our group (Bednarczyk et al., 2016) showed that an increase in broiler weight is achieved when DN and BI are administered via *in ovo* or in-water. These results are in agreement with other previous studies which reported enhanced growth performance in birds supplemented with mannooligosaccharides as a growth promoter (Oyofo et al., 1989; Newman, 1994).

Table 10.2 - Number and mortality of broiler chickens.

	C[1]	BI[2]	DN[3]	SEM	Significance
n. chick/house	24,360	23,933	24,766	344	ns
n. chick/m^2	21.2	21.3	21.5	0.2	ns
n. chicken final/house	23,493	22,995	23,639	326	ns
Mortality 7 day (%)	1.36	1.94	2.07	0.23	ns
Total mortality (%)	3.56	3.92	4.55	0.33	ns

[1] C = Control, *in ovo* injection of physiological saline; [2] BI = Bi^{2}tos; [3] DN = DiNovo®.
Significance: ns = *P* > 0.05.

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Table 10.3 - *Productive performance, feed intake, feed conversion ratio and European broiler index of each chicken house.*

	C[1]	BI[2]	DI[3]	SEM	Significance
Livestock BW (kg)	52,253.00	52,314.67	53,001.33	1,003.32	ns
BW/m^2 (kg)	46.04	47.32	46.00	0.86	ns
BW average (kg)	2.30	2.36	2.32	0.03	ns
FI (kg)	89,938.0	88,340.0	90,920.0	2,031.7	ns
FCR	1.72	1.68	1.71	0.02	ns
EBI	328.2	347.3	325.6	6.60	ns

[1] C = Control, *in ovo* injection of physiological saline; [2] BI = Bi^{2}tos; [3] DI = DiNovo®.
Significance: ns = $P > 0.05$.

Slaughter traits of 15 broiler chickens (males and females) for each treatment are presented in Table 10.4. Compared with the C group, chickens from treated groups were significantly heavier (BI = +9.7%, $P < 0.05$; DN = +15.6%, $P < 0.01$) and showed a higher ($P < 0.01$) carcass weight and carcass yield. There were no statistically significant differences ($P > 0.05$) between the prebiotic groups. Pruszynska-Oszmalek et al. (2015) also found a significant increase in the final BW of 34 d old chickens which were *in ovo* injected with Bi^{2}tos. Treated birds had a higher breast muscle weight (BI = +12.3%, $P < 0.05$; DN = +16.9%, $P < 0.01$) in comparison with those of the C group; whereas no significant differences ($P > 0.05$) were observed between the prebiotic groups. Breast muscle yield was similar ($P < 0.05$) among the groups. According to our previous study (Maiorano et al., 2012) it can be assumed that the application of prebiotics has a positive impact on muscle weight. Moreover, Maiorano et al. (2012) found a slightly greater thickness (diameter) of muscle fibers in chickens *in ovo* injected with prebiotics or synbiotics compared with those of the control group.

As expected, males were heavier (+14.6%, $P < 0.01$) and showed a higher carcass and breast weights ($P < 0.01$) and carcass yield ($P < 0.05$); while breast muscle yield was similar between sex ($P > 0.05$).

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Table 10.4 - Effect of prebiotics and sex on slaughter traits

	C[1]	BI[2]	DI[3]	Female	Male	SEM	P	S
		Prebiotics (P)		Sex (S)			Significance	
Final BW (g)	2,151.9^{Bb}	2,360.3^a	2,488.1^A	2,204.6	2,526.7	0.03	**	**
Carcass weight (g)	1,452.3^B	1,646.4^A	1,724.7^A	1,504.5	1,762.7	0.02	**	**
Carcass yield (%)	67.45^B	69.70^A	69.30^A	68.19	69.76	0.25	*	*
Breast muscle (g)	497.74^{Bb}	559.21^a	581.70^A	515.81	591.83	10.67	*	**
Breast muscle (%)	34.15	33.89	33.79	34.19	33.57	0.31	ns	ns

[1] C = Control, *in ovo* injection of physiological saline; [2] BI = Bi^{2}tos; [3] DI = DiNovo®
Significance: ns = $P > 0.05$; * $P < 0.05$; ** $P < 0.01$.
Means in a row with different letters are significantly different at: $^{a,b}P < 0.05$, $^{A,B}P < 0.01$.

Figure 10.4 (a, b) showed the effects of prebiotic treatments on the lipid oxidation of breast meat. Overall, the TBARS values were lower in the meat of C birds in comparison with the prebiotic groups along the entire storage time. However, significant differences ($P < 0.05$) were only observed between the C and BI on d 4 (Figure 10.4a). In all experimental groups, the TBARS values were similar between 0 and 2 d of storage (Figure 10.4b), followed by an increase of TBARS values after 4 d of storage with different magnitude in all groups. In particular, a significant increase of TBARS values were observed in the C group from the 4th d of storage ($P < 0.01$) when compared to time 0, with the highest ($P < 0.01$) TBARS content on d 6 when compared to d 0 and d 2 of storage. The meat samples of both DN and BI were more marked by lipid oxidation from d 4 of the aerobic storage. In the BI group, the TBARS values on d 4 and 6 of storage were significantly higher when compared to d 0 ($P < 0.01$) and d 2 ($P < 0.05$) of storage. DN group showed a similar trend ($P < 0.01$). Recently, it has been shown that domestic birds are particularly susceptible to oxidative stress as a result of the genetic selection toward larger breast muscles, increased total BW, and the faster growth rates (Fellenberg and Speisky, 2006; Sihvo et al., 2014). This assumption may help explain why the meat from chickens of prebiotic groups, that had a bigger/heavier breast muscle, showed meat more susceptible to oxidation. Further research is warranted to elucidate and to confirm this result.

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In this work, conducted under commercial conditions, new data describing the effect of *in ovo* administration of BI (non-digestive trans-galactooligosaccharides) and DN (*Laminaria* spp. extract containing laminarin and fucoidan) prebiotics are reported. Prebiotics were associated with significant improvements in a number of parameters, including body weight, carcass weight, carcass yield and breast muscle weight. Males had significantly higher slaughter traits compared to females, except for breast muscle yield. Meat from prebiotic treated birds displayed a higher lipid oxidation levels compared to that from untreated ones along the entire storage time. However, the highest TBARS detected values are below the TBARS levels associated with meat rancidity (from 0.5 to 1.0 mg/kg muscle for pork and lamb, from 0.6 to 2.3 mg/kg muscle for beef) (Ripoll et al., 2011; Kasapidou et al., 2012).
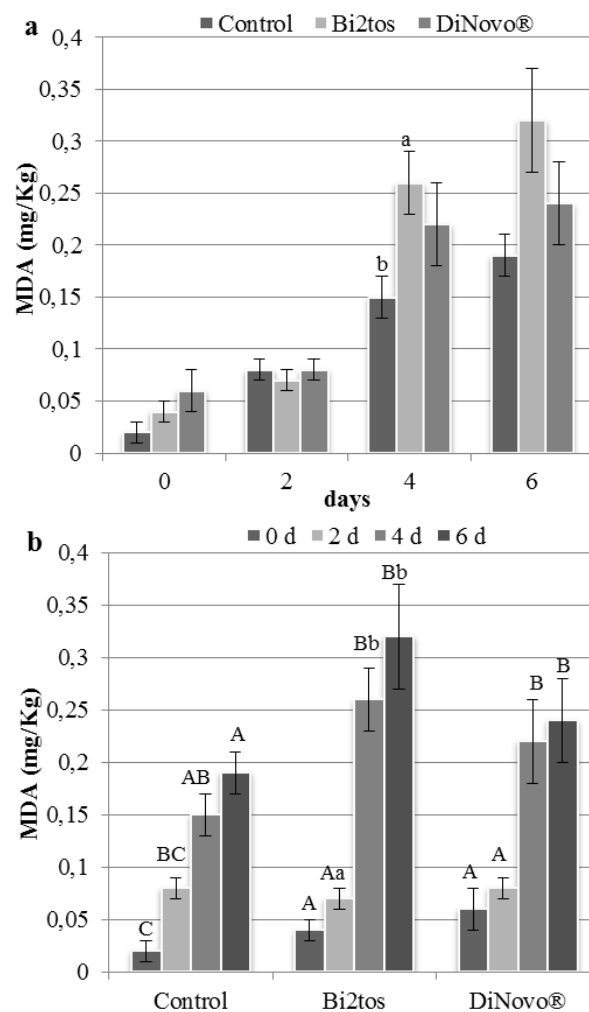
Figure 10.4 - *Number of chickens for each chicken house and each treatment*

10.4 Conclusions

The results of this investigation, conducted under commercial conditions, showed that *in ovo* administration of BI (non-digestive trans-galactooligosacharides) and DN (dietary *Laminaria*-derived laminarin and fucoidan) prebiotics was associated with significant improvements in a number of parameters of relevance to commercial poultry production. Males had significant higher slaughter traits compared to females, except for breast muscle yield. Meat from *in ovo* treated birds displayed a higher lipid oxidation levels compared to that from untreated ones along the entire storage time. However, in this study, the values of meat from all experimental groups at 6 days of storage are below the critical value of 0.5 recognized for the production of rancid odour influencing negatively the taste of meat (Kasapidou et al., 2012). It must be taken into account that the harmful phenomena of lipid oxidation goes beyond the actual impact on food quality and reaches the potential influence of oxidized foods on consumer health.

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ADP Adenosine Diphosphate

AGP Antibiotic Growth Promoters

ATP Adenosine Triphosphate

AVEC Association of Poultry Processors and Poultry Trade in the EU countries

BI Bi^{2}tos

BW Body Weight

BWG Body Weight Gain

CDC Centers for Disease Control and Prevention

CFU Coloning Forming Units

DFD Dark Firm and Dry

DFM Direct Feed Microbials

DG SANCO Directorate General for Health and Consumer Affairs

DM Dry Matter

DN DiNovo®

DPM Deep Pectoral Muscle

EBI European Broiler Index

EC European Council

ECM Extracellular Matrix

ED Embryonic Day

EDTA Ethylenediaminetetraacetic acid

EFSA European Food Safety Authority

ER Endoplasmatic Reticulum

EU European Union

FAO Food and Agriculture Organisation on the United Nations

FCR Feed Convertion Ratio

FDA Food and Drug Administration

FI Feed Intake

FOS Fructo-oligosaccharises

GAG Glucosaminoglycans

GI Gastrointestinal Tract

GOS Galacto-oligosaccharises

HFBA Heptafluorobutyric acid

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HLP hHydroxylysylpyridinoline

HMB β-hydrohy-β-methylbutyrate

HP Hydroxyproline

HPLC High-Performance (high-pressure) Liquid Chromatography

IBD Infectious Bursal Disease

IMCT Intramuscular Connective Tissue

MD Marek's Disease

MDA Malondialdehyde

MMPs Matrix Metalloproteinases

MOS Mannan-oligosaccharises

ND Newcastle Disease

NRC National Research Council

PM Pectoral muscle

PSE Pale, soft and exudative

RFO Raffinose Family Oligosaccharises

SCFA Short Chain Fatty Acids

SCFA Short Chain Fatty Acids

TBA Thiobarbituric Acid

TBARS Thiobarbituric Acid Reactive Substances

TIMPs Tissue Inhibitors of Matrix Metalloproteinases

TLC Thin Layer Cromatography

UK United Kingdom

US United States

WBP Water Binding Potential

WHC Water Holding Capacity

WHO World Health Organisation

WOAH World Organization for Animal Health

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