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Effects of different probiotics and synbiotics and mode of their administration on productive performance, carcass traits and meat quality in broiler chickens

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To Francesca. It was a honour to be your friend.
You will always be with me.

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ABSTRACT

In recent years, the key role of gut microbiota in several aspects of physiology and animal welfare, is increasingly evident. In the poultry industry, antibiotics have been used for a long time, both as therapeutic agents to treat diseases, and also as growth promoters (AGPs) in order to enhance health and performance of birds without therapeutic aims. However, this approach has led to undesired effects, such as the development of antibiotic resistance, and the presence of antibiotic residues in poultry meat and poultry products. In this context the use of AGPs was banned by the European Union from January 1, 2006 (EC regulation No 1831/2003). The ban of AGPs has contributed to increased incidence of enteric diseases (e.g., salmonellosis and campylobacteriosis), resulting in higher mortality rates and lower productivity, as well as increased risks of food-borne diseases in humans, causing serious economic damage to the poultry industry. Since the ban of AGPs in EU, several feed additives have been tested in broiler chickens in order to improve health and performance. Among others, in the post-antibiotics era, probiotics, prebiotics and synbiotics (combination of pro- and prebiotics) offer a natural and safe solution to replace AGPs through the modulation of the activity of the gastrointestinal microbiota, and thus, are considered beneficial to the host animal. The conventional administration of these bioactives in feed and/or water in the first hours/days post-hatching, could lead to conflicting results due to the environmental conditions, such as the individual feed and water intake, the quality of water, and other factors. Moreover, these bioactives should be administered as early in life as possible, minimizing the environmental variables that can compromise their efficacy. To reduce the effect of these factors, *in ovo* injection technology of probiotics, prebiotics and synbiotics has been developed. The method allows the accurate and precise delivery of the bioactive substance at very low doses to all embryos at early stage of development, minimizing the effect of environmental factors and influencing the microbiome structure in newly hatched chicks.

This thesis, which involved two different research works, has aimed to evaluate the effects of different bioactives (probiotics, prebiotics and their combination), administerd in feed (Trial 1) or *in ovo* (Trial 2) on productive performance and meat quality traits (physico-chemical characteristics, intramuscular collagen properties, total lipids content, cholesterol content, fatty acids composition) in broiler chickens.

The first experiment aimed to assess the effects of a probiotic preparation and a synbiotic combination supplemented in feed on economic impact (European Broiler Index, EBI), performance, carcass traits and meat quality in broiler chickens. 360 one-day-old female chicks (Ross 308) were randomly allotted to 3 dietary treatments: basal diet (Control, **C**); basal diet with 1% of Lavipan® (JHJ Sp. z o. o., Gizałki, Poland) (**L**) consisting of *L. lactis* IBB500, *C. divergens* S1, *L. casei* ŁOCK 0915, *L. plantarum* ŁOCK 0862 and *S. cerevisiae* ŁOCK 0141; basal diet with a combination of Lavipan® (1%) with RFO (0.8%) (**LR**). Both formulations were supplemented for the first 7 days of chick's life. Chickens were reared in 30 floor pens (10 replicate pens/treatment, 12 chicks/pen). To provide commercial conditions, the poultry house was filled with 9000 as-hatched chicks. Animals were fed *ad libitum* with commercial diets and had free access to water. Mortality for the overall experimental period was calculated for each pen replicate. Body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR), at 10, 21 and 40 days of age, were calculated on pen basis. At 41 days of age, 10 randomly chosen birds per treatment, between more big, were weighed and slaughtered. The yields of carcass, breast and legs were calculated. pH, color and water holding capacity (WHC) were measured at 24 hours *post mortem* on right pectoral muscle (PM). Total lipids and fatty acids analyses on left PM were carried out. Data were evaluated by one way ANOVA. Scheffé's test was applied to compare the differences among means. The mortality was lower in groups fed with supplementation of probiotic (1 %) and synbiotic (2.27 %) compared with C group (5 %), however, the differences were not significant ($P > 0.05$). The BWG within the first 10 days of life was affected by dietary probiotic and synbiotic formulation ($P < 0.05$). In general, both L and LR groups were characterized by better BWG values in comparison with C (207.89, 208.99, and 197.63 g/bird, respectively; $P = 0.037$). No significant differences in the BWG were found for the rest of the rearing period even if the treated groups showed a slightly higher ($P > 0.05$) BWG compared to C. FI within the first 10 days of life was affected by the treatment; it was higher ($P < 0.05$) for chickens of L group compared to C. The total amount of FI was slightly higher ($P > 0.05$) both in L and LR groups in comparison with C. In the present research, FCR was similar between experimental groups for the whole rearing period ($P > 0.05$). EBI was better both in L and LR groups compared with C; however treatment with L was associated with a higher ($P < 0.05$) value (+2.6%) in comparison with C. The final body weight was similar ($P > 0.05$) between experimental groups. The dietary probiotic and synbiotic supplementation had no effects ($P > 0.05$) on carcass weight and carcass yield, as well as on PM weight, PM yield, and legs weight and legs yield, as well as physico-chemical traits (pH, color and WHC). The total content of lipids, SFA, MUFA and PUFA, as well as the individual fatty acids were not

affected (P > 0.05) by the treatment. Dietary probiotic and synbiotic supplementation had no effects (P > 0.05) on all calculated nutritional ratios (n-6/n-3, PUFA/SFA). Atherogenic and thrombogenic indices were similar (P > 0.05) between experimental groups as well.

The results indicate that, taking into account the effects on growth performance, the economic impact could be relevant if considered to feed a high number of animals reared in commercial conditions with basal diet with 1% of Lavipan® and basal diet with a combination of Lavipan® (1%) with 0.8% RFO.

The aim of the second trial was to evaluate the effect of two different synbiotics administered *in ovo* on performance, carcass traits and meat quality in broiler chickens. On day 12 of incubation, 5850 eggs (Cobb 500FF) were randomly divided into 3 experimental groups and were *in ovo* injected with either: 0.2 ml of a physiological saline solution (Control, **C**); 0.2 ml of a synbiotic formulation containing 2 mg/embryo of Bi^{2}tos (Clasado BioSciences Ltd., Sliema, Malta), trans-galactooligosaccharides enriched with 10^5cfu/embryo of *Lactobacillus salivarius* IBB3154 (**SYN1**) or 0.2 ml of a synbiotic formulation containing 2 mg/embryo of raffinose family oligosaccharides (RFO) enriched with 10^5 cfu/embryo of *Lactobacillus plantarum* IBB3036 (**SYN2**). After the injection, the injection hole was covered with a drop of organic glue and the incubation was continued until hatching. Among the hatched chickens, 2040 males (680 per each group) were randomly chosen and reared in a commercial poultry house. Chickens were raised in pens (n = 75/pen) with 8 pen replicates per treatment for effect on performance. Moreover, separate pens for sampling (n = 10 birds per pen: 8 replications per each experimental group) were included in the experimental design. Animals were fed *ad libitum* with commercial diets according to their age and had free access to water. Mortality for the overall experimental period was calculated for each pen replicate. FI and FCR were calculated on a pen basis. At 42 days of age, two birds per pen (16 birds per treatment) were randomly chosen from the separate pens for sampling and slaughtered. At slaughter, hot carcass weight was recorded and carcass yield was calculated. PM was removed from each carcass and weighed; its percentage was calculated based on hot carcass weight. pH and color on the right PM were recorded at 45 minutes and 24 hours *post mortem*; in addition, water holding capacity (WHC) at 24 hours was measured on the right PM. Intramuscular collagen, total lipids, cholesterol and fatty acids analysis on left PM were carried out. Data were analyzed by one way ANOVA. Scheffé's test was applied to compare the differences among means. Mortality rate was slightly higher (P > 0.05) in C group compared with synbiotics. *In ovo* administration of synbiotics did not affect (P > 0.05) both FI and FCR. The final BW was similar between experimental groups (P > 0.05). No effects were found also for values of carcass weight and carcass yield (P > 0.05), as well as in case of PM

weight and PM yield between experimental groups (P > 0.05). pH value measured 45 minutes and 24 hours *post mortem* were not significantly affected (P > 0.05) by both synbiotic treatments. The lightness of meat (L*) measured in PM at 45 minutes *post mortem* was affected by synbiotics; it was higher (P < 0.01) for meat from chickens of SYN1 group in comparison with SYN2. No differences (P > 0.05) were detected for L* measured at 24 hours between the three experimental groups. Redness (a*) and yellowness (b*) measured 45 minutes and 24 hours *post mortem* were similar (P > 0.05) among groups. Synbiotic treatment did not affect (P > 0.05) WHC of breast muscle. Collagen concentration (μg/mg), collagen maturity (mol of HLP/mol of collagen) and HLP concentration (μg/mg) were not significantly (P > 0.05) influenced by *in ovo* synbiotic administration.Treatment of synbiotics reduced (P = 0.061) the lipid content compared with control group, markedly (P < 0.05) with synbiotic SYN2. The total SFA content was affected (P < 0.01) by *in ovo* administration of synbiotics; it was markedly increased (P < 0.01) with SYN1 treatment in comparison with C and SYN2. The total amount of MUFA was similar between C and SYN1, but it was lower (P < 0.05) in SYN1 compare with SYN2. The PUFA content was markedly influenced (P < 0.01) by the synbiotic treatment being reduced (P < 0.01) by administration of SYN1 compared to C and SYN2; while similar values (P > 0.05) were found between C and SYN2. *In ovo* treatment was associated also with significant effects on n-3 long chain PUFA derivatives: eicosapentaenoic acid (EPA, C 20:5 n-3), was increased with SYN2 (P <0.05) compared with SYN1 and reduced with SYN1 (P < 0.01) compared to C; docosahexaenoic acid (DHA, C 22:6 n-3), was increased with SYN1 in comparison with SYN2. Docosapentaenoic acid (C 22:5 n-3) was not affected (P > 0.05) by treatment. The n-6/n-3 PUFA ratio was influenced by the synbiotic administration (P = 0.039), with slightly higher values in SYN1 and SYN2 groups compared with C group (P > 0.05). The PUFA/SFA ratio was affected by *in ovo* treatment with synbiotics (P < 0.01): meat from SYN1 had lower value of PUFA/SFA ratio compared with C and SYN2 groups (P < 0.01). SYN1 administration resulted in an increased (P < 0.01) of both atherogenic and thrombogenic indices compared with C and SYN2. Synbiotics did not affect (P > 0.05) the cholesterol content of PM.

The results indicate that *in ovo* administration of synbiotics did not negatively affect productive performance and physicochemical properties of meat. However, *in ovo* administration of *Lactobacillus salivarius* + Bi^{2}tos (SYN1) affected negatively the fatty acids profile of meat and the values of atherogenic and thrombogenic indices, indicating a higher risk of incidence of pathogenic phenomena. Whereas, SYN2 (*Lactobacillus plantarum* + Lupin RFO) did not affect fatty acids profile and nutritional properties of chicken meat.

RIASSUNTO

In anni recenti, il ruolo chiave del microbiota intestinale è diventato sempre più rilevante in diversi aspetti della fisiologia e del benessere animale. Nell'industria avicola, gli antibiotici sono stati utilizzati per lungo tempo, sia come agenti terapeutici nel trattamento delle malattie, sia come promotori di crescita per migliorare la salute e le performance degli animali. Questo approccio ha portato a conseguenze negative, come ad esempio lo sviluppo del fenomeno dell'antibiotico-resistenza ed alla presenza di residui di antibiotici nella carne e nei prodotti derivati. In questo contesto, l'uso degli antibiotici auxinici è stato vietato nell'Unione Europea dal 1° Gennaio 2006 (Regolamento CE N. 1831/2003) Conseguentemente al divieto, si è verificato un aumento dell'incidenza delle malattie enteriche negli animali (salmonellosi, campilobatteriosi), con aumento della mortalità e calo della produttività, nonché un incremento dei casi di tossinfezioni alimentari nell'uomo. Tutto questo, ha portato ad ingenti danni economici per l'industria avicola. Successivamente al divieto, diversi additivi alimentari sono stati testati nell'allevamento avicolo. Tra questi, probiotici, prebiotici e simbiotici (combinazione di probiotici e prebiotici), offrono una naturale e sicura alternativa agli antibiotici auxinici, attraverso la modulazione del microbiota intestinale, migliorando lo stato di salute degli animali. La somministrazione convenzionale di queste sostanze nell'alimento e nell'acqua, nelle prime ore o giorni post-schiusa, è soggetta a risultati contrastanti dovuti a variabili incontrollabili, come ad esempio il consumo individuale di alimento e acqua, la qualità dell'acqua, il processo di lavorazione delle materie prime per la produzione del mangime ed altri fattori. Inoltre, al fine di ottenere i risultati desiderati, questi bioattivi dovrebbero essere somministrati agli animali il prima possibile, al fine di minimizzare l'effetto dei fattori ambientali che possono comprometterne l'efficacia. Per questi motivi, è stata sviluppata la tecnologia dell'iniezione *in ovo* di probiotici, prebiotici e simbiotici. Questo approccio permette una precisa ed accurata somministrazione della sostanza bioattiva, a dosaggi molto bassi, in fase embrionale ad un precoce stadio di sviluppo, minimizzando l'effetto dei fattori ambientali ed influenzando la struttura del microbioma dei pulcini.

Questa tesi, che ha coinvolto due differenti sperimentazioni, ha avuto lo scopo di valutare l'effetto di differenti probiotici e formulazioni simbiotiche, somministrate nell'alimento (Esperimento 1) o *in ovo* (Esperimento 2), sulle performance produttive e le

caratteristiche qualitative della carne (proprietà fisico-chimiche, proprietà del collagene intramuscolare, contenuto di lipidi totali e colesterolo, profilo degli acidi grassi) di pollo.

Il primo studio ha avuto come obiettivo quello di valutare gli effetti di una preparazione probiotica e di una formulazione simbiotica somministrate nell'alimento, sull'efficienza economica dell'allevamento (European Broiler Index, EBI), le performance produttive e le caratteristiche qualitative della carne di pollo. 360 pulcini femmine di un giorno di età (Ross 308) sono stati casualmente sottoposti a 3 trattamenti alimentari: dieta base (Controllo, **C**); dieta base con aggiunta di 1% di Lavipan® (JHJ Sp. z o. o., Gizałki, Polonia) (**L**), un probiotico commerciale costituito da *L. lactis* IBB500, *C. divergens* S1, *L. casei* ŁOCK 0915, *L. plantarum* ŁOCK 0862 e *S. cerevisiae* ŁOCK 0141; dieta base con aggiunta di una formulazione simbiotica costituita da 1% di Lavipan® con RFO (oligosaccaridi della famiglia del raffinosio) (0.8%) (**LR**). Entrambe le formulazioni sono state somministrate ai pulcini per i primi 7 giorni di vita. I polli sono stati allevati in un'azienda commerciale (PiastPasze Sp. z.o.o., Olszowa, Polonia) secondo le raccomandazioni previste dall'Unione Europea in materia di benessere animale (direttiva 86/609/CEE). I polli sono stati allevati in gabbie collettive (n = 12 polli per gabbia: 10 repliche per ciascun gruppo sperimentale). Al fine di creare un ambiente di allevamento simile ad un allevamento commerciale, sono stati aggiunti in azienda 9000 pulcini di un giorno di età. Gli animali sono stati alimentati *ad libitum* con un mangime commerciale adeguato alla loro età ed hanno avuto accesso libero all'acqua. La mortalità totale è stata calcolata per gabbia. L'incremento di peso giornaliero, l'assunzione di cibo e l'indice di conversione alimentare (ICA) a 10, 21 e 40 giorni, sono stati calcolati per gabbia. A 41 giorni di età, tutti gli animali sono stati pesati individualmente e contestualmente sono stati scelti 10 animali per trattamento, casualmente tra i più pesanti, per essere macellati. Al momento della macellazione è stato registrato il peso della carcassa, del petto e delle cosce e calcolate le relative rese. A 24 ore *post mortem*, sul muscolo pettorale destro è stato registrato il pH e colore, e misurata la capacità di ritenzione idrica (WHC). Il muscolo pettorale sinistro è stato messo sottovuoto e congelato (-20°C) fino al momento delle analisi dei lipidi totali e del profilo degli acidi grassi. I dati ottenuti sono stati sottoposti all'analisi statistica mediante ANOVA ad una via. Le differenze tra le medie sono state valutate mediante il test di Scheffé. La mortalità è risultata più bassa nei gruppi alimentati con probiotico (1%) e simbiotico (2,27%) rispetto al gruppo di controllo (5%), tuttavia, le differenze non sono risultate significative ($P > 0,05$). L'incremento di peso giornaliero nei primi 10 giorni di vita è stato influenzato dalla supplementazione di probiotico e simbiotico ($P < 0,05$). In generale, i gruppi L ed LR sono stati caratterizzati da migliori valori di incremento di peso nei primi 10 giorni di sperimentazione rispetto al gruppo di controllo (207,89, 208,99

e 197,63 g/animale, rispettivamente; $P < 0,05$). Nessuna differenza significativa è stata riscontrata per il resto del periodo di allevamento, anche se i gruppi trattati hanno mostrato un incremento di peso leggermente più alto ($P > 0,05$) rispetto al controllo. L'assunzione di alimento nei primi 10 giorni di vita è stata influenzata dal trattamento, risultando più alta ($P < 0,05$) per i polli del gruppo L rispetto al controllo. L'assunzione di alimento per tutto il periodo sperimentale è risultata leggermente più elevata ($P > 0,05$) nei gruppi L ed LR rispetto al gruppo di controllo. I valori di ICA sono risultati simili ($P > 0,05$) tra i gruppi sperimentali per tutto il periodo di osservazione. I valori dell'EBI sono risultati migliori nei gruppi L ed LR rispetto al controllo; tuttavia, il trattamento con L è stato associato con valori più elevati ($P < 0,05$; +2,6%) rispetto al gruppo di controllo. Il peso alla macellazione è risultato simile ($P > 0,05$) tra i gruppi sperimentali. La supplementazione probiotica e simbiotica non ha avuto effetti ($P > 0,05$) sul peso della carcassa, del muscolo pettorale e delle cosce e sulle relative rese. Anche le proprietà fisico-chimiche della carne (pH, colore e WHC) non sono state influenzate dal trattamento ($P > 0,05$). Il contenuto totale di lipidi, acidi grassi saturi (SFA), monoinsaturi (MUFA) e polinsaturi (PUFA), così come il contenuto dei singoli acidi grassi, non è stato influenzato ($P > 0,05$) dal trattamento. Il probiotico e il simbiotico non hanno avuto effetti ($P > 0,05$) sugli indici nutrizionali calcolati (n-6/n-3, PUFA/SFA, indici aterogenico e trombogenico).

I risultati di questo studio indicano che, tenendo in considerazione gli effetti sulle performance di crescita, l'impatto economico della supplementazione dietetica con Lavipan® (1%) e con Lavipan® (1%) arricchito con RFO (0,8%), potrebbe essere rilevante se si considera di allevare un elevato numero di animali in condizioni commerciali.

L'obiettivo del secondo studio è stato quello di valutare l'effetto di due differenti simbiotici somministrati *in ovo* sulle performance produttive e le caratteristiche qualitative della carne di pollo. Al 12° giorno di incubazione, 5850 uova (Cobb 500FF) sono state divise casualmente in tre gruppi sperimentali, cui sono stati somministrati, mediante tecnica di iniezione *in ovo*, differenti bioattivi: **C**, gruppo di controllo iniettato con 0,2 ml di soluzione fisiologica; gruppo **SYN1**, iniettato con 0,2 ml di una formulazione simbiotica contenente 2 mg/embrione di Bi^{2}tos (Clasado BioSciences Ltd., Sliema, Malta), un prebiotico commerciale costituito da *trans*-galatto-oligosaccaridi, arricchita con 10^5ufc/embrione del batterio probiotico *Lactobacillus salivarius* IBB3154; gruppo **SYN2**, iniettato con 0,2 ml di una formulazione simbiotica contenente 2 mg/embrione di oligosaccaridi della famiglia del raffinosio (RFO), arricchita con 10^5 ufc/embrione del batterio probiotico *Lactobacillus plantarum* IBB3036. Entrambi i batteri sono stati forniti dalla banca microbiologica dell'Istituto di Ricerca "Biochemistry and Biophysics" di Varsavia (Polonia). Dopo

l'iniezione, il foro è stato chiuso con colla organica e le uova sono state nuovamente incubate fino alla schiusa. Tra i pulcini nati, 2040 maschi (680 per ciascun gruppo sperimentale) sono stati scelti casualmente ed allevati in un'azienda commerciale (PiastPasze Sp. z.o.o., Olszowa, Polonia) secondo le raccomandazioni previste dall'Unione Europea in materia di benessere animale (direttiva 86/609/CEE). I polli sono stati allevati in gabbie collettive (n = 75 polli per gabbia: 8 repliche per ciascun gruppo sperimentale), per la valutazione degli effetti del trattamento sulle performance produttive. Inoltre, sono state incluse nel disegno sperimentale gabbie separate per il campionamento (n = 10 polli per gabbia: 8 repliche per ciascun gruppo sperimentale). Gli animali sono stati alimentati *ad libitum* con un mangime commerciale adeguato alla loro età ed hanno avuto accesso libero all'acqua. La mortalità totale è stata calcolata per gabbia per tutto il periodo sperimentale. L'assunzione di alimento e l'indice di conversione alimentare (ICA) sono stati calcolati per gabbia. A 42 giorni di età, 2 polli per gabbia (16 per ciascun gruppo sperimentale) sono stati scelti casualmente dalle gabbie per il campionamento e macellati. Alla macellazione, è stato registrato il peso della carcassa e calcolata la resa. Il muscolo pettorale è stato rimosso da ogni carcassa e pesato; la resa è stata calcolata in funzione del peso della carcassa. Il pH e il colore sono stati registrati sul muscolo pettorale destro a 45 minuti e 24 ore *post mortem*; inoltre, la capacità di ritenzione idrica (WHC) è stata misurata sul muscolo pettorale destro a 24 ore *post mortem*. Il muscolo pettorale sinistro è stato messo sottovuoto e congelato (-20°C) fino al momento delle analisi chimiche sulle proprietà del collagene intramuscolare, contenuto di lipidi totali e colesterolo, e profilo degli acidi grassi. I dati ottenuti sono stati sottoposti all'analisi statistica mediante ANOVA ad una via. Le differenze tra le medie sono state valutate mediante il test di Scheffé. Il tasso di mortalità è risultato leggermente più alto ($P > 0,05$) nel gruppo di controllo rispetto ai simbiotici. La somministrazione *in ovo* dei simbiotici non ha influenzato ($P > 0,05$) l'assunzione di alimento e l'indice di conversione alimentare. Il peso alla macellazione è risultato simile fra i gruppi sperimentali ($P > 0,05$). Il trattamento non ha avuto effetti significativi anche per quanto riguarda il peso e la resa della carcassa ($P > 0,05$), così come sul peso e sulla resa del muscolo pettorale ($P > 0,05$). Il pH misurato a 45 minuti e 24 ore *post mortem* non è stato influenzato ($P > 0,05$) da entrambe le formulazioni simbiotiche. Il valore di luminosità (L*) della carne registrato sul muscolo pettorale a 45 minuti *post mortem* è stato influenzato dai simbiotici, risultando più alto ($P < 0,01$) per la carne dei polli del gruppo SYN1 rispetto al gruppo SYN2. Il valore di L* misurato a 24 ore è risultato simile fra i gruppi sperimentali ($P > 0,05$). L'indice del rosso (a*) e l'indice del giallo (b*) misurati a 45 minuti e 24 ore *post mortem* sono risultati simili ($P > 0,05$) tra i gruppi. Il trattamento con i simbiotici non ha influenzato ($P > 0,05$) la WHC del muscolo pettorale. Le proprietà del collagene

intramuscolare non sono state significativamente influenzate (P > 0,05) dalla somministrazione *in ovo* dei simbiotici. Il trattamento ha ridotto (P = 0,061) il contenuto lipidico rispetto al gruppo di controllo, marcatamente (P < 0,05) nel caso del simbiotico SYN2. Il contenuto totale di SFA è stato influenzato (P < 0,01) dalla somministrazione *in ovo* dei simbiotici, risultando marcatamente più elevato (P < 0,01) nel gruppo SYN1 rispetto al gruppo di controllo e SYN2. Il contenuto totale di MUFA è risultato simile tra il gruppo di controllo e SYN1, ma più basso (P < 0,05) nel gruppo SYN1 rispetto al gruppo SYN2. Il contenuto di PUFA è stato significativamente influenzato (P < 0,01) dai simbiotici, essendo stato ridotto (P < 0,01) dalla somministrazione del SYN1 rispetto al gruppo di controllo e SYN2. Valori simili (P > 0,05) sono stati invece osservati tra il gruppo di controllo e SYN2. Inoltre, il trattamento *in ovo* è stato associato con effetti significativi sul contenuto di PUFA a lunga catena della serie n-3: l'acido eicosapentaenoico (EPA, C 20:5 n-3) è stato incrementato nel caso del gruppo SYN2 (P < 0,05) rispetto al gruppo SYN1 e ridotto nel gruppo SYN1 (P < 0,01) rispetto al gruppo di controllo; il contenuto di acido docosaesaenoico (DHA, C 22:6 n-3) è risultato più elevato (P < 0,05) nel gruppo SYN1 rispetto al SYN2. Il contenuto di acido docosapentaenoico (DPA, C 22:5 n-3) non è stato influenzato (P > 0,05) dal trattamento. Il rapporto n-6/n-3 è stato influenzato dal trattamento (P = 0,039), con valori leggermente più alti nei gruppi SYN1 e SYN2 rispetto al gruppo di controllo (P > 0,05). Il rapporto PUFA/SFA è stato influenzato dalla somministrazione *in ovo* dei simbiotici (P < 0,01): la carne dei polli del gruppo SYN1 ha presentato valori più bassi di tale rapporto rispetto al gruppo di controllo e SYN2 (P < 0,01). Inoltre, la somministrazione del simbiotico SYN1 ha aumentato (P < 0,01) gli indici aterogenico e trombogenico rispetto al gruppo di controllo e SYN2. I simbiotici non hanno influenzato (P > 0,05) il contenuto di colesterolo del muscolo pettorale.

I risultati indicano che la somministrazione *in ovo* dei simbiotici non ha avuto effetti negativi sulle performance e le proprietà fisico-chimiche della carne. Tuttavia, la somministrazione del simbiotico SYN1 (*Lactobacillus salivarius* + Bi^{2}tos) ha avuto effetti negativi sul profilo acidico della carne e sui valori degli indici aterogenico e trombogenico. Mentre, il simbiotico SYN2 (*Lactobacillus plantarum* + RFO) non ha avuto effetti sul profilo acidico e sulle proprietà nutrizionali della carne.

In conclusione, i risultati di questa tesi indicano che la supplementazione nell'alimento dei probiotici ha avuto effetti più marcati nei giovani animali in crescita, migliorando l'incremento di peso giornaliero e l'efficienza economica della produzione del pollo da carne (EBI). In merito agli aspetti nutrizionali, i simbiotici (SYN1 e SYN2) somministrati *in ovo* hanno ridotto il contenuto lipidico della carne. Il simbiotico SYN1 (*Lactobacillus salivarius* +

Bi^{2}tos) ha influenzato negativamente il profilo acidico della carne e i valori degli indici aterogenico e trombogenico, indicando un più elevato rischio di insorgenza di fenomeni patologici.

Quindi, è possibile affermare che l'uso di questi bioattivi, con differenti vie di somministrazione, è sicuramente una valida opzione al fine di migliorare lo stato di salute e le performance degli animali, risultando in importanti vantaggi economici. D'altra parte, la somministrazione *in ovo* dei simbiotici potrebbe rappresentare una valida alternativa alla convenzionale somministrazione nei mangimi. Questo metodo consente una precisa somministrazione dei bioattivi a bassi dosaggi e ad un precoce stadio di sviluppo embrionale, garantendo un corretto sviluppo della microflora intestinale, aspetto importante al fine di evitare infezioni nel primo periodo post-schiusa, ed eliminando i costi della prolungata somministrazione nell'alimento. Inoltre, la somministrazione *in ovo* potrebbe presentare ulteriori vantaggi economici in ragione della possibilità di testare questa tecnologia in combinazione con i vaccini, o con la somministrazione di altre tipologie di nutrienti. Questo, potrebbe aprire in un prossimo futuro, alla possibilità di utilizzare questa tecnologia su scala industriale.

Alla luce di quanto affermato, sono necessarie ulteriori ricerche per poter migliorare le conoscenze riguardo gli effetti della somministrazione *in ovo* dei simbiotici sulle performance e le caratteristiche qualitative della carne di pollo, sia in condizioni sperimentali che di campo. Inoltre, vi è la necessità di ricerche future anche al fine di selezionare la giusta combinazione di queste sostanze bioattive, per ottenere formulazioni più funzionali. Infine, è sicuramente necessario approfondire gli studi, per meglio comprendere gli effetti di questi bioattivi sulle vie metaboliche degli animali, come ad esempio la sintesi dei lipidi e degli acidi grassi.

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µ micro
AGPs antibiotics growth promoters
AI atherogenic index
ALA α-linolenic acid
ANOVA analysis of variance
ATP adenosine triphosphate
BW body weight
BWG body weight gain
CE competitive exclusion
CFU coloning-forming unit
CLA conjugated linoleic acids
COMb carboxymyoglobin
DFD dark, firm and dry
DHA docosahexaenoic acid
DMb deoxymyoglobin
DPA docosapentaenoic acid
DPM deep pectoral myopathy
EBI European broiler index
EFA essential fatty acids
EPA eicosapentaenoic acid
FA fatty acids
FCR feed conversion ratio
FI feed intake
FOS fructo-oligosaccharides
GALT gut-associated lymphoid tissues
GIT gastrointestinal tract
GOS galacto-oligosaccharides
HDL high density lipoprotein
HLP hydroxylysilpyridinoline
HPLC high-performance (high-pressure) liquid chromatography
IMC Intramuscular collagen
IMCT intramuscular connective tissue
L:D hours light:hours darkness in a photoperiod
LA linoleic acid
LAB lactic acid bacteria
LDL low density lipoprotein
MALT mucosa-associated lymphoid tissues
MMb metmyoglobin
mmHg millimeter of mercury
mmol millimoles
mol moles
MUFA monounsaturated fatty acids
NDO non-digestible oligosaccharides
OMb oxymyoglobin
pH_u ultimate pH
PM pectoral muscle
PSE pale, soft and exudative
PUFA polyunsaturated fatty acids
RFO raffinose family oligosaccharides

SCFA short chain fatty acids
SFA saturated fatty acids
TI thrombogenic index
TOS *trans*-galacto-oligosaccharides
WB wooden breast
WHC water holding capacity
WS white striping

PART 1. INTRODUCTION (Literature review)

Chapter 1
Trends in poultry meat production and consumption: an overview

Meat is a food with a high nutritional value of great importance for the psychophysical development and maintenance of health and human well-being. It is an important source of some micronutrients such as iron, selenium, vitamins A, B12 and folic acid. These micronutrients are either not present in plant derived food or have poor bioavailability. In addition, meat, as a protein rich and carbohydrate low product, contributes to a low glycemic index which is assumed to be beneficial with respect to overweight, the development of diabetes and cancer. As an essential part of a mixed diet, meat ensures adequate delivery of essential micronutrients and amino acids and is involved in regulatory processes of energy metabolism (Biesalski, 2005).

The world food economy is being increasingly driven by the shift of diets and food consumption patterns towards livestock products. In developing countries, where almost all world population increases take place, consumption of meat has grown at an average rate of 5.1% *per annum* since 1970 (Alexandratos and Bruinsma, 2012). According with the report OECD/FAO (2016), the global meat production is projected to be 16% higher in 2025 than in the base period (2013-2015). This compares with an increase of almost 20% in the previous decade. Developing countries are projected to account for the vast majority of the total increase, through a more intensive use of protein meal in feed rations. Globally, 10% of meat output will be traded in 2025, up from 9% in 2015, with most of the increase coming from poultry meat.

Poultry meat has become one of the most important consumer products worldwide, with very different levels of development, in diverse form (Magdelaine et al., 2008). The market orientation of the poultry meat sector is facilitated by the characteristics of poultry meat production that cumulates many advantages compared to other meat sectors. First of all, the short production cycle enables producers to respond quickly to market signals, allowing for rapid improvements in genetics, animal health, and feeding practices. The shorter duration of the production cycle grants much more flexibility and reactivity to the producers to adapt. Moreover, poultry meat is characterized by many additional advantages compared to meats of other species: (i) price competitiveness compare to pork, beef or lamb meat; (ii) absence of religious restrictions; (iii) low fat content and good and balanced protein content (AVEC,

2016). Poultry meat, is the primary driver of the growth in total meat production in response to expanding global demand of more affordable animal protein compare to red meats. Low production costs and lower product prices have contributed to making poultry the meat of choice both for producers and consumers in developing countries.

Over the decade 2000-2010, the poultry sector was the most dynamic, showing the greatest growth of all meat sectors as reflected in world consumption (FAO, 2010). The most common sources of poultry meat are popular domestic Galliformes, such as chickens and turkeys (87% and 6% of total poultry production, respectively). Other sources of commercial poultry meats come from other species, such as ducks, geese, pigeons, and quails.

Globally, the production of the most important poultry products had a remarkable increase. As an example, between 1995 and 2005, the world production of poultry meat increased from 55.2 to 82.8 million tons (Scanes, 2007). The leading countries in poultry meat production are United States, China, Brazil and Russia (Table 1.1).

Table 1.1 Main poultry producing countries worldwide (‘000 tons carcass weight).

	2010	**2011**	**2012**	**2013**	**2014**	**2015**
EU						
France	1.749	1.864	1.849	1.872	1.826	1.867
Germany	1.623	1.681	1.695	1.714	1.775	1.796
Italy	1.221	1.232	1.261	1.259	1.261	1.307
Netherlands	739	806	838	848	941	1.057
Poland	1.586	1.706	1.920	1.970	2.210	2.430
Spain	1.281	1.278	1.251	1.299	1.390	1.435
United Kingdom	1.568	1.558	1.607	1.606	1.587	1.625
Third countries						
Argentina	1.643	1.694	1.710	1.825	1.979	2.048
Brazil*	12.797	13.352	13.155	12.828	13.227	13.600
China	17.215	17.776	18.672	18.913	19.150	19.300
India	2.226	2.268	2.309	2.358	2.651	2.786
Japan	1.416	1.377	1.443	1.449	-	-
Mexico	2.715	2.798	2.826	2.838	2.880	2.988
Russia	2.549	2.875	3.285	3.448	3.973	4.180
USA	19.608	19.796	19.944	20.235	20.450	21.064

Source: AVEC Annual report, 2016.

*: only chicken and turkey meat.

Note: Partial provisional or estimated. Mostly gross domestic production.

Chicken meat remains the world’s most popular meat. The global production (Table1.2) of broiler meat is forecast to increase 1% to a record of 90.4 million tons as expansion by the United States, Brazil, India, and the EU more than offset a significant decline by China. US production is forecast to increase 2% to a record of 18.7 million tons as

lower feed prices spur expansion. Exports are expected to rise 5% to 3.1 million tons (USDA, 2016a).

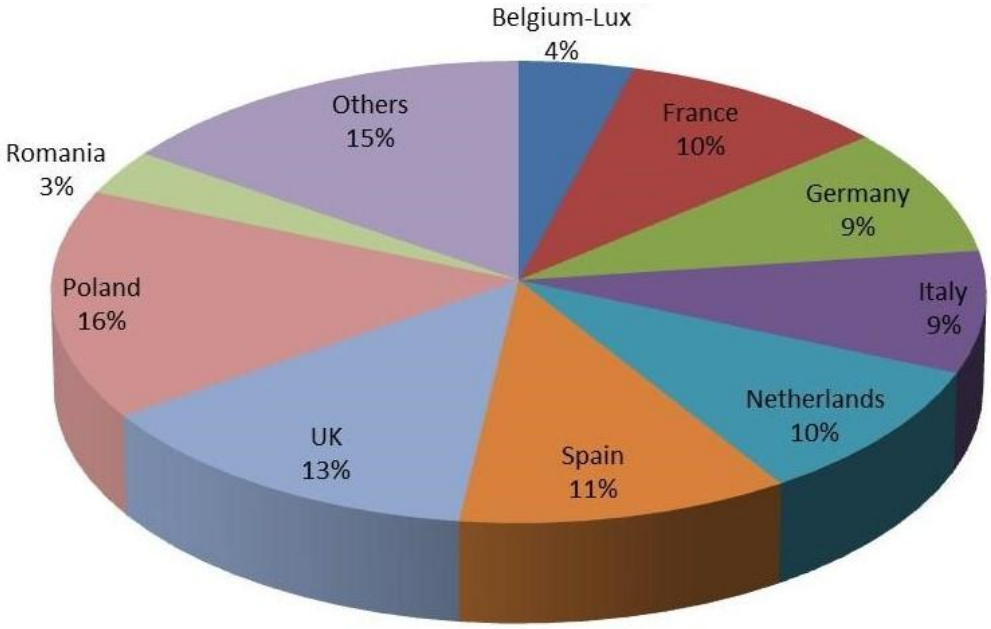
Table 1.2 Broiler meat production in selected countries (1.000 metric tons, ready to cook equivalent).

	2012	**2013**	**2014**	**2015**	**2016**
Argentina	2.014	2.060	2.050	2.080	2.100
Brazil	12.645	12.308	12.692	13.146	13.605
China	13.700	13.350	13.000	13.400	12.700
European Union	9.660	10.050	10.450	10.810	11.070
India	3.160	3.450	3.725	3.900	4.200
Indonesia	1.540	1.550	1.565	1.625	1.640
Mexico	2.958	2.907	3.025	3.175	3.270
Russia	2.830	3.010	3.260	3.600	3.750
Thailand	1.550	1.500	1.570	1.700	1.780
Turkey	1.723	1.758	1.894	1.909	1.900
USA	16.621	16.976	17.306	17.971	18.283
Others	14.866	15.480	16.018	15.378	15.250
Total	**83.267**	**84.399**	**86.555**	**88.694**	**89.548**

Source : USDA, 2016a.

The European Union is one of the world's top producers in poultry meat and a net exporter of poultry products. Over the years, the market organization for poultry sector was improved to ensure the development of the sector, the quality of the products and consumers protection while harmonizing the entire market. The leading countries in broiler meat production are Poland (16%), UK (13%), closely followed by Spain (11%), Netherlands (10%) and France (10%). These five countries ensure 60% of the EU production of poultry meat (Figure 1.1).

Figure 1.1 Main broiler producing countries in EU (*Source*: USDA, 2016b).



The strongest increase in production (1.1 % a year) is expected in the EU-N13, due largely to sustained productivity gains in Hungary, Poland and Romania. In a context of relatively low feed prices throughout the outlook period, strong domestic and world demand will together contribute to an expected growth of total EU production to 14.1 million tons by 2025.

Global annual meat consumption per capita is expected to reach 35.3 kg retail weight equivalent (r.w.e.) by 2025. Over the next decade global meat consumption will raise by 1.3 kg r.w.e. mainly in poultry. This increase will largely be located in developing countries, despite consumption will continue to increase slightly in developed countries. Poultry will surpass pig meat as the favoured animal protein during the outlook period. Contrary to red meat, and thanks to its healthy image and affordability, poultry meat is the only meat of which EU consumption is expected to increase, to reach 22.8 kg per capita by 2025 and growing at an annual rate of 0.3 % (FAO, 2016). In the US, which is a mature market for meat products, chicken has become the most popular protein meat surpassing beef for the first time in 100 years in 2014. Brazil shows also a huge growth in poultry meat consumption per capita from 29.9 kg in 2000 to 45 kg in 2014 (AVEC, 2016).

While all sources show that total meat consumption in the EU-28 has been negatively impacted by the economic downturn, broiler meat, which is the cheapest source of protein, was less affected. In the EU-28, sales of cheaper cuts (legs and wings) also increased faster than sales of more expensive parts, such as breasts or whole birds. This trend is expected to

extend into 2017 in the absence of any economic recovery. In several EU countries, such as Germany, France and Poland, the switch to broiler meat is enhanced by the belief that it is a healthier and leaner meat and more convenient to cook and prepare. It is also considered easier to prepare for catering and restaurant use than other meats. The consumption of organic, GMO-free, and free-range broilers is also on the increase in most EU-28 countries and especially in Austria, Germany and the Netherlands. (USDA, 2016b). In Table 1.3 is presented the broiler meat consumption in European Union and third countries in the period 2010-2015.

Table 1.3 Broiler meat consumption in selected EU and third countries (kg/head).

	2010	**2011**	**2012**	**2013**	**2014**	**2015**
EU						
Austria	12.6	13.1	13.3	13.3	13.9	14.0
France	14.8	15.2	15.8	16.2	16.9	17.5
Germany	10.9	11.4	11.1	11.7	11.8	12.1
Italy	11.5	11.6	11.7	11.7	11.9	12.5
Netherlands	18.8	18.4	18.4	18.5	.	.
Portugal	.	.	.	.	26.0	.
United Kingdom	22.2	21.7	22.0	22.5	22.5	22.9
EU-15	**16.7**	**16.8**	**17.1**	**17.2**	**18.0**	**18.4**
Third countries						
Argentina	36.5	38.2	42.0	41.9	41.3	42.9
Brazil	46.3	47.8	46.0	44.1	44.3	45.0
China	9.1	9.4	9.8	44.1	9.2	9.2
India	2.2	2.3	2.5	2.7	2.9	3.0
Iran	22.3	23.3	.	.	.	.
Japan	16.3	16.5	17.4	17.4	17.6	17.7
Mexico	28.5	29.1	29.5	30.01	29.8	30.3
South Africa	29.0	28.9	30.2	29.5	29.1	30.0
United Arab Emirates	59.1	.	.	.	.	.
USA	43.5	43.7	42.4	43.2	43.9	46.6

Source: AVEC, Annual report 2016.

Note: Mainly estimated official data on chicken consumption of only a few countries available. Because of shrinking database continuation of earlier time series is not always possible.

Concerning global trade, exports by major traders are forecast to climb 5% to a record 11.4 million tons. While shipments by both top two suppliers (Brazil and the United States) are forecast to grow, Brazil's increase will be higher because of its access to the Chinese market and its relatively weak Brazilian real. Although the Middle East remains a key destination for Brazilian shipments with Saudi Arabia ranked first, China is expected to be Brazil's leading growth market in 2017. Exports account for over 30% of Brazilian production, compared to only 16% for the United States, and thus exports are critical to the vitality and growth of Brazil's poultry industry (USDA, 2016a). According with the EU

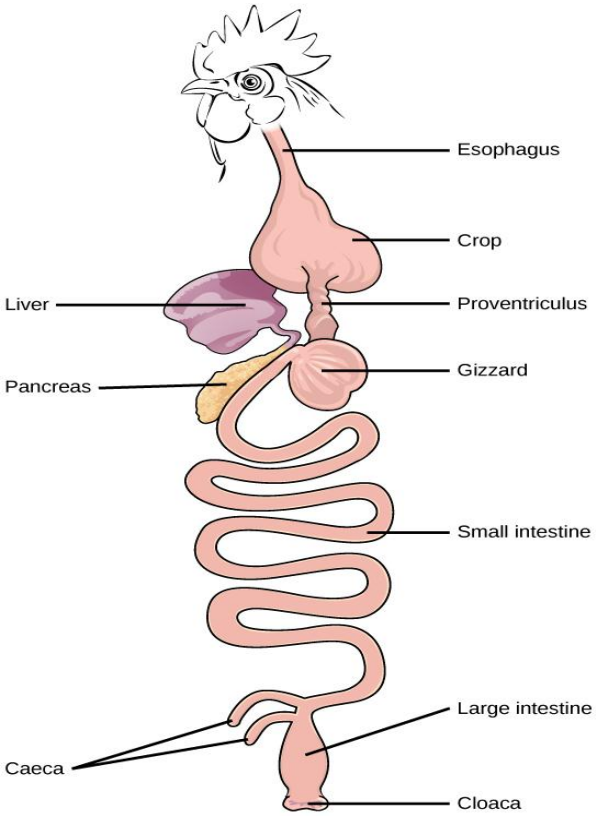
Agricultural Outlook (2015-2025), world import demand for poultry meat is expected to remain very strong, but to increase more slowly (by 3.2% a year over the next decade, as compared with 5.0% over the previous 10 years), to reach 17 million tons in 2025. The additional demand is shared almost equally by the Middle East, Sub-Saharan Africa and Asia. EU exports will continue to rise moderately, by an average of 1.4% a year until 2025, reaching 1.6 million tons, despite the absence of export refunds. A specific feature of the trade in poultry meat is that the EU is exporting lower-quality and cheaper cuts (such as legs and wings) and importing cuts with higher added value (such as breasts and cooked preparations).

Chapter 2

The poultry gastrointestinal tract (GIT)

The digestive system of birds has been subjected to strong variations during evolution, from the point of view of morphology, digestive strategy and metabolic capability, to match the different nutrient content and physical attributes of foods available in natural habitat (Klasing, 1999). The organs of the gastrointestinal tract (GIT) of the bird include the beak, mouth, salivary glands, tongue (but not teeth), pharynx, esophagus, crop, proventriculus, gizzard, intestines, ceca, rectum and cloaca. The length of the different organs vary with size of the bird, type of nutrition and other factors (Sturkie, 1976). In general, the digestive system of poultry (Figure 2.1) is similar to other animal species. However, the GIT of the bird has some specific peculiarities, with specific features.

Figure 2.1 The general structure of the digestive system of the chicken (*Source*: www.boundless.com/biology/textbooks/boundless-biology-textbook/animal-nutrition-and-the-digestive-system-34/digestive-systems-195/vertebrate-digestive-systems-748-11981/).



2.1 General structure and function

2.1.1 Beak, tongue, oral cavity and pharynx

The beak, tongue and oral cavity function in grasping, testing, mechanical processing, and lubricating and propelling the food to the esophagus (Klasing, 1999). Chickens, as with most of birds, obtain feed with the use of their beak. The mouth contains glands which secrete saliva which wets the feed to make it easier to swallow; the saliva also contains some enzymes which start the digestion of the food (Jacob and Pescatore, 2011). There is no sharp line of demarcation between the mouth and pharynx and there is no soft palate in most of birds. The oral cavity consists of stratified squamous epithelium (Sturkie, 1976). Compare to humans, the rapid transit of food through the mouth, the absence of mastication, the relatively low production of saliva and low numbers of taste receptors, result in a poor taste acuity (Klasing, 1999).

2.1.2 Esophagus and crop

The esophagus is a flexible tube that connects the mouth with the rest of the GIT. Its length is approximately 15-20 cm in the adult chicken. The esophagus is characterized by an external longitudinal and internal circular muscles and by anabundant presence of mucous glands.

The crop is an outside pocket of the esophagus and is located outside the body cavity in the neck region. The size and shape vary according to the eating habits of the species and the structure is essentially the same as the esophagus (Sturkie, 1976). The crop has a very important function and can be considered a storage organ for ingested food. This is essential for birds, because the stomach region (proventriculus and gizzard) does not have a large storage capacity. The crop is not characterized by a strictly nutritional role and enzyme secretion, and considerable absorption is not reported. However, in this tract, a considerable hydration occurs, which may aid the grinding and enzymatic digestion in the subsequent parts of the GIT. Moreover, some kind of exogenous enzymes and other compounds activated by moisturization could be able to exert their effect in the crop (Svihus, 2014).

2.1.3 Proventriculus and gizzard

The proventriculus and gizzard are the true stomach compartments of birds. The proventriculus, or glandular stomach, is a fusiform organ where digestion begins, and varies in size with the species. This organ is lined with a mucous membrane, which contains the

gastric glands. As it occurs in the human stomach, hydrochloric acid and digestive enzymes (e.g., pepsin) are secreted and added to the feed (Sturkie, 1976; Svihus, 2014).

The gizzard, or muscular stomach, has an important additional function in grinding feed material. Especially in the granivorous species, is characterized by a massive muscular development and a thick keratinoid-like lining which aid in the grinding process; consumed feed and the digestive juices from salivary glands and the proventriculus pass into the gizzard for grinding, mixing and mashing (Sturkie, 1976; Jacob and Pescatore, 2011).

2.1.4 Small intestine

The small intestine is the site of most of the digestive processes where takes place all the absorption of nutrients (Svihus, 2014). The small intestine of poultry is relatively simple and short, but highly efficient (Dibner and Richards, 2004). It is divided into 3 parts: 1) *duodenum*, 2) proximal small intestine (*jejunum*), 3) and distal small intestine (*ileum*). In relation to body length, the intestines of birds are shorter than those of mammals; however, there is a strong variation in the length, in relation to food habits (Sturkie, 1976). The shape and function of this part of the GIT is less variable compare to the more anterior digestive organs, because the physical nature of the different foods is already reduced to a uniform and fluid chyme (Klasing, 1999). The epithelium of the intestine usually consists of a simple columnar cells with many goblet cells and contains villi and intestinal crypts (Sturkie, 1976; Klasing, 1999). The villi contain a rich capillary bed, which picks up the absorbed nutrients and transfers them to the portal blood vessels. Goblet cells secrete abundant mucous which protect the intestinal epithelium from digestive enzymes and abrasion by the digesta. The mucous is particularly thick in the proximity of the anterior *duodenum*, because of the protection of the villi from the strong acidity of the digesta leaving the gizzard. Two muscle layers, the inner circular and outer longitudinal, surround the intestine and are responsible for mixing the digesta and propelling it through the tract (Klasing, 1999).

The *duodenum* is the first tract of small intestine. It receives digestive enzymes and bicarbonate (to neutralize hydrochloric acid from proventriclous) from the pancreas, and bile from the liver via the gall bladder. The released nutrients are absorbed mainly in the *jejunum* and *ileum*; the Meckel's Diverticulum marks the end of the *jejunum* and the start of the *ileum* (Jacob and Pescatore, 2011).

The *jejunum* has a key role, because the most of nutrients are digested and absorbed in this tract (Svihus, 2014). This is highlighted by the fact that the empty weight of this tract is usually 20 to 50% higher than the *ileum* (Hetland and Svihus, 2001; Rodgers et al., 2012).

The *ileum* is the last part of the small intestine and ends at the ileo-ceco-colic junction. In this segment occurs some digestion and absorption of fat, protein and starch, but this site also plays a role for water and mineral absorption. However, it has been shown that the *ileum* plays an important function for digestion and absorption of starch in fast-growing broiler chickens (Svihus, 2014).

2.1.5 Ceca, large intestine and cloaca

The pair of ceca in domesticated poultry is another unique feature of the avian digestive system. The ceca are situated at juncture of the small and large intestines. In some species (granivorous) they are large, prominent and paired; in other species they can be single, rudimentary, or absent (Sturkie, 1976). In Galliformes, the ceca are usually long and well developed with a constricted proximal portion, measured by Clarke (1978) to be one to two millimeters wide in three weeks old chickens, and join the colon just distal to the muscular ring separating the ileum from the colon. From the histological point of view, intestinal ceca are similar to the small and large intestine and they can have different functions based on the many morphological and histological types found in birds and the wide range of cecal sizes that occur (Clench, 1999). The proximal region of the ceca has been found to contain numerous villi, lymphoid cells and many goblet cells (Svihus et al., 2013). One of the most important function of villi is to filter materials, allowing fluid and fine particles to enter the cecal lumen while excluding bigger and solid substances. In domestic fowl, these villi have also important absorptive functions. Because a cecum is blind-ended, its contents can be retained for longer periods than would be possible in the small or large intestine, where digesta move relatively rapidly (Clench, 1999); the retention time in the ceca is usually longer, as indicated by the fact that cecal content is not significantly reduced after 24 hours of food deprivation in broilers (reviewed in Svihus, 2014). At various time and under different conditions, ceca are the site for: (i) fermentation, further digestion and absorption of nutrients. During fermentation, are produced several fatty acids as well as B vitamins; (ii) production of immunoglobulins and antibodies; (iii) microbial action of both anaerobic and aerobic bacteria, fungi and other organisms; and (iv) utilization and absorption of water (Clench, 1999; Jacob and Pescatore, 2011). It was estimated that 36% of the water and 75% of the sodium of renal origin is absorbed by the lower digestive tract, where the ceca is the most important organ. Moreover, it is also possible that the ceca can play a role in recycling of renal nitrogen. The functionality of the ceca is strongly influenced by diet, and the ceca enlarge as a consequence of an increased amount of fermentable material in the diet (Svihus, 2014).

The large intestine extends from the ceca to the cloaca. Despite the name, it is shorter and small in diameter compared with the large intestine of mammals; there is no sharp line of demarcation between the rectum and colon as in mammals. Histologically, is similar to small intestine, except for differences in size of villi that are shorter and richer in lymphoid follicles. The large intestine is the site where the last water re-absorption occurs (Sturkie, 1976; Klasing, 1999; Jacob and Pescatore, 2011).

The cloaca is the common opening into which empties the large intestine (*coprodaeum*), and the urinary and reproductive tracts (*urodaeum*) and has a much larger diameter compare to the rectum. *Coprodaeum* and *urodaeum*, in turn, empty into the *proctodaeum*, which opens externally through the anus. At the junction of the large intestine and the cloaca is a dorsal projection known as the bursa of Fabricius, a prominent lymphoid organ. The rectum enters midventrally into the *coprodaeum* region of the cloaca, which serves as a storage for urine and feces. The *coprodaeum* is separated by the *urodaeum* by a mucosal fold that receives the ureters and the oviduct, or the deferent ducts in males. Moreover, this fold allows the passing and the expulsion of feces, avoiding the contamination of *ureodaeum* and *proctodaeum*; the fold closes during egg laying and ejaculation to prevent fecal contamination of the egg or semen, respectively (Sturkie, 1976; Klasing, 1999).

2.1.6 Accessory organs

The most important accessory organs of the avian digestive system are liver, gall bladder and pancreas. The liver, characterized by two lobes of nearly equal size, has a primary digestive role in the production of bile and bile salts. Bile is a detergent involved in the digestion of lipids and absorption of fat soluble vitamins. Bile acids and salts, phospholipids, and cholesterol are secreted into the bile canaliculi and collected by the bile ducts. The gall bladder, the storage organ of bile produced in the liver, is present in some species, but is absent in others (Klasing, 1999). The pancreas lies within a loop of the *duodenum*. The digestive enzymes produced in the tubulo-acinar glands of the pancreas, are collected into ducts and are primarily involved in protein digestion; avian pancreatic juice contains enzymes similar to those of mammals, including amylase, lipases, trypsin, chymotrypsin, carboxypeptidases A, B, and C, deoxyribonuclease, ribonucleases, and elastases. The pancreas also produces bicarbonate, which buffers the intestinal pH (Klasing, 1999; Jacob and Pescatore, 2011).

2.2 Physical and functional development of the GIT in neonatal poultry

In the last decades, drastic changes in the growth rate, development and meat yield of fast growing meat-type chickens have occurred. As the time it takes modern broilers to achieve market size decreases, the period of embryonic development becomes a larger part of the productive life of bird and thus, the incubation has played an important role in improving growth efficiency. Consequently, anything that supports or limits growth and development during the incubation period will have a marked effect on overall performance and health of modern hybrids for meat production (Hulet, 2007; De Oliveira et al., 2008).

Poult mortality, embryonic, and neonatal growth are known to be influenced by several factors include stressors, such as temperature and hatchery servicing procedures, incubation temperature, time of removal from the incubator, prolonged holding without feed or water after hatching, genetics and poult sex (Christensen et al., 2007).

According to Moran (2007), embryonic development can be divided in three main steps as follows:

1) *Establishment of germ*: this first phase includes the first week of incubation, which is characterized by the formation of the egg compartments (amnion, chorion, allantois, and yolk sac), that are necessary for the survival of the developing embryo. Considering that at this stage blood cells are immature and chorionic vascular system is poorly developed, oxygen supply is provided by anaerobic glycolysis of the limited amounts of accessible glucose present in the egg. Success of this transition is substantially dependent by albumen integrity. Long holding, poor holding, or both conditions before incubation, not only damage formation of a fully functional chorioallantoic, but adversely define the albumen sac that concurrently forms at the small end, and recovery of its nutrients;

2) *Embryo completion*: this stage of embryonic development is characterized by a fully developed chorioallantoic, which is able to provide adequate O_2-CO_2 exchange and support the rapid embryonic growth. In this phase, deficiencies of pantothenic acid and riboflavin may occur in practice, and accentuated deaths at 12 days of incubation are observed. While, the embryo is structurally complete by 14 days of incubation, providing the wherewithal to support the transition to an independent chick in the last 7 days, is very important as well;

3) *Preparation for emergence*: this last phase is characterized by oral consumption of the amnion by the embryo, accumulation of glycogen reserves in muscle and liver tissues and glycogenolysis, initiation of pulmonary respiration, abdominal internalization of remaining yolk, shell pipping, and emergence. During this period, strong physiological and metabolic

changes occur, and any disturbance may markedly affect embryonic survival and later performance (reviewed in De Oliveira et al., 2008).

Feeding the hen properly, with essential nutrients, takes up a fundamental importance. Subsequently, a synchronized progression of events ensues to ultimately realize a viable chick. Ensure an adequate access to O_2, diversify energy sourcing, and accommodating metabolic patterns, is particularly important and could dramatically alter with each event. The importance of these events is highlighted by an accentuation of deaths at each transition that emphasize the magnitude of changes (Moran, 2007).

The emergence phase of embryonic development, is the most important because of the physiological and metabolic aspects that take place during this stage. Current knowledge on poultry embryo development towards hatch, underlines many tissues that are most affected by changes during this period (De Oliveira et al., 2008). The development of the GIT is one of the most important metabolic pathways in poultry embryos before hatching.

The GIT acts as a selective barrier between the tissues of the bird and its luminal environment. This barrier is composed of a physical, chemical, immunological and microbiological components. Several factors associated with diet and pathogens can negatively affect the balance among the different components of the chicken gut and, consequently, affect health status and performance of birds (Yegani and Korver, 2008).

2.2.1 Morphological and functional changes of the GIT

The digestive system is the main nutrient supply organ. The early development of the digestive function will enable the hatched chick to better utilize nutrients, grow efficiently and achieve its genetic potential. The first week after hatch is the most critical moment in the life of animal. After the emergence from the egg, the digestive and immune systems are still immature and not prepared to cope with environmental challenges. First of all, is necessary to consider the transition from yolk to oral nutrition; the residual nutrient supply from yolk present in the peritoneal cavity, is depleted in broiler chicks and poults within 4 to 5 days (reviewed in Sell, 1996). Moreover, there are substantial changes of the digestive tract and organs and the development of the immune function. The capacity to digest food and absorb and transport nutrients, is limited during the early life of animals. To achieve the genetic potential, the newly hatched chick must quickly adapt to digest and utilize nutrient in efficient way, from complex exogenous dietary sources (Ravindran, 2003).

During incubation, the sterile yolk supply the needs of the bird and are delivered from the yolk sac via the bloodstream (Dibner et al., 2008). It consists mainly of lipids and proteins, while the external feed consists of carbohydrates, lipids and proteins. This change

demands appropriate redistribution of the cellular mechanisms that catalyse the digestion or hydrolysis and absorption of food components. As the chicks make the metabolic and physiological transition from egg nutrition to exogenous feed, the yolk lipids, such as oleic acid, are quickly absorbed (this occurs close to hatch), whereas dietary carbohydrates and amino acids are not well utilized because of limitations in digestive and absorptive capacity. Thus, the digestive system plays an essential role in determining the development potential of the hatched chick. Nutrient digestion, absorption, assimilation and incorporation into developing and growing tissues, including skeletal muscle, depend directly on the functional capabilities of the intestinal epithelial layer. To achieve optimal muscle growth rates, and ultimately muscle and meat yields in poultry, it is essential to maximize enterocyte development, function and performance. Early functions of the GIT are vital for the chicken's growth performance. Therefore, it is critical to achieve optimal intestinal development and functional capacity (Uni, 2006). As shown by several studies, avian species with high growth rates are characterized by a rapid early development of the GIT and digestive organs; the consequence of such data, could be that potential inefficiencies in early GIT growth could limit the phenotypic expression in birds with a high genetic potential (reviewed in Ravindran, 2003).

As shown from the results obtained by Uni et al. (2003), with the progression of incubation, the body weight (BW) of the embryo increases, as well as the weight of the small intestine. However, small intestinal weight increased at a much greater rate than BW, which shows a little increase close to hatching. The intensified growth rate of the small intestine is clearly shown by the weights of the intestine as a proportion of the embryo weight. In fact, during the last 3 days of incubation, this ratio increased from approximately 1% on day 17 to 3.5% at hatch. The morphology of the small intestine also changed rapidly. Histology indicated that the intestine including the external muscular layers and the villi were growing rapidly. At day 15, villi were rudimentary; however, on day 17, villi at different stages of development were observed.

An additional organ of the embryo which is critical from the metabolic point of view, is the liver. As in all animals, the liver is the most metabolically active tissue of the embryo. It is the only organ in which all metabolic pathways and metabolic enzymes are active, and some of them are present exclusively in the liver. The embryonic hepatic tissue is essential to produce glucose through gluconeogenesis during periods of limited oxygen supply just before hatch. The liver shows extensive growth during the last stage of incubation, growing proportionally faster than the rest of the embryo to accommodate its increased metabolic demand by the late term embryo. To keep energy homeostasis, hepatic cells are under fine

control of circulating hormones, including insulin, glucagon, corticosteroids and thyroid hormones (reviewed in De Oliveira et al., 2008).

Consequently to the intake of exogenous feed, the GIT and associated organs are characterized by a rapid development to assimilate the ingested nutrients (Uni et al., 1998). In the days following hatching, the weights of proventriculus, gizzard and small intestine increase more rapidly in relation to BW compare to other organs and tissues. This higher growth is maximal in chicks at 4 to 8 days of age, and afterwards there is a relative decline (Ravindran, 2003). A two to fourfold increase in intestinal length was observed until 12 days of age, while the weight of the three intestinal segments (*duodenum*, *jejunum* and *ileum*) increased seven to tenfold. Villi increase in size and number, giving them a greater absorptive surface per unit of intestine. This rapid morphological development immediately after hatch involves differing rates of increase in villus volume in the *duodenum*, *jejunum* and *ileum* (Uni, 2006). While duodenal villus growth is almost complete by day 7, *jejunum* and *ileum* development continues beyond 14 days of age. Uni et al. (1998) have shown that the volume of jejunal villus was initially lower than duodenal villus volume, but become greater after day 10, while ileal villus volume was lower throughout. Crypt depth increased two to threefold with age and was greatest in the *duodenum*. Increases with age were greatest in the *duodenum* and lowest in the *ileum*. Enterocyte density ranges from 200.000-280.000 cells/cm^2 in all three segments of the small intestine and changes little with age. However, an increase in the total number of enterocytes per villus is observed with age, resulting from the remarkable increase in villus length (Uni, 2006).

Concerning the development of associated digestive organs, the allometric growth of the pancreas reached a maximum of about fourfold compare to body growth at 8 days of age and after that time declined to approximately 2.5 times at day 23. For the first two weeks after hatch the relative liver weight increased faster compare to BW (reviewed in Ravindran, 2003). After hatch, enzymatic activity in the small intestine changes to adapt to the substrate presented. The secretion of digestive enzymes by pancreas and small intestine, increase after hatch although the rate of increase is different for different enzymes (Noy and Sklan, 1997). Activities of lipase, amylase and proteases all increase during the first week of life. Pancreatic amylase activity increase threefold between 1 and 10 days post hatch, while trypsin and lipase activities increase five to six fold (Nir et al., 1993). Similarly, the total activity per gram of intestine increase steadily for maltase and sucrase, which are important enzymes in carbohydrate digestion (Uni et al., 1998).

2.2.2 GIT associated immune system

In addition to functioning as the primary site of digestion and absorption, the GIT plays a key role as an important barrier between the external environment and the bird. The immune system of birds is a complex composed of several cells and soluble factors that cooperate to produce a protective immune response (Yegani and Korver, 2008). The correct fulfilment of the immunological function is of crucial importance for poultry, because the most of commercial flocks are raised under intensive rearing system, and in these conditions the flocks are exposed to a rapid spread of infectious agents and disease outbreaks (Sharma, 2008).

The immune system of the bird is partially developed at hatch. Lymphoid organs represent the main structural category of the immune system. The primary lymphoid organs, the thymus (site of T-lymphocyte production and differentiation) and the bursa of Fabricius (site of B-lymphocyte production and differentiation), are both present, and are populated by lymphoid tissue. The migration of lymphocytes to the thymus occurs in many steps, beginning at day 6 of embryogenesis (Dibner et al., 2008; Yegani and Korver, 2008). Functional immune cells leave the primary lymphoid organs and populate secondary lymphoid organs. Gut-associated lymphoid tissues (GALT) are an example of secondary lymphoid organs (Sharma, 2008). In chickens, these kind of tissues includes the bursa of Fabricius, cecal tonsils, Peyer's patches and lymphoid aggregates in the *urodeum* and *proctodeum* (Yegani and Korver, 2008). These structures undergo a rapid development concurrently with the development of the digestive structures and functions. The GALT are a component of the mucosa-associated lymphoid tissues (MALT), which also includes bronchial, salivary, nasopharyngeal, and genitourinary lymphoid tissues. MALT develop with specific features that reflect their role as the first line of defence on mucosal surfaces. The distribution of lymphoid structures is not uniform across different segments of the intestine. The chicken's foregut is relatively poor in lymphoid follicles, but numerous follicles are present in the hindgut, and these are abundant especially in the ceca (Friedman et al., 2003; Sklan, 2005).

In contrast with other immune systems associated with lumens, the GALT interacting with two types of antigenic molecules: 1) harmless antigens, which are basically nutrients that should not evoke immune responses; 2) antigens derived from intestinal or external pathogens that should evoke protective immune responses (Friedman et al., 2003).

Material ingested contains nutrients, non-nutrients, and beneficial or potentially harmful organisms and material; the GIT selectively allow the nutrients to cross the intestinal

wall, while preventing the deleterious components of the diet from crossing the intestinal barrier. Immune responses to gastrointestinal antigenic stimulation could have negative effects on feed efficiency, are energetically expensive, and deviate nutrients away from production (Korver, 2006).

The correct balance between immune activation and regulation is necessary to maintain intestinal homeostasis and avoid intestinal inflammation (Chambers and Gong, 2011).

Chapter 3
The poultry gastrointestinal microbiota

3.1 Composition and development of the intestinal microbiota

According with the definition of Freter (1992), the intestinal microbiota is "the usually complex mixture of bacterial populations that colonize a given area of the GIT in individual human or animal hosts that have not been affected by medical or experimental intervention or disease". In recent years, has become evident that the gut microbiota influences the health and physiology of the vertebrate hosts, with recognized roles in several aspects of animal physiology, such as nutrition, development of intestinal morphology and digestive function, as well as immunity (Kohl, 2012; Waite and Taylor, 2015). Birds represent an interesting study model in the context of the roles of intestinal microorganisms, because they are characterized by an extremely complex and unique diets, physiological traits and developmental strategies (Kohl, 2012).

In domestic fowl, the intensive selection over the last six or seven decades has resulted in chickens that convert food in body mass very efficiently, making them the most efficient farm animals in terms of the input required to produce high quality meat protein. Consequently, it is clear that nutrients utilization requires an interaction between the biochemical functions provided by the chicken and the microbiota present within the GIT. The intestinal microbiota includes several kinds of microorganisms housed in the digestive tract, such as bacteria, fungi and protozoans. Bacterial populations, which are the predominant microorganisms, represent a wide range of interacting metabolic and morphologic type; the chicken GIT comprised over 900 species of bacteria (Gabriel et al., 2006; Stanley et al., 2014). The most predominant genera found in both chicken and turkey are *Clostridium*, *Ruminococcus*, *Lactobacillus*, and *Bacteroides,* but with different distribution between the two bird species (Pan and Yu, 2014).

The GIT of the chick has been considered to be sterile at hatch. However, it was recently shown that the microbial colonization of the digestive system, may start during the last stage of embryonic development. Some authors (reviewed in Chambers and Gong, 2011) reported the presence of bacteria of different genera in the embryo cecum; Pedrosa (2009) showed a viable and morphologically diverse bacteria community within embryos intestines since day 16 of incubation. The newly hatched chicks are initially exposed to microorganisms

arising from the surface of the egg shell, which is populated by bacteria from the intestine of hen and the surrounding environment. Therefore, the microbial inoculum of the early stage of the post hatch period is of critical importance for the establishment of the gut microbial community (Rinttilä and Apajalahti, 2013).

Following hatch, microbial colonization of the digestive tract evolves very rapidly in a process called ecological succession (Chambers and Gong, 2011); the gut microflora vary along the length of the digestive tract. In chickens, the main sites of bacterial activity are the crop, the caeca, and to a lesser degree, the small intestine (Gabriel et al., 2006). The crop has a characteristic microflora that consist of lactobacilli and enterococci, with small numbers of other species, such as micrococci and yeast (Dibner and Richards, 2004). In the gizzard and proventriculus, the low pH is responsible for the reduction in the bacterial population (Gabriel et al., 2006). The microbial community is established in the small intestine within approximately two weeks (Lan et al., 2005). The *duodenum* is not a favourable environment for the development of microorganisms, because of the presence of many enzymes, high concentration of antimicrobial compounds such as bile salts, and reflux movements from the *jejunum* to the gizzard, which result in a rapid change in environmental conditions. In the *ileum*, the environment allows a better bacterial growth, because of the lower enzyme and bile salt concentration. Generally, the main genera of bacteria within the chicken small intestine are *Lactobacillus*, *Enterococcus* and *Clostridium*, with some bacteria from the family *Enterobacteriaceae* (Gabriel et al., 2006; Brisbin et al., 2008); *L. aviarius* and *L. salivarius* are the predominant species associated with the mucosa of the upper intestinal tract (Chambers and Gong, 2011).

Compare to the small intestine, the caecal microbial community, is established at a later age; microbial communities establishment in the caecum needs 6-7 weeks (Lan et al., 2005). In the ceca, the slow turnover of contents facilitates bacterial development and results in an increase in their number. The ceca contain a more diverse community of bacteria, both facultative and strictly anaerobic, including genera of *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Escherichia*, *Fusobacterium*, *Lactobacillus*, *Streptococcus* and *Campylobacter*. Occasionally, *Pseudomonas aeruginosa* has been isolated (Lan et al., 2005; Gabriel et al., 2006; Brisbin et al., 2008). After the chick has been fed for one day, the numbers of lactobacilli in the caeca are quite variable; by the third day, however, large numbers of bacteria are present throughout the GIT and some of these species are only transient (Lan et al., 2005). The density of bacteria in the *ileum* and *cecum* of broiler chicks one day post hatch can reach 10^8 and 10^{10}, respectively, increasing to 10^9 and 10^{11} per gram of digesta during the first three days post hatching and remain relatively stable for the following

30 days (Apajalahti et al., 2004). In the crops of broilers, counts of lactobacilli increased from about 10^6 to 10^9 CFU/g digesta between 1 and 7 days and ranged between 10^8 and $10^{8.5}$ thereafter to 42 days of age (reviewed in Chambers and Gong, 2011). The microbiota becomes fully developed when birds are close to 40 days (Lan et al., 2005). The proximal small intestine contains approximately 10^3-10^5 bacterial cells/g digesta, the distal small intestine harbors 10^8-10^9 bacterial cells/g digesta, while the density of bacterial cells in the ceca can reach 10^{12}/g digesta (Brisbin et al., 2008).

3.2 Functions of the intestinal microbiota

Extensive interactions occur between poultry host and its gut microbiome. These interactions are manifested particularly through modulation of host gut morphology and physiology, exchange of nutrients and immunity.

The presence of microbiota has many impacts on the digestive system of the host. It produces short chain fatty acids (SCFA) via fermentation and stimulates digestive system development by increasing size and tissue components of the guts (Chambers and Gong, 2011). The microbial population is distributed throughout the GIT, and the caecum is the major region for anaerobic bacteria (Lan et al., 2005). The diet derived complex carbohydrates not degraded in the small intestine, such as non-starch polysaccharides and resistant starch, are the main sources of carbon and energy for the commensal microbiota in the lower intestine. Therefore, the intestinal microbiota has an important catalytic potential, which leads to the formation of microbial metabolites with beneficial or adverse health effects (Rinttilä and Apajalahti, 2013). Many intestinal bacteria can hydrolyze indigestible dietary polysaccharides, oligosaccharides and disaccharides to their compositional sugars, which can then be fermented by intestinal bacteria, yielding mainly lactic acid and SCFA, primarily acetate, propionate and butyrate (Lan et al., 2005; Rinttilä and Apajalahti, 2013; Pan and Yu, 2014). The host's intestinal enzymes are not able to metabolize the non-digestible carbohydrates which commensal bacteria metabolize to produce SCFA; therefore, they provide the host with added energy supply (Chambers and Gong, 2011).

SCFA, especially butyrate, represent an important energy source for intestinal epithelial cells and are known to regulate cellular differentiation and proliferation, thereby increasing intestinal tissue weight. The epithelium provides a highly selective barrier that prevents the passage of toxic and proinflammatory molecules; therefore, the contribution of butyrate and other SCFA to epithelial development is essential to maintain the normal intestinal barrier functions. Moreover, SCFA can regulate intestinal blood flow, regulate mucin production and affect intestinal immune responses. In addition to energy-yielding

activity, the formation of SCFA reduces pH of the intestinal environment, that could inhibit acid-sensitive pathogenic bacteria such as members of the family *Enterobacteriaceae* (Lan et al., 2005; Rinttilä and Apajalahti, 2013; Pan and Yu, 2014). Some authors (reviewed in Rinttilä and Apajalahti, 2013) have shown that in humans up to 95% of the SCFA produced during carbohydrate fermentation are used by the host, providing 5 to 15% of the total energy requirements; pigs may obtain 30% of their energy from SCFA produced by the hindgut fermentation, while ruminants can obtain almost all their energy from SCFA produced in the rumen. Moreover, gut microbiota also contribute to host nitrogen metabolism through the synthesis of glucose or proteins and may also contributes to the absorption of minerals like sodium in the ceca and colon, and to synthetize some vitamins, such as B, K and E (Gabriel et al., 2006).

The GIT microbiota is involved in protection against adverse microorganisms. The most important mechanism through which commensal bacteria prevent or reduce colonization of pathogens is called "*competitive exclusion*" (CE). This concept describe the phenomenon associated with the mechanism used by bacteria already present in the gut to maintain their presence in this environment, avoiding the colonization of the same intestinal sites by other kinds of microorganisms. The CE can be expressed in different ways. The mechanisms used by commensal bacteria to prevent or reduce colonization of pathogens include the competition for nutrients or the occupation of attachment sites on the mucosal surface of the intestine (Chambers and Gong, 2011). Some types of beneficial bacteria create a microenvironment hostile to other bacterial species by the production of antimicrobial metabolites. Some authors (reviewed in Brown, 2011) reported that lactobacilli and *B. cereus* produce various metabolites which have inhibitory effects on pathogenic bacteria; *in vitro* experiments have been demonstrated that *B. cereus* produces some bacteriocins with inhibitory action against range of bacteria like *Bacillus*, *Klebecella*, *Pseudomonas*, *Proteus*, *Salmonella*, *Shigella*, *Staphylococcus*, *Vibrio* species and *E. coli*. Lactobacilli present in the crop, produce a large amount of lactic acid deleterious to coliforms and most other bacteria; SCFA produced by bacteria also have bacteriostatic and even bactericidal effects, variable according to the type of acids and bacteria (Gabriel et al., 2006).

Digestive tract is the most important source of microorganisms and important interactions between these non-self-cells and host immune system takes place in the GIT. The GALT is the first line of protection against pathogens and is distributed along all digestive tract of the chicken. It plays an essential role in making specific antibodies, and microbial communities that inhabit the GIT, can stimulate this immune response and thus, fortify the defensive mechanisms of the host (Lan et al., 2005). The role of GIT microbiota in carrying

out this function is multifactorial. The intestinal microflora is involved in the development and regulation of the immune response by influencing the number, distribution and degree of activation of cell populations of the intestinal immune system. Bacteria stimulate innate immunity by activating phagocytosis and cytokine synthesis by macrophages (Gabriel et al., 2006) and provide non-inflammatory protection of the mucosal membrane through immune modulation, increasing or decreasing the amounts of mediators secreted by immune system cells associated with the intestine and by stimulating T helper and regulatory cells (Chambers and Gong, 2011).

3.3 Factors affecting the gut microbiota

The gut microbiota can be affected by several factors. Among them, age is the most important factor during the microbiota development. In fact, in young birds, the number and the diversity of the microorganisms, and their specificity in the different parts of the digestive system, increase rapidly with age (Chambers and Gong, 2011). Other factors are the animal strain, sex and the rearing environment. The bedding material used in chicken houses is usually mixed with chicken excreta and thus harbors a complex microbial community, especially intestinal bacteria, that can have a potential impact on chicken intestinal microbiota. Moreover, increased breeding density and heat stress seem to increase pathogens bacteria (Gabriel et al., 2006; Pan and Yu, 2014).

Another important factor that is necessary to consider, is the role of the diet, that has a critical potential impact on the intestinal microbiome in poultry, because some dietary components that are not digested and absorbed, act as a substrates for the growth of intestinal bacteria (Pan and Yu, 2014). The interactions between the composition of the diet and microbiota can affect the mucosal architecture and the mucus composition of the GIT (Lan et al., 2005). Several feed additives used in poultry diet can affect the gut microbiome and some of them are able to modulate the intestinal microbiota aiming to reduce enteric pathogens. A class of feed additives that has a critical impact on the modulation of the intestinal microbiota is antibiotic growth promoters (AGPs). The use of these substances in broilers diet, has been a common practice in the past years for promoting growth or preventing diseases. However, it has beenr reported that the presence of antibiotics can alter the stability of the intestinal microbiota and can reduce the population of *Lactobacillus* in the intestines (Lan et al., 2005). Moreover, due to the growing concern about antibiotic resistance, which has become a worldwide health problem, there is a trend to the ban on the use of AGPs. Most AGPs are banned in the European Union, and the United States has started to reduce the use of these compounds, with a possible ban in the near future (Pan and Yu, 2014).

In poultry production, the ban on the use of AGPs is of increasing interest in realizing the role of commensal microbiota on the host health. Understanding this relationship is of essential importance for achieving future sustainability and for improving the efficiency and environmental acceptability of poultry production. Thus, the alternatives to the use of antibiotics which can control diseases and promote growth of chickens are of great interest (Lan et al., 2005; Pan and Yu, 2014).

Chapter 4
Antibiotics growth promoters (AGPs)

The most important aim of animal production is the development of safe foods for human consumption without overlooking animal welfare and environmental safety (Gaggìa et al., 2010). An increasingly significant concern is to obtain animal products with high qualitative standards. A way to get the improvement of these products is supported by the use of feed additives.

These compounds include a wide class of products. According with the current legislation (EC 1831/2003, Art. 2 (2a)), "feed additives" means substances, micro-organisms or preparations, other than feed material and premixtures, which are intentionally added to feed or water in order to perform, in particular, one or more of the functions mentioned in Article 5(3). According with Article 5(2), a feed additive shall not: a) have an adverse effect on animal health, human health or the environment; b) be presented in a manner which may mislead the user; c) harm the consumer by impairing the distinctive features of animal products or mislead the consumer with regard to the distinctive features of animal products. As mentioned by Article 5(3), the feed additive shall: a) favourably affect the characteristics of feed; b) favourably affect the characteristics of animal products; c) favourably affect the color of ornamental fish and birds; d) satisfy the nutritional needs of animals; e) favourably affect the environmental consequences of animal production; f) favourably affect animal production, performance or welfare, particularly by affecting the gastro-intestinal flora or digestibility of feedingstuffs, or g) have a coccidiostatic or histomonostatic effect. According with Article 4, antibiotics, other than coccidiostats or histomonostats, shall not be authorized as feed additives.

4.1 Definition and mode of action

Poultry is one of the fastest growing sources of meat worldwide, representing about one-fourth of all the meat produced in the year 2000 (Apata, 2009). In case of intensive rearing system, where animals are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses because of increased mortality in flocks, lost productivity and contamination of poultry meat and poultry products for human consumption (Patterson and Burkholder, 2003; Kabir, 2009). The improvement of feeding and health management techniques, has

involved the use of antibiotics as therapeutic agents to treat diseases, and antibiotics as growth promoters (AGPs) added to poultry feed at low doses, in order to improve growth and feed efficiency, without any therapeutic aims.

The growth promoter effect of antibiotics was discovered in the 1940s, when it was observed an improved growth in animals fed with dried mycelia of *Streptomyces aureofaciens* containing residues of chlortetracycline (reviewed in Castanon, 2007). Other preliminary indications of a positive effect on productive performance in swine and poultry were reported by Jukes et al. (1950) and Moore et al. (1946). The United States Food and Drug Administration approved the use of these substances as animal feed additives without veterinary prescription in 1951 (Jones and Ricke, 2003); in the 1950s and 1960s, each European country adopted its own regulations concerning the use of antibiotics in animal feeds (Castanon, 2007).

These substances improved feed efficiency and animal growth and reduced morbidity and mortality due to clinical and subclinical diseases. It is not totally clear how AGPs exert their beneficial effects. Experiments with germ-free chickens have seemed to indicate that the action of the growth promoters is mediated by their antibacterial effect (Butaye et al., 2003). It is widely accepted that the growth-promoting effect is connected to the interactions with the intestinal microbiota (Pan and Yu, 2014). The various groups of antibiotics act in different ways to reduce the numbers of specific bacteria in the gut, and thereby increase the efficiency of nutrient utilization. Several hypotheses have been proposed to describe the mode of action of AGPs: (i) reduction or elimination of the activity of pathogenic bacteria that may cause subclinical infections, thus allowing the host to achieve production levels closer to their potential; (ii) elimination of bacteria that produce toxins that reduce the growth of the host animal; (iii) stimulation of the growth of microorganisms that synthesize several kinds of nutrients; (iv) reduction of the growth of microorganisms that compete with the host animal; (v) increased absorptive capacity of the small intestine through a decrease in the thickness of the intestinal wall. This may be due to the loss of mucosa cell proliferation in the absence of luminal SCFA derived from microbial fermentation. The reduction in gut wall has been used to explain the improved nutrient digestibility observed with AGPs (Dibner and Richards, 2005; McDonald et al., 2011).

4.2 Public health concerns and ban of AGPs in European countries

Between 1960 and 2000 the world's poultry production has almost quadrupled (Millet and Maertens, 2011). The worldwide increase in the use of AGPs in poultry and livestock production has led to the problem of bacterial antibiotic resistance development during the past years. Latest scientific trials have shown that resistance to antibiotics is not only due to the ability of some kinds of bacteria to survive to antibiotics, but also to the capacity to transmit acquired resistance to their progeny through extrachromosomal DNA fragments called plasmids (Apata, 2009). The wider use of antibiotics as feed additives in the long run can contribute to the development of resistant bacteria to medicines used to treat infections. The resistant genes of these microorganisms represent a risk for humans if they are transferred to people. Because of this, the World Health Organization (1997) and the Economic and Social Committee of the European Union (1998) established that the use of antibiotics as additives in food animals is a public health issue (reviewed in Castanon, 2007). In the European Union, the first country that banned AGPs was Sweden, which prohibited the use in feedingstuffs of additives belonging to the groups of antibiotics in 1986. Following, other member states banned on their territories the use of some antibiotics; Denmark withdrew the glycopeptide avoparcin in 1995, and in 1996, Germany declared that this glycopeptide produce resistance to glycopeptides used in human medicine. In 1998 Finland banned spiramycin because of its use in human medicine, and virginiamycin was banned in Denmark in the same year because two streptogramins were important in human medicine from the clinically point of view (Castanon, 2007; Phillips, 2007).

Subsequently, as a result of the above steps, Directive 97/6 banned the use of Avoparcin from April 1, 1997 and spiramycin, virginiamycin and bacitracin zinc were withdrawn by Regulation 2821/1998 from June 30, 1999. On January 1, 1999, Sweden applied the safeguard clause for the antibiotics still authorized as feed additives, including also those still permitted in poultry production: flavophospholipol and avilamycin. This initiative, together with the conclusions of the World Health Organization (1997) and of the Economic and Social Committee of the European Union (1998), led to the total ban for antibiotics as growth promoters in the European Union with the Regulation 1831/2003, which declared that antibiotics, other than cocciodiostats and histomonostats, could be marketed and used as feed additives only until December 31, 2005; from January 1, 2006, those substances would be deleted from the Community Register of authorized feed additives (Castanon, 2007). This political decision was taken on the basis of the precautionary principle that enables rapid response in the face of a possible danger to human, animal or plant health, or to

protect the environment. In particular, where scientific data do not permit a complete evaluation of the risk, recourse to this principle may, for example, be used to stop distribution or order withdrawal from the market of products likely to be hazardous. The precautionary principle is detailed in Article 191 of the Treaty on the Functioning of the European Union. It aims at ensuring a higher level of environmental protection through preventative decision-taking in the case of risk (http://eur-lex.europa.eu).

4.3 Consequences of the ban of AGPs

The ban has certainly had a considerable effect on livestock production. The prohibition on marketing and use led to a drastic reduction in antibiotic consumption, and thus the decrease of the risk of transferring to humans microbes with resistant genes to antibiotics (Castanon, 2007; Millet and Maertens, 2011). A study regarding the situation of Denmark, Norway and Sweden revealed that the ban did not lead to increased use of therapeutic antimicrobials per animal, except for piglets in Denmark (Grave et al., 2006). In this country, the use of antibiotics in animal production, fell from 206.000 kg in 1994 to 94.000 kg in 2001. Moreover, data from Denmark, Germany and Holland, show that the ban has also had a strong effect on resistance rates in enterococci in the faecal flora of man and animals. Similarly, the virginiamycin resistance rate in *E. faecium* has dropped from about 60% to 30% in chickens and to 5% in chicken meat since the ban in 1997-1998 (Phillips et al., 2004). In Sweden, as a result of the ban and increased attention to disease prevention and correct use of antimicrobials, the total use of these compounds decreased by approximately 55% in the period 1986-1999, and a relatively low prevalence of antimicrobial resistance has been maintained (Wierup, 2001).

Another important consequence of the ban, is the request of the improvement of the hygienic conditions of farms. It was demonstrated that under good production conditions it's possible to obtain competitive production results for the rearing of poultry without the continuous use of antibiotics (Wierup, 2001; Engster et al., 2002). The ban of antibiotics in animal feedstuffs will have also important effects in the international market of poultry meat, because the European Union only imports foods obtained from animals that were not fed with antibiotics, in application of the precautionary principle allowed by the World Trade Organization (Castanon, 2007).

However it's clear that the use of AGPs was accompanied by health promotional or prophylactic effects (Casewell et al., 2003). The prohibition of the use of these compounds in poultry production, has contributed to increased incidence of enteric diseases such as salmonellosis and campylobacteriosis, resulting in higher mortality rates and lower

productivity, as well as increased health risks for the operators in the poultry sector and increased relevance of contamination of poultry meat and poultry products. Despite efforts to improve other aspects of husbandry, the use of veterinary antibiotics for therapeutic purposes, which are identical to those used in human medicine, has increased, and this could represent a risk to human health in relation to resistance in *Salmonellae*, campylobacters and zoonotic strains of *E. coli* (Casewell et al., 2003). This aspect has led animal scientists to invest in research for innovative strategies and alternative products trying to transform the difficulties arising from the ban into opportunities (Millet and Maertens, 2011). Several safer natural compounds have been studied to replace antibiotics, such as herbs and their extracts (Jayasena and Jo, 2013; Rossi et al., 2013; Falowo et al., 2014; Cardinali et al., 2015), biomolecules from microalgae (Yaakob et al., 2014; Michalak et al., 2015; Moroney et al., 2015), probiotics and prebiotics (Patterson and Burkholder, 2003; Gaggìa et al., 2010) and organic acids (Grashorn, 2010; Mani-López et al., 2011; Papatsiros et al., 2012).

In this way consumer perception of animal production may improve as a result of the ban. In the long term, countries that adopted the ban could see economic advantages. Moreover, perhaps producers from non-EU countries will be forced to give up AGPs in order to sell their products to European Union and other markets (Dibner and Richards, 2005).

Chapter 5
Novel alternatives to AGPs: probiotics, prebiotics and synbiotics

The microbiota within the GIT can be considered a metabolically active organ with its wide biodiversity in terms of species and the high number of cells that can reach 10^{14} (Gaggìa et al., 2010). Recently, the growing interest concerning the intestinal microbiota has highlighted its key role in health and disease. The metabolic activity of microorganisms allows the absorption of nutrients, promote gastric development, stimulate epithelial cells proliferation and differentiation, and has protective functions against pathogens (Guarner and Malagelada, 2003; Sekirov et al., 2010). Consequently the ban on the use of AGPs, it was necessary to find viable and safe solutions for the prevention of enteric diseases and the improvement of productive performance of animals. There are several strategies and methods to modulate the intestinal microbiota in order to improve its competitiveness. Among others, the administration of probiotics, prebiotics and synbiotics (combination of probiotics and prebiotics) offer a natural method to reduce pathogens bacteria or enhance the population of beneficial bacteria through the modulation of the activity of the gastrointestinal microbiota, and are, accordingly, considered beneficial to the host animal, improving its performance and well-being (Gaggìa et al., 2010; Chambers and Gong, 2011; Uyeno et al., 2015).

5.1 Probiotics

5.1.1 Definition and main types of probiotic microorganisms

Many definitions have been suggested to establish what is a probiotic. Fuller (1989) defined a probiotic as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance”. More recently, FAO/WHO (2002) have defined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. According to Fuller (1989) a good probiotic should be: (i) a strain which is capable of exerting a beneficial effect on the host animal, for example increasing growth or resistance to diseases; (ii) non-pathogenic and non-toxic; (iii) present as viable cells, preferably in large numbers; (iv) capable of surviving and metabolizing in the gut environment; resistant to low pH and organic acids; (v) stable and capable of remaining viable for periods under storage and field conditions.

Probiotic microorganisms exert their beneficial effects on the host through different ways. They compete with pathogenic bacteria for energy sources and nutrients, therefore, reducing their proliferation in the GIT. Moreover, bacterias in the gastrointestinal environment antagonize pathogens through the production of antibacterial compounds such as cytokines, bacteriocins and hydrogen peroxide which inhibit growth of pathogenic microorganisms. Lactic acid-producing probiotics (e.g., *Lactobacillus* spp. and *Bifidobacterium* spp.) may exert an antimicrobial effect on pathogens by reducing the local pH of the gut lumen (Azizpour et al., 2009; Brown, 2011; Mizock, 2015). Among *Lactobacillus* spp., *L. plantarum* is most often associated with bacteriocin production. Several types of bacteriocins have been isolated from various *L. plantarum* strains (Fooks and Gibson, 2002). Some probiotics produce nutrients and growth factors which are stimulatory to beneficial microorganisms of the gut microbiota (Chaucheyras-Durand and Durand, 2010). Probiotics are also able to improve the immunity of the host thanks to their capacity of adhesion to the intestinal mucosa that enables to create a natural barrier against pathogens microorganisms. Moreover, probiotics have anti-inflammatory properties (Brown, 2011) and exert antitoxin effects (Oelschlaeger, 2010).

Probiotics are identified by their genus, species, and strain level. The genera *Lactobacillus* and *Bifidobacterium* are the most widely used probiotics and include a large number of species and strains characterized by important properties in an applied context (Turpin et al., 2010; Felis and Dellaglio, 2015). They are both saccharolytic bacteria that are able to ferment carbohydrates to lactic acid that inhibits growth of pathogens. Moreover, metabolites produced from fermentation can be used by many kinds of anaerobic bacteria to produce beneficial SCFA (Mizock, 2015).

5.1.1.1 Genera Lactobacillus

Lactic acid bacteria (LAB) represent a group of Gram-positive bacteria united by certain morphological, metabolic, and physiological characteristics. They are non-sporulating, non-respiring but aerotolerant cocci or rods, which produce lactic acid as one of the main fermentation products of carbohydrates. According with the current taxonomic classification, LAB belong to the phylum *Firmicutes*, the class *Bacilli*, and the order *Lactobacillales*. They are chemoorganotrophic, requiring rich media to grow (Felis and Dellaglio, 2015). The multiplicity of the environmental niches of lactobacilli is reflected in the diversity and the heterogenic phylogeny of the genus that comprises over a hundred different species. Among these, there are several well-characterized and biologically, technologically, and commercially relevant species such as *L. acidophilus*, *L. casei*, *L. delbrueckii* subsp.

bulgaricus, *L. plantarum*, *L. rhamnosus*, and *L. salivarius* (Barrangou et al., 2012). Microorganisms of the genus *Lactobacillus* are found in numerous different ecological niches in nature. These bacteria are a part of the natural microbiota of humans and animals. They are found in various niches within the host such as the GIT, urogenital tract, oral cavity, and skin; lactobacilli are also naturally present in plants and soil. These bacteria have been used for the production of fermented foods for centuries. Actually, lactobacilli are present in different foods and have an excellent record for safety; they can be found in a wide variety of fermented foods, especially in dairy products, such as yogurt, cheese and fermented milks (Gomes and Malcata, 1999; Barrangou et al., 2012).

Several species of lactobacilli have been recognized as probiotics due to their wide range of health-promoting effects in humans (Turpin et al., 2010). Lactobacilli have also been investigated and used as probiotics for farm animals such as poultry, pigs and cattle (Gaggìa et al., 2010).

5.1.1.2 Genera Bifidobacterium

Bifidobacteria are non-motile, non-sporulating, anaerobic, or microaerophylic Gram-positive bacteria. The name of the genera *Bifidobacterium*, is derived from the fact that they typically appear as bifid, branched, or Y-shaped rods. According to the taxonomic classification, bifidobacteria belong to the phylum *Actinobacteria*, the class *Actinobacteria*, subclass *Actinobacteridae*, and the order *Bifidobacteriales*. Bifidobacteria are microorganisms of primary importance in the active and complex ecosystem of the intestinal tract of humans and other warm-blooded animals. They are distributed in various ecological niches in the human gastrointestinal and genitourinary tracts, and the exact ratio is determined mainly by age and diet (Gomes and Malcata, 1999). In the intestinal tracts of animals and humans, bifidobacteria are considered one of the most important genera. Their presence in high numbers is associated with good health status of the host. It is generally accepted that bifidobacteria are helpful in maintaining appropriate balance of the microbiota in the GIT, reducing the risk of pathogen infection. Bifidobacteria are very promising probiotics. However, it is accepted that probiotic properties are species and/or strain specific and that the probiotic effects of a specific strain must not be extrapolated to other strains (reviewed in Medina et al., 2007). They are frequently used in food and pharmaceutical preparations and their application in animal feeding is of increasing interest (Gaggìa et al., 2010).

5.1.2 Application of probiotic microorganisms

The health effects of probiotic microorganisms and their application in humans are well known. Probiotics can potentially provide an important means in reduction of disorders associated with the GIT, such as diarrhea and inflammatory diseases and bowel syndromes (Azizpour et al., 2009; Goldin, 2011). Probiotics have activity against *Helicobacter pylori*, a Gram-negative bacteria responsible for gastritis, peptic ulcers and gastric cancer (Azizpour et al., 2009). Moreover, there is some evidence that probiotics may lower total serum cholesterol and/or low-density lipoprotein (LDL) cholesterol, and can influence the etiology of colon cancer and possibly tumors at other sites. Probiotics are also involved in the treatment of urogenital infections and allergic reactions, and have some positive effects in oral health (Goldin, 2011; Meurman and Stamatova, 2012; Mizock, 2015).

In livestock, probiotics are important not only for the prevention of diseases, but also to improve the livestock production. Particularly, they can be used in modulation of the gastrointestinal ecosystem, digestive processes, growth stimulation, and in the prevention and therapy of digestive tract diseases in young farm animals. *L. acidophilus*, *L. casei*, *L. helveticus*, *L. lactis*, *L. salivarius*, *L. plantarum*, *Enterococcus faecium*, *E. faecalis*, and *Bifidobacterium* spp., are most frequently used as veterinary probiotics (Bomba et al., 2012). The most common probiotics for monogastric animals mainly aiming the hindgut (caecum, colon) which is characterized by an abundant and diverse microbial population (Chaucheyras-Durand and Durand, 2010).

The poultry industry has become one of the largest and most important sectors of livestock production worldwide. Nowadays, the most important target is the safety and the healthiness of poultry meat and poultry products, minimizing the impact of poultry industry on the environment. In order to reach these objectives, keeping at the same time high productivity levels of farms, probiotics can be used as a replacement of AGPs. In poultry production, probiotic microorganisms have important effects on modulation of gut microbiota and pathogen inhibition, on growth performance and also on meat quality and egg production. Various probiotic strains have been studied to evaluate the effects on the avian intestinal microbiota, concerning its modulation and the protection against different kinds of pathogens; particularly, the research on the effect of feeding *Lactobacillus* spp. to broilers has received an increasing interest. Studies have focused on strains previously selected *in vitro* because of their adhesion properties and antimicrobial activity (Patterson and Burkholder, 2003).

Lan et al. (2004) have shown that two strains of *Lactobacillus* (*L. agilis* and *L. salivarius*) enriched the *Lactobacillus* flora in broiler chicken *jejunum* and *cecum* increasing

the amount of *Lactobacillus* spp. inhabiting the intestine. Probiotic strains maintained the natural stability of bacterial microbiota. Mountzouris et al. (2007) studied the effect of many species of probiotic bacteria (*Lactobacillus reuteri, L. salivarius, Enterococcus faecium, Bifidobacterium animalis, Pediococcus acidilactici*) isolated from the intestinal tract of healthy chickens. Probiotics modulated the composition and the enzymatic activities of cecal microflora, resulting in a meaningful probiotic effect. The application of probiotics in poultry production is strongly associated with the mechanism of competitive exclusion. Many studies have shown the possibility to control and reduce *Salmonella* colonization with different probiotic cultures (reviewed in Gaggìa et al., 2010). Higgins et al. (2008) have shown that a *Lactobacillus*-based probiotic preparation strongly reduced *Salmonella enteritidis* in challenged neonatal broiler chicks. Moreover, several studies have established the protective effect of competitive exclusion on the host, against pathogens such as *Salmonella typhimurium, S. gallinarum, Campylobacter jejuni* and *Clostridium perfrigens* (reviewed in Bomba et al., 2012). Many authors reported important effects of probiotics also on immune response (Kabir et al., 2004; Koenen et al., 2004; Khaksefidi and Ghoorchi, 2006; Wang et al., 2015). It is important to consider the implications of the use of probiotics regarding productive performance. The results from Kabir et al. (2004) showed a higher weight gain and carcass yield in broiler chicks fed with probiotics. Peng et al. (2016) reported an improved average daily gain and feed conversion ratio in broilers supplemented with probiotics (*Lactobacillus plantarum* B1).

In layer hens, the supplementation of probiotics can increase egg production, egg weight and eggshell thickness and decrease egg yolk cholesterol, serum cholesterol and triglyceride levels (Kurtoglu et al., 2004; Mikulski et al., 2012).

In swine production, the weaning and post-weaning period (separation from the mother, drastic change of diet, transport to a production farm) is the most critical moment regarding the occurrence of microbial imbalances in the GIT and infections (Gaggìa et al., 2010). All these factors can have adverse effects on the balance of the intestinal microbiota and on immune response (Modesto et al., 2009). In neonatal piglets, probiotics are useful to sustain the development of the gut microbiota, to prevent diarrheal diseases and to stimulate the immunological functions. In the weaning and post-weaning period, probiotics can stimulate growth and prevent post-weaning diarrhea (Bomba et al., 2012). The administration of probiotics in gestating sows, has shown a positive impact on average live weight and feed intake, observing simultaneously a greater size and vitality of piglets (reviewed in Chaucheyras-Durand and Durand, 2010). Takashi et al. (2007) have shown that the supplementation of the diet of neonatal pigs with a strain of *Lactobacillus plantarum* result in

an increase of total gut population of lactobacilli in weaned pigs. The administration of lactobacilli and bifidobacteria immediately after birth, support the colonization of a beneficial commensal microbiota reducing the incidence and severity of necrotizing enterocolitis and lowering colonization density of the potential pathogen *Clostridum perfringens* (Siggers et al., 2008). Deng et al. (2013) have observed that the co-administration of two strains of *Bacillus subtilis* and *Lactobacillus salivarius* results in a stronger mucosal immunity. Many studies have shown the positive effects of probiotic administration (different species and strains of *Lactobacillus*) on growth performance and feed intake of pigs in the weaning period. The treatment with a strain of *L. plantarum* was associated with a better daily weight gain and feed conversion ratio, and improved pork quality (Suo et al., 2012). In grower and finisher pigs, the administration of a probiotic containing *Bacillus licheniformis* and *B. subtilis* significantly improved weight gain, feed conversion ratio and carcass quality (Alexopoulos et al., 2004). Moreover, Ross et al. (2012) observed an improved fatty acids profile of pig meat after probiotic administration (*Lactobacillus amylovorus* and *Enterococcus faecium* mixed colture).

In ruminants, the use of probiotics has been widely studied in the pre-ruminant's life and in adult animals, considering both the health status of the host. The most commonly used probiotics in ruminants are yeasts (*Saccharomyces cerevisiae*), that mainly affect the microbial population dynamics in the rumen and the utilization of nutrients. LAB are another important group of probiotics. Probiotics can improve the milk yield in dairy animals, increase the weight gain, and improve nutrient digestibility (FAO, 2016). In calves, probiotics such as LAB or *Bacillus* species generally target the lower intestine and represent an interesting means to stabilize the gut microbiota and decrease the risk of pathogen colonization. Neonatal-calf diarrhea, most often caused by enterotoxigenic *E. coli*, is an important cause of morbidity and mortality in young ruminants; the use of yeasts or LAB, can reduce the incidence of diarrhea in calves (Gaggìa et al., 2010; Uyeno et al., 2015). Moreover, probiotics can be involved in the reduction of methane production (reviewed in Bomba et al., 2012).

5.1.3 Safety of probiotics

The microbial genera and species used as probiotics in animal feed are generally considered safe. In Table 5.1 are presented the expected health-promoting characteristics and safety criteria of probiotics.

Probiotic use is not without risks. Probiotic microbes in feed can pose a serious risk related to the transfer of antibiotic resistance genes/determinants, and the production of enterotoxins, deleterious metabolic activities and excessive immune stimulation in susceptible

individuals (Musa et al., 2009; FAO, 2016). In Europe, the European Food Safety Authority (EFSA) has introduced the concept of Qualified Presumption of Safety (QPS). EFSA has been using this concept as a generic risk assessment tool to assess the safety of a microorganism intended to deliberately enter the food chain. According to this concept, if microorganisms of certain taxonomic groups either do not pose any safety risk or the risk can be clearly defined and eliminated, the group can be designated as a group with QPS status. Any particular microorganism intended to be introduced into the food chain, which can be unequivocally identified and have QPS status, may not be the subject of a detailed pre-market safety assessment other than satisfying predetermined specific qualifications (EFSA, 2007).

Lactobacillus and *Bifidobacterium* are probably the safest probiotic microbes, because they have been safely traditionally used in different fermented food, and are naturally present in the GIT and other sites in humans and animals. However, some very rare cases of infections (e.g., endocarditis, lactobacillaemia) have been reported in immunocompromised people (FAO, 2016). EFSA has published a list of microorganisms, which possess a known historical safety, proposed for QPS status (Table 5.2).

Table 5.1 Expected characteristics and safety criteria of probiotics.

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

Source: Gaggìa et al., 2010.

Table 5.2 List of taxonomic units proposed for QPS status.

Gram-Positive Non-Sporulating Bacteria[a]			
Species			**Qualifications**
Bifidobacterium adolescentis	*Bifidobacterium bifidum*	*Bifidobacterium longum*	
Bifidobacterium animalis	*Bifidobacterium breve*		
Corynebacterium glutamicum			QPS status applies only when the species is used for production purposes.
Lactobacillus acidophilus	*Lactobacillus farciminis*	*Lactobacillus paracasei*	
Lactobacillus amylolyticus	*Lactobacillus fermentum*	*Lactobacillus paraplantarum*	
Lactobacillus amylovorus	*Lactobacillus gallinarum*	*Lactobacillus pentosus*	
Lactobacillus alimentarius	*Lactobacillus gasseri*	*Lactobacillus plantarum*	
Lactobacillus aviaries	*Lactobacillus helveticus*	*Lactobacillus pontis*	
Lactobacillus brevis	*Lactobacillus hilgardii*	*Lactobacillus reuteri*	
Lactobacillus buchneri	*Lactobacillus johnsonii*	*Lactobacillus rhamnosus*	
Lactobacillus casei	*Lactobacillus kefiranofaciens*	*Lactobacillus sakei*	
Lactobacillus crispatus	*Lactobacillus kefiri*	*Lactobacillus salivarius*	
Lactobacillus curvatus	*Lactobacillus mucosae*	*Lactobacillus sanfranciscensis*	
Lactobacillus delbrueckii	*Lactobacillus panis*	*Lactobacillus zeae*	
Lactococcus lactis			
Leuconostoc citreum	*Leuconostoc lactis*	*Leuconostoc mesenteroides*	
Pediococcus acidilactici	*Pediococcus dextrinicus*	*Pediococcus pentosaceus*	
Propionibacterium freudenreichii			
Streptococcus termophilus			

[a]Absence of acquired antibiotic resistance should be systematically demonstrated unless cells are not present in the final product.

Bacillus			**Qualifications**
Species			
Bacillus amyloliquefaciens	*Bacillus lentus*	*Bacillus pumilus*	Absence of emetic food poisoning toxins with surfactant activity. Absence of enterotoxic activity[b].
Bacillus atrophaeus	*Bacillus licheniformis*	*Bacillus subtilis*	
Bacillus clausii	*Bacillus megaterium*	*Bacillus vallismortis*	
Bacillus coagulans	*Bacillus mojavensis*	*Geobacillus stearothermophillus*	
Bacillus fusiformis			

[b]When strains of these QPS units are to be used as seed coating agents, testing for toxic attività is not necessary, provided that the risk of transfer to the edible part of the crop at harvest is very low.

Yeasts			**Qualifications**
Species			
Debaryomyces hansenii			
Hanseniaspora uvarum			
Kluyveromyces lactis	*Kluyveromyces marxianus*		
Pichia angusta	*Pichia anomala*		
Saccharomyces bayanus	*Saccharomyces cerevisiae*	*Saccharomyces pastorianus (synonym of Saccharomyces carlsbergensis)*	S. cerevisiae, subtype S. boulardii is contraindicated for patients of fragile health, as well as for patients with a central venous catheter in place. A specific protocol concerning the use of probiotics should be formulated.
Schizosaccharomyces pombe			
Xanthophyllomyces dendrorhous			

Source: EFSA, 2007.

5.2 Prebiotics

5.2.1 Definition and main types of prebiotic compounds

Among the alternatives proposed to replace AGPs, prebiotic compounds represent another interesting way to manipulate the gut ecosystem. Gibson and Roberfroid (1995) defined a prebiotic as a "non-digestible food ingredient which beneficially affects the host by selectively stimulating the growth of and/or activating the metabolism of one or a limited number of health-promoting bacteria in the intestinal tract, thus improving the host's microbial balance". Latest definitions, describe a prebiotic as a "selectively fermented ingredient that allows specific changes, in both the composition and activity in the gastrointestinal microbiota which confers benefits to the host" (FAO/WHO, 2002).

Classification of a food ingredient as a prebiotic requires scientific demonstration that the ingredient (Gibson and Roberfroid, 1995): (i) resists gastric acidity, hydrolysis by mammalian enzymes, and absorption in the upper gastrointestinal tract; (ii) is fermented by the intestinal microflora; (iii) selectively stimulates the growth and/or activity of intestinal bacteria potentially associated with health and well-being.

Although several types of prebiotics such as peptides, proteins and lipids can be used, oligosaccharides are the most important because they can be hydrolyzed and fermented by gut bacteria. They occur naturally in foods such as leeks, asparagus, chicory, Jerusalem artichokes, garlic, onions, wheat, oats, and soybeans. Currently, candidate prebiotic compounds encompass several non-digestible oligosaccharides (NDO) including, among others, fructo-oligosaccharides (FOS, oligofructose, inulin), lactulose, galacto-oligosaccharides (GOS) and *trans*-galacto-oligosaccharides (TOS). Legumes are also a good source of oligosaccharides known as α-galactosides or the raffinose family of oligosaccharides (RFO), which are utilized by bifidobacteria (Patterson and Burkholder, 2003; Martìnez-Villaluenga et al., 2005; Gaggìa et al., 2010). Another important group of prebiotics are β-glucans, which are polysaccharides mainly found in oat and barley bran (El Khoury et al., 2012).

5.2.1.1 Fructo-oligosaccharides (FOS)

Fructans are one of the most popular prebiotics supplements available, comprising 61% of the publications on the topic of prebiotic supplementation (Barry et al., 2009). Inulin, its respective hydrolysates, and oligofructose that are commonly found in several kinds of plants, are a classic example of prebiotics and have been tested in many experimental studies

(Patterson and Burkholder, 2003). These compounds can be extracted from plant sources, microbial synthesis, and enzymatic hydrolysis of polysaccharides. FOS are comprised of short chain 1 to 2 linked fructose polymers that can be generated commercially either by inulin hydrolysis or enzymatic conversion of sucrose or lactose (Ricke, 2015). Because FOS include β-linkages as part of their chemical structures, they can resist adsorption and enzymatic degradation in the upper part of the GIT and reach the ceca, where the most of fermentation occurs in chickens (Park et al., 2013). Inulin and FOS are considered selective because they are fermented by LAB and bifidobacteria, which beneficially affect the host as probiotic microorganisms (Ricke, 2015).

5.2.1.2 Galacto-oligosaccharides (GOS) and trans-galacto-oligosaccharides (TOS)

GOS are one of the most commonly produced prebiotic oligosaccharides worldwide. They can be obtained through the enzymatic conversion of lactose (milk sugar) by the enzyme β-galactosidase. Lactose is a disaccharide that consists of β-D-galactose and β-D-glucose bonded through a β1-4 glycosidic linkage and is usually purified from cow's milk whey (Nauta et al., 2010). *Trans*-galacto-oligosaccharides (TOS) are produced by β-galactosidases having transgalactosylation activity. TOS are water-soluble, and contain galactose units with β-linkages that prevent hydrolytic digestion (Van Loo and Vancraeynest, 2008; Barry et al., 2009). The application of GOS have an important role in the composition of infant milk formula and infant foods, and proved to be promising concerning the formulation of specialized foods for the elderly and hospitalized people. The use of these compounds is taking growing interest also as regards the livestock feed and pet food industries (reviewed in Torres et al., 2010).

5.2.1.3 Raffinose family oligosaccharides (RFO)

Raffinose (α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) is found in several vegetables and whole grains and can be easily extracted from cottonseed (a coproduct of cottonseed oil production) and soy whey, a coproduct of soybean oil and soy milk production (Hernandez-Hernandez et al., 2011). RFO (α-galactosides) are another important class of water-soluble carbohydrates in plants. These compounds are soluble low-molecular-weight oligosaccharides represented by raffinose, stachyose, verbascose, and other oligosaccharides formed by α-(1→6)-galactosides linked to C-6 of the glucose moiety of sucrose. RFO can be simply and rapidly isolated and purified from legume extracts, including lupins, to be used as ingredients during the production of several functional foods such as

milk, to enhance the numbers and acidification activity of bifidobacteria during manufacture of probiotic-fermented milk products (Gulewicz et al., 2000; Martìnez-Villaluenga et al., 2005). Different studies have demonstrated the low digestibility of these oligosaccharides and their effect on gut microbiota. However, they can give rise to the production of undesirable levels of flatulence (Hernandez-Hernandez et al., 2011). The mechanism of action of these compounds can contribute to disease prevention in plants as well as in animals and humans (Van den Ende, 2013). *In vivo* study has shown that α-galactosides from lupins stimulate bifidobacterial growth and indirectly immune response in rats (Martìnez-Villaluenga et al., 2008). *In ovo* studies have demonstrated the potential prebiotic effect of α-galactosidase derived from lupin seeds (Martìnez-Villaluenga et al., 2004).

5.2.1.4 β-glucans

Glucans are glucose polymers, classified according to their interchain linkage as being either α- or β-linked. β-glucans are a heterogeneous group of non-starch polysaccharides, consisting of D-glucose monomers linked by β-glycosidic bonds. The macromolecular structure of β-glucans depends on both the source and method of isolation. The main sources are cereals, yeast, mushrooms, seaweeds and some bacteria (El Khoury et al., 2012; Lam and Cheung, 2013). Increased interest in β-glucans arises from their functional and bioactive properties. Cereal β-glucans have been demonstrated to have prebiotic properties owing to their ability to pass undigested through the GIT, where they act as a substrate for microbial fermentation and selectively stimulate the growth and activity of a small number of beneficial bacteria (Gibson, 2004). In humans their beneficial role in insulin resistance, dyslipidemia, hypertension, and obesity is being continuously documented. The fermentability of β-glucans and their ability to form highly viscous solutions may constitute the basis of their health benefits (El Khoury et al., 2012). It was shown that supplementation of the diet with high-viscosity βeta-glucans, can produce a prebiotic effect in the ceca of rats increasing the number of lactobacilli (Snart et al., 2006).

5.2.2 Application of prebiotic compounds

Dietary modulation of the human gut flora has been carried out for many years. In humans, prebiotic addition to the diet has brought positive aspects to the gut microbial balance, increase the mineral absorption, and are involved in lowering of triacylglycerol and total cholesterol concentration and/or the total and LDL cholesterol concentration. Moreover, these compounds are involved in the reduction of the risk of cancer (de Vrese and

Schrezenmeir, 2008; Al Sheraji et al., 2013). In human studies, FOS and TOS have clearly demonstrated to enhance the growth of beneficial bacteria species belonging to the genera *Lactobacillus* and *Bifidobacterium*, when the amount in the diet is around 5-20 g/day (reviewed in Macfarlane et al., 2006).

In recent years, prebiotics have been commonly used in *in vivo* feeding experiments with a wide range of companion and livestock species, such as poultry, pigs, cattle and horses, to study the effects on gut microflora, immunomodulation of the host, suppressive effects on the enteric and systemic infections by pathogens, performance indices, quality of animal products and on animal welfare (Alloui et al., 2013). The main effect of prebiotics in the animal organism is to stimulate the resident microbiota of the host to proliferate, to stop pathogens bacteria, and to share health benefits to the host. Prebiotics are selectively fermented by beneficial microflora into SCFA. High fermentation activity and high concentration of SCFA is correlated with a lower pH, which is associated with a suppression of pathogens and increased solubility of some kinds of nutrients. This mechanism may inhibit some pathogens and reduce colonization of some species like *Salmonella* and *Campylobacter*, changing environment favorable for beneficial GIT bacteria such as *Bifidobacteria* and *Lactobacillus* (Alloui et al., 2013; Park et al., 2013; Dhama et al., 2014). In addition, prebiotics increase intestinal enzyme secretion, are involved in diminution of ammonia and phenol products, and promotion of resistance to pathogenic bacteria proliferation in the gut (Yusrizal and Chen, 2003b).

Other important prebiotic effects have been investigated concerning the effect on lipid metabolism. There have been several animal experiments that have indicated the potential of prebiotics to positively influence serum lipid levels (reviewed in Macfarlane et al., 2008). Depending on the type of diet and the genetic background of the animals, the effect of prebiotics on lipid metabolism may be present either in the liver (improvement of steatosis) or in the serum (decrease in triglyceridemia), or both (Delzenne and Neyrinck, 2008). One of the most important link between the effect of prebiotics inside the gut and their effect on lipid homeostasis, is the production of SCFA. Intestinal breakdown of prebiotics leads to the production of acetate, propionate, and butyrate, which are almost completely absorbed along the digestive tract. Whereas butyrate is widely metabolized by enterocytes, propionate and acetate can reach the liver through the portal vein. When acetate enters the hepatocyte, it is mainly activated by the cytosolic acetyl-CoA synthetase2, and then enters the cholesterolgenesis and lipogenesis pathways. Conversely, propionate is a competitive inhibitor of the protein devoted to the entrance of acetate in liver cells, a phenomenon which contributes to a decrease in lipogenesis and cholesterolgenesis. This suggest that one role of

prebiotics is to alter these breakdown products produced during fermentation (Teitelbaum, 2010). In Table 5.3 are described the most important characteristics of prebiotics and their beneficial effects on animal organism.

Table 5.3 Characteristics of ideal prebiotics and their desiderable properties.

Prebiotics	
Properties	**Positive effects**
Not hydrolyzed or absorbed	Higher SCFA production
Selectively stimulate growth of one or a limited number of beneficial bacteria	Better biomass and stool bulking
Beneficially modify the intestinal microbiota activities	Enhanced vitamin B synthesis
Positively modulate host defence system	Improved mineral absorption
	Cancer prevention
	Decrease in blood cholesterol level
	Decreased ammonia and urea excretion
	Lower excreta content of skatole, indole, phenol, etc.

Source: Alloui et al., 2013.

In poultry, oligosaccharides reach the hindgut and alter lower intestinal tract physiology and function, which could be beneficial in preventing bacterial contamination of broiler carcasses (Orban et al., 1997). The diet supplementation with prebiotic oligosaccharides such as FOS, could improve the gut microbial population, including a reduction in *Salmonella* colonization (Alloui et al., 2013). Several studies have shown that beneficial bacteria such as *Bifidobacteria* and *Lactobacillus* were increased in the large intestine of broilers when supplemented with FOS (reviewed in Park et al., 2013). The supplementation with FOS is also involved in decreasing cecal indole and phenol concentrations (Cao et al., 2005). In broiler chickens, performance parameters have been evaluated with prebiotic supplementation. Body weight gain, feed conversion ratio and carcass weight were improved in the most of studies (Xu et al., 2003;Yusrizal and Chen, 2003a; Józefiak et al., 2008; Yang et al., 2008). The addition of fructans to broiler diets may also reduce the volatile ammonia contents of feces (Yusrizal and Chen, 2003b). This has interesting environmental implications. A lower ammonia level in broiler stables also has health benefits, as ammonia can irritate the upper respiratory tract and subsequently result in secondary bacterial infections (Van Loo and Vancraeynest, 2008). Although GOS have been less investigated in the poultry industry compare to FOS, it was shown that GOS

preferentially stimulated bifidobacteria and significantly modified intestinal microflora in broiler chickens (Jung et al., 2008). Glucans have a strong immunomodulating activity that was well studied in livestock (reviewed in Ganguly, 2013). It was shown that health, growth and general performance of broiler chickens may be improved by the use of β-glucans (Paul et al., 2012). Cox et al. (2010) have shown that a yeast-derived β-glucan significantly reduced lesion severity and is capable of altering immune-related gene expression profiles, favoring an enhanced T helper type-1 cell response during coccidiosis, although did not influence performance. Other results (Shao et al., 2013) have shown that dietary addition of β-glucans can alleviate intestinal mucosal barrier impairment in broiler chickens challenged with *Salmonella typhimurium*.

As has been observed in poultry, most changes in intestinal microbiota occur in swine in response to prebiotic supplementation. Several authors reported increased bifidobacteria and lactobacilli, observing changes in microbial ecology. Generally decreased populations of enterobacteria, clostridia, coliforms, and *E. coli* were observed with prebiotic supplementation (reviewed in Barry et al., 2009). Prebiotics also offer possibilities to reduce the excretion of nitrogen into the environment. Piglets consuming a diet enriched in fermentable carbohydrates in the form of sugar beet pulp, native wheat starch, lactulose, and inulin, were shown to have a reduced protein fermentation along the GIT and reduced ammonia concentrations in the feces (Awati et al., 2006).

The use of prebiotics in ruminants is limited due to the presence of rumen, which represents a huge fermentation organ in which prebiotics would be completely hydrolyzed, and thus would not reach more distal areas of the GIT where they can exert their beneficial activities (Van Loo and Vancraeynest, 2008). However, enhancements in rumen-protective technologies may allow these compounds to be used in feedlot and dairy cattle, considering also that several classes of non-digestible oligosaccharides are found in plant cell wall in nature including feeds normally used for livestock feeding (Gaggià et al., 2010).

5.2.3 Safety of prebiotics

Prebiotics are natural food ingredients present in edible plants and are part of the traditional diet. Several toxicological studies have shown that prebiotics, especially chicory FOS do not increase morbidity or mortality or cause reproductive or target-organ toxicity (Al-Sheraji et al., 2013). These compounds are not mutagenic, carcinogenic or teratogenic (Carabin and Flamm, 1999). The only biological effects observed have been attributed to their action as non-digestible, fermentable carbohydrates causing self-limited gastrointestinal

distress (Al-Sheraji et al., 2013). The gastrointestinal symptoms are usually dose-dependent (Briet et al., 1995).

5.3 Synbiotics

Synbiotics refer to nutritional supplements combining probiotics and prebiotics in a synergistic form. The principal reason for using a synbiotic is the concept that a probiotic, without its prebiotic substrate does not survive well in the digestive system. Without the necessary source of nutrients for the probiotic, it will have a more important intolerance for oxygen, low pH, and temperature (Alloui et al., 2013). Synbiotics are relatively recent among additives used in livestock nutrition. However, results on *in vivo* trials are promising, showing a synergistic effect coupling probiotics and prebiotics in the reduction of food-borne pathogenic bacterial populations (Gaggìa et al., 2010).

In poultry, several studies have suggested that performance can be enhanced when using both probiotics and prebiotics. As an example, Fukata et al. (1999) found in broilers that a probiotic and FOS each reduced intestinal *Salmonella enteritidis* colonization when used singly, but their combination was more effective. A considerable increase in the bifidobacteria, lactobacilli and total anaerobes populations has been shown when feeding a diet containing a combination of GOS and *Bifidobacterium lactis* but no effect on body weight, feed intake and feed conversion ratio was observed (Jung et al., 2008). Awad et al. (2009) studied the effect of a dietary treatment with a synbiotic product (a combination of *E. faecium*, a prebiotic derived from chicory, and immune modulating substances derived from sea algae) on broiler chickens. Body weight, average daily weight gain, carcass yield percentage, and feed conversion rate were significantly increased compared with the control, whereas no increase in organ weight was found, with exception for the small intestine; a significant increase in the villus height in both *duodenum* and *ileum* was also observed.

Therefore, the use of synbiotics could represent an important and synergistic strategy to improve gut health of chickens from the first days of life and control pathogen release in the environment, decreasing the risk of food-borne infections in humans.

Chapter 6
Influence of probiotics, prebiotics and synbiotics on meat quality in broiler chickens

The attention of consumers regarding the quality and safety of meat is keeping a growing interest, and the demand of safe and qualitative meat production on the poultry market has considerably increased in recent years. The producers are increasingly focused to the use of natural and safe feed supplements, which positively affect animal health, increase their productivity, and improve quality of the production (Bomba et al., 2012).

There are several reports that state the possibility to influence meat quality in broiler chickens through the use of natural compounds such as probiotics, prebiotics and their combination. Several studies have shown that probiotic microorganisms can improve sensory characteristics and some physico-chemical properties of meat. Some trials showed that the enrichment of diets with yeast could favorably improve the quality of meat from broilers. As an example, meats from broiler chickens fed a diet containing chromium-enriched *S. cerevisiae* or *S. cerevisiae* cell wall and extract exhibited increased tenderness (Zhang et al. 2005) and increased water holding capacity (Lee et al. 2002). Also a study conducted by Zheng et al. (2015) has shown a significantly less cooking loss and drip loss in breast meat of broiler chickens fed *E. faecium* compared to control birds. Pelicano et al. (2003) observed a decrease in color (lightness) and increase in pH of breast muscle 5 hours after slaughter in birds fed supplemented with probiotics. Moreover, the sensory analysis showed a better meat flavour and general aspect 72 hours after slaughter in case of concomitant use of probiotics in water and feed. Additionally, it has been demonstrated that some cultures of lactic acid bacteria can have a protective effect on chicken meat, resulting in a reduced growth rate of *Listeria monocytogenes* and *Salmonella enteritidis*, without spoilage effect and reduction of the nutritional values (reviewed in Gaggia et al., 2011).

Also prebiotics provided interesting results concerning chicken meat quality. The results of a study conducted by Park and Park (2011) have shown that the addition of inuloprebiotics as an alternative for antibiotics to broiler diets can improve the quality and the storability of chicken meat. They found a higher water holding capacity, moisture content, L^* (lightness) and b^* (yellowness) values in breast muscle of chicken fed inuloprebiotics (250 g ton^{-1}). Moreover, the TBARS values after the 3rd day at low temperature storage in thig muscle of broilers were lower compare to the control and those fed antibiotics. Additionally,

concerning sensory evaluation, the scores of taste and flavour, color, juiciness texture and total acceptability of chicken meat were improved in case of birds fed inuloprebiotics. Abdel-Raheem and Abd-Allah (2011) found that meat from broiler chickens feed with diet supplemented with prebiotics was more tender and juicy. Interestingly, these natural bioactives can also affect the fatty acids profile of chicken meat. In fact, Kalavathy et al. (2006) reported that *Lactobacillus* cultures supplemented in feed reduced the oleic acid (C18:1) levels of the liver, muscle and carcass but increased the arachidonic acid (C20:4) in the liver. Supplementation of *Lactobacillus* cultures also increased slightly the total polyunsaturated fatty acids (PUFA) in the liver. Salma et al. (2007) observed a greater ratio of unsaturated fatty acids to saturated fatty acids in muscles of broilers fed with 0.04% of *Rhodobacter capsulatus* supplemented diet compared to control birds. A more recent work (Hossain et al., 2012) showed that the administration of a plant extract (*Alisma canaliculatum*) combined with different strains of probiotics decreased the levels of arachidonic acid, docosahexaenoic acid (C 22:6 n-3), PUFA, and n-6 fatty acids in breast meat from broilers.

There are several reports that evaluated the effects of synbiotics on meat quality of broiler chickens. As an example, results obtained by Abdurrahman et al. (2016) showed that the supplementation of prebiotic (dahlia tuber powder as inulin source) and probiotic (*Lactobacillus* spp.) affected breast meat color increasing L* and b* values; moreover, the content of fat and cholesterol in meat were decreased by the supplementation of combined prebiotic and probiotic. On the other hand, Raksasiri et al. (2015), who investigated the effects of a synbiotic supplementation (Jerusalem artichoke and BACTOSAC-P), did not found significant effects on meat quality traits taken into account (pH, color, cooking loss, drip loss, shear force, chemical composition). Aristides et al. (2012) observed that chickens fed diets enriched with synbiotics (*S. cerevisiae*, *L. acidophilus*, *L. casei*, *B. bifidum*, mannanoligosaccharides and fructoligosaccharides) showed a reduction in the development of pale, soft and exudative (PSE) meat and also a decrease in lipid oxidation. Also Hossain et al. (2012) found an improved oxidative stability in meat from broilers fed diets supplemented with a medicinal plant (*A. canaliculatum*) and different strains of probiotic microorganisms.

Although results on *in vivo* trials are promising, showing a synergistic effect coupling probiotics and prebiotics, especially in the reduction of food-borne pathogenic bacteria, the acquisition of data on the efficacy of synbiotic products as feed additives in poultry needs further investigation (Gaggìa et al., 2010). The application of probiotics, prebiotics and synbiotics considering meat quality traits in broiler chickens could have interesting

implications, considering the increasing attention of consumers towards healthy meat products obtained with natural methods.

Chapter 7

In ovo technology

7.1 Introduction

The first experiment concerning *in ovo* delivery of exogenous material was reported in the 1980s for vaccination against Marek's disease (Sharma and Burmester, 1982). Over the years, further tests have been conducted on the experimental injection of small amounts of drugs, vaccines and nutrients into the egg during incubation. These early developments have led to increased research into *in ovo* techniques for poultry, for improved starting weights, better feed utilization, faster growth and higher final weights (Kadam et al., 2013).

The early feeding of chicks has become increasingly important because of several observations. The GIT is relatively immature at hatch. Thus, appropriate dietary strategies during the first week of life are relevant to achieve the maximum genetic potential of the modern broiler. Meat-type broilers are capable of achieving 70 g/day until 40 days. This achievement requires emphasis on early phase nutrition (Ravindran, 2003; Noy and Uni, 2010). Chicks are usually held in the incubator after hatching, until post-hatching treatments such as sex determination, vaccination, beak trimming and transport. This process takes at least 24 hours and in some cases more than 48 hours prior to placement. In this time the early hatching chicks are at a disadvantage because of the prolonged fasting period and potential dehydration it causes (Noy and Uni, 2010). The lack of access to feed during this time leads to a depression in intestinal function and bird performance, which may not be overcome at later stage in life (Uni et al., 1998; Bigot et al., 2003). Although nutrients in the residual yolk are supposed to support the chick during the fasting period, they represent an insufficient contribution to the nutritional requirements for both maintenance and growth of broiler chicks (Bigot et al., 2003). Holding chicks for 36 hours with no access to feed or water resulted in a 100-200 g lower BW at 40 days compare to chicks with immediate access to feed (Noy and Sklan, 1997). Moreover, delayed feeding results in an increased mortality rate of about 5%, impaired muscle development (Willemsen et al., 2010) and seems to affects immunological capacities (Dibner et al., 1998).

Feed is not sterile and contains several antigens, so the earlier the feed passes through the GIT, the sooner the proliferating stem cells will meet environmental antigens, which may help to create a wider antibody repertoire (Uni, 1998). The increases in BW (Noy and Sklan,

1998) and development of the immune system (Friedman et al., 2003) can be obtained by early access to feeding. These growth responses have been attributed to the stimulation of the GIT function and the consequent improvements of nutritional maturity. High-quality diets containing many supplements have been developed for provision to the chicks once they reach the farm (Noy and Sklan, 1999). Generally, feeding immediately post-hatch has been shown to be more beneficial than feeding high-quality diets after a delay in feeding (Bhuiyan et al., 2011).

In ovo feeding of nutrients could represent a more effective option. This approach has been developed by Uni and Ferket (2003), which introduced the concept of administrating high volume (0.4-1.2 ml) of nutrients to the amniotic fluid of chicken and turkey eggs so as to "feed" the embryo, which consumes the amniotic fluid prior to hatch. Supplying embryos with exogenous nutrients *in ovo* may improve hatchability, increase hatched chick weight, and/or the final body weight of broilers through modulating embryo gut morphology. This study was distinct from other methods of *in ovo* nutrient administration, for which reason, it was patented (US patent, 6.592.878).

Several studies have demonstrated the positive effects of *in ovo* feeding: improved immune response to enteric antigens (Kadam et al., 2013), improved intestinal development and digestive capacity (Tako et al., 2005; Smirnov et al., 2006), increased growth rate and feed efficiency (Kornasio et al. 2011; Gholami et al., 2015), increased muscle development and breast meat yield (Kornasio et al. 2011). Various potential nutrient supplements can be used for *in ovo* feeding and many of these substances have been tested for their contribution to hatchability and production performance. Among others, there are vitamins and minerals (Oliveira et al., 2015; Yair et al., 2015), carbohydrates (Cheled-Shoval et al., 2011; Zhai et al., 2011), fatty acids and other modulators (Foye et al., 2007; Liu et al., 2012).

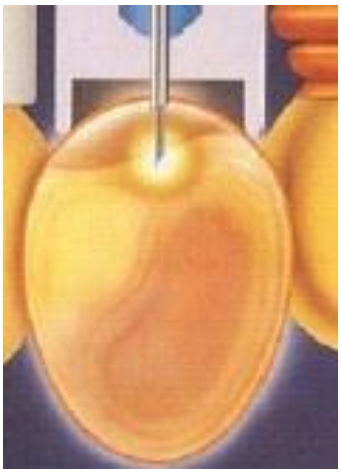
If early feeding is beneficial for early development of the newly hatched chicks, feeding the embryo during incubation would be expected to enhance hatchability and development of the digestive tract and increase body weight and nutritional status of the hatchling. The potential benefits of this method could be stronger if both practices are combined, minimizing the adverse effects of post-hatch holding of chicks. *In ovo* feeding is specifically targeted at the amnion, from where nutrients are delivered to the small intestine just prior to hatch (Uni and Ferket, 2004).

7.2 *In ovo* administration of probiotics, prebiotics and synbiotics

In recent years, the key role of the intestinal microbiota on immune function, host nutrition, and in general on health status and productive performance of poultry, is increasingly recognized. Among the different alternatives proposed to replace AGPs in poultry production, probiotics, prebiotics and synbiotics are able to modulate the activity of the intestinal microbiota, improving the health status of the host animal (Gaggìa et al., 2010).

In case of poultry, these compounds are conventionally added to feed and/or water at first hours/days post-hatching. The main concern about the use of these bioactives is their efficient administration under fully controlled conditions. In fact, in-feed or in-water supplementation depend on the amount of individual feed and/or water intake, the quality of water (chlorinated) and other environmental factors. Consequently, consumed dose of bioactives can be variable in the first hours/days after hatching. Moreover, during early post-hatching period, the possible infection of chicks by harmful bacteria, cannot be overlooked. Additionally, to achieve desired efficacy, these bioactives have to be administered to the animals as early in life as possible (Bednarczyk et al., 2011, 2016). Thus, *in ovo* approach for the injection of bioactives directly into the air chamber of the incubating egg was developed (Gulewicz and Bednarczyk, Polish patent Nb. 197726). The method allows the accurate and precise delivery of the bioactive substance at very low doses to all embryos at early stage of development, minimizing the effect of environmental variables and influencing the microbiome structure in newly hatched chicks (Bednarczyk et al., 2016; Maiorano et al., 2017) (Figure 7.1).

Figure 7.1 Schematic representation of *in ovo* technology.



The recent literature indicates encouraging results concerning *in ovo* administration of prebiotics (Pilarski et al., 2005; Bednarczyck et al., 2011, 2016; Maiorano et al., 2017) and

synbiotics (Maiorano et al., 2012; Sławińska et al., 2014), when the bioactives are delivered *in ovo* on day 12 of incubation. It was shown that this stage of embryonic development is the optimal moment for the injection, because the allantocorion is completely developed and highly vascularized, allowing the transfer of the bioactive solution from the air chamber to the GIT of the embryo (Martìnez Villaluenga et al., 2004).

Despite the GIT of chicks has been considered sterile at hatch, it was recently shown that the microbial colonization of the digestive system, may start during the last stage of embryonic development. Some studies (reviewed in Chambers and Gong, 2011) reported the presence of bacteria of different genera in the embryo cecum; Pedrosa (2009) showed a viable and morphologically diverse bacteria community within embryos intestines since day 16 of incubation. Thus, the inoculation of bioactives to the embryo can promote very early the development of a beneficial microflora prior to hatch. It was shown that a single *in ovo* injection with prebiotics on the day 12 of incubation leads to an increase in the number of bifidobacteria at the time of hatch, although this fact ensures the long-term maintenance of a high level of bifidobacteria in the intestinal tract (Martìnez Villaluenga et al., 2004; Bednarczyk et al., 2016). The application of bioactives to the chicken diet can be successfully replaced by injecting these compounds *in ovo* at very low doses. *In ovo* route of bioactives delivery can replace prolonged and costly supplementation of the broiler chickens with these bioactive compounds (Bednarczyk et al., 2016).

Chapter 8

Poultry meat: chemical composition, technological properties, sensory attributes and safety aspects

According to the European legislation, the term meat refers to the edible parts removed from the carcass of domestic ungulates including bovine, porcine, ovine and caprine animals, as well as domestic solipeds. Additionally, poultry, lagomorphs, wild game, farmed game, and small and large wild game, are included (European Comission, 2004).

Meat has exerted a crucial role in human evolution and is an important component of a healthy and well balanced diet due to its nutritional richness (de Castro Cardoso Pereira and dos Reis Baltazar Vicente, 2013). The perception of the role of meat in the diet, can take on different meanings depending on the context. In developing countries, meat and its availability represent a critical factor in order to improve the nutritional status and avoid the problems connected to malnutrition. This aspect cannot be overlooked, considering that the demographic development will bring the world's population to touch 9 billion in 2050, with possible problems in the availability of high quality dietary protein sources. On the other hand, in developed countries, meat is increasingly considered among the factors responsible of the increased incidence of some diseases, such as cardiovascular diseases and cancer. In this regard, more and more attention is paid in respect of lipid component, in terms of content and quality of fat. This vision obscured the recognition of the essential role of proteins and micronutrients of meat on the nutritional quality of the global human diet. The prospected correlation between high meat intake and human health problems has led to a reduction in meat consumption. In this context, the worldwide production and consumption of chicken meat have suffered to a lesser degree these negative consequences, because of the nutritional characteristics of this meat type. Because of the high protein content, low fat content, high content of mineral substances, low level of saturated fatty acids and cholesterol, chicken meat is one of the most significant meat type used in human diet (Cavani et al., 2009).

Poultry meat quality is affected by a wide variety of factors, including age, sex, genotype, rearing conditions and production practices (Berri, 2004). The quality of meat was the subject of interest of both scientists and technologists specializing in meat processing. The quality of meat is represented by the sum of all nutritional, sensory, hygienic, technological and processing characteristics. Because of this fact, to completely describe the quality of meat, chemical, physical, technological and sensory analyses are necessary. Each type of

analysis contributes specific and important information on overall meat quality (Liu et al., 2004).

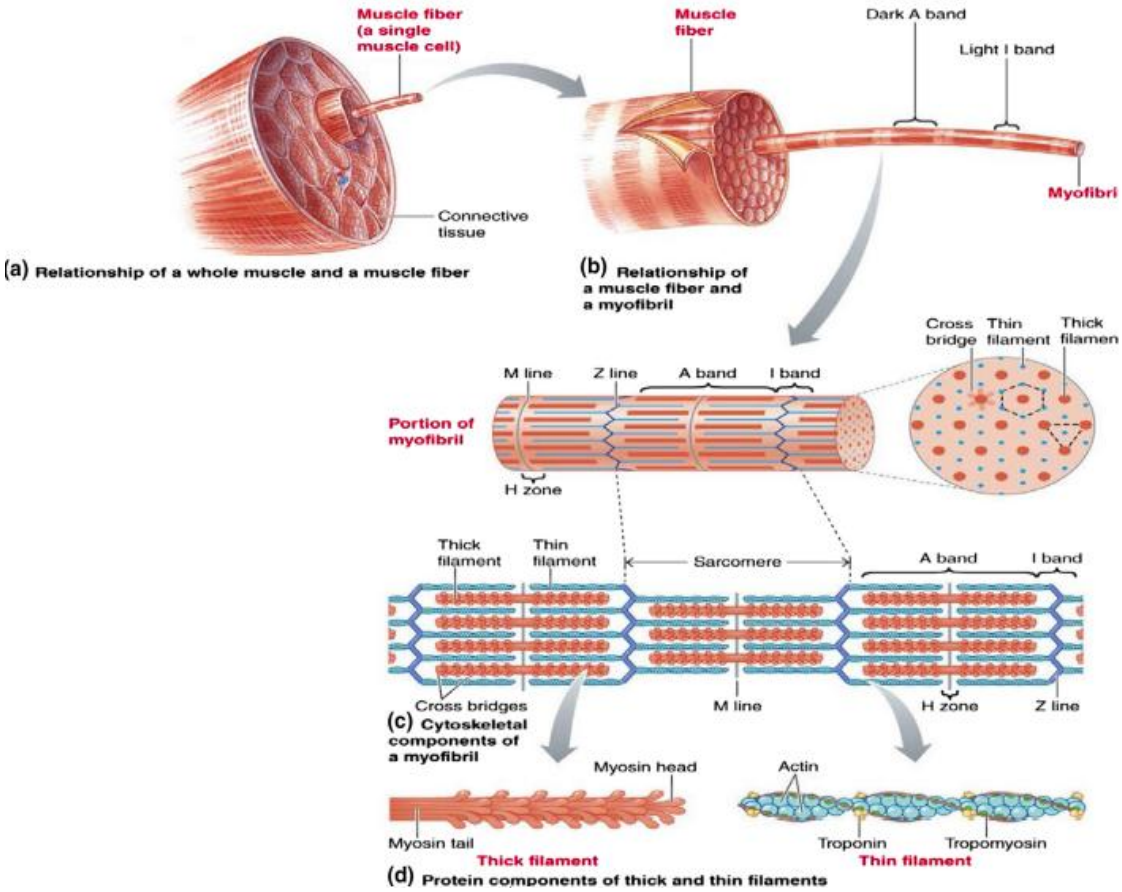
8.1 The muscle structure

The muscular system provides the mechanical activity for the animal in the form of mobility of the different parts of the skeleton or its appendages, movement of materials along tubular organs such as the alimentary canal, air passages and blood vessels, and pumping of the blood through the circulatory system by heart (Dingle, 1991). The skeletal and muscular systems are very closely linked and are often referred to as the musculoskeletal system (Biressi et al., 2007). Birds have three types of muscle: smooth, cardiac, and skeletal. Smooth muscle is controlled by the autonomic nervous system and is found in the blood vessels, gizzard, intestines, and organs. Cardiac muscle is the specialized muscle of the heart, while, skeletal muscle (also called striated muscle), is the muscle that forms the shape of the chicken and is used for the chicken's voluntary movements (Jacob and Pescatore, 2013). In the vertebrate, body skeletal muscle is the most abundant tissue (Biressi et al., 2007). Skeletal muscle formation begins during embryogenesis, including stem and progenitor cell maintenance, lineage specification and terminal differentiation. In adult period, skeletal muscles are composed of bundles of multinucleated myofibers which are distributed tendon to tendon and provided contractile activity in skeletal muscle (Shahjahan, 2015). Moreover, skeletal muscle is one of the most dynamic and plastic tissues of the body. Among skeletal muscles, in case of poultry, on attention deserve breast and leg muscle. Since chickens do not fly, breast muscles are not used as often as they would if chickens could fly. The breast meat of chicken is frequently referred to as “white meat”. White meat is white because of the minimal activity of these muscles. The thigh and leg muscles are a source of meat that typically referred to as “dark meat”. This kind of meat is dark because the muscles are used for sustained activity. The higher activity of leg muscles increases their need for oxygen. The darker color of more active muscles comes from myoglobin, a chemical compound in the muscle, which is important for oxygen transport. Other species of poultry capable of flight (duck, geese) have dark meat throughout (e.g., breast, thigh, and drumstick) (Jacob and Pescatore, 2013).

Skeletal muscle has a complex hierarchical structure (Figure 8.1). Muscle is mainly composed of water (75%), and other substances including inorganic salts, minerals, proteins, fat, and carbohydrates. Except the chemical composition of muscles, also their role deserve on attention. Skeletal muscle contributes significantly to multiple bodily functions. From a metabolic point of view, the roles of skeletal muscle include a contribution to basal energy

metabolism, serving as a storage for important substrates (e.g., amino acids), the production of heat for the maintenance of core temperature, and the consumption of the majority of oxygen and fuel used during physical activity. From a mechanical perspective, the main function of skeletal muscle is to convert chemical energy into mechanical energy to generate force and power, maintain posture, and produce movement that affects activity, allows for participation in social and occupational settings, maintains or enhances health, and contributes to functional independence (Frontera and Ochala, 2014).

Figure 8.1 Structure of skeletal muscle (*source*: Frontera and Ochala, 2014).



The architecture of skeletal muscle is characterized by a particular and well-described arrangement of muscle fibers (also referred to as myofibers or muscle cells) and associated connective tissue. At the whole muscle level, the size of a muscle is determined mostly by the number and size of individual muscle fibers. Chicken skeletal muscle can be further divided into five distinct muscle fiber types: type I slow-contracting "red" fibers, type IIA and IIB fast contracting "white" fibers, and a type IIIA and IIIB which are slow, tonic "intermediate" fibers. In chicken, type I muscle fibers are found in the soleus muscle that requires a sustained

level of activity for activities such as walking and standing (McKee, 2003). Moreover, the type I red fibers are slow oxidative and contain large amounts of myoglobin, contains many mitochondria, has many blood capillaries and generate adenosine triphosphate (ATP) by the aerobic system, hence called as oxidative fibers. It splits ATP at a slow rate and slow contraction velocity. Hence, they are resistant to fatigue. Type IIA fibers are fast oxidative (also called fast twitch A). They contain many mitochondria. Moreover, they have high capacity for generating ATP by oxidation, split ATP at a very rapid rate and high contraction velocity (Khatiwada et al., 2011). Type IIA fibers are found in muscles that are fast-moving and repetitive in action; therefore, they do not fatigue very easy. Type IIA fibers are found in muscles such as the Sartorius (red) (McKee, 2003). The type IIB fibers contain low myoglobin content, few mitochondria, few blood capillaries (Khatiwada et al., 2011). Type IIB muscle fibers are fast-contracting but are more easily fatigued in comparison to both type I and type IIA muscle fibers. Type IIB fibers have higher levels of ATP and glycogen and are found primarily in pectoral muscle, posterior *latissimus dorsi* and to some degree in the Sartorius (white). Type IIIA and IIIB slow-tonic fibers are not found in mammals but are found in muscles such as the *plantaris* and *anterior latissimus dorsi* of the avian species (McKee, 2003).

Each muscle fiber contains myofibrils proteins that are responsible for the formation of a gel network in meat (Lantto et al., 2006). Myofibrils consist of thick and thin filaments which are organized into a contractile unit called a sarcomere and surrounded by a basal lamina (Shahjahan, 2015). The myofibril is a scaffold for spatial distribution of the proteins that integrate force production and transmission. Myofibrils are connected to intermediate filaments, transverse tubules, and sarcoplasmic reticulum, and microtubules. Near the periphery of the cell the Z-bands form costameric attachments that connect myofibrils with a cytoskeletal array of proteins beneath the sarcolemma (Sanger et al., 2005). Myofibrils are composed of long proteins including actin, myosin, and titin, and other proteins that hold them together. Actin is a family of highly conserved contractile proteins found in all eucaryotic cells. Multiple actin isoforms have been identified, and some of these proteins are related with diverse functions such as muscle contraction, cytoskeletal structure, cell motility, and chromosome movement. Differences in amino acid sequence between the various actin filaments have shown that at least six different polypeptides are expressed in birds and mammals. The best-studied actin, the α isoform, is a major constituent of the contractile apparatus in skeletal muscle (Chang et al., 1984; Yamin and Morgan, 2012). Examination of the structure of muscle actin has indicated that the amino acid sequence of this protein is very highly conserved. This suggests that a high degree of structural integrity is necessary for actin

function (Anderson, 1976). Considering meat quality, on special attention deserves an interaction of actin and myosin. The binding of actin and myosin to form actomyosin, protects myosin against further denaturation. Thus, it is suggested that the rapid rate of *post mortem* glycolysis in poultry reduces the sensitivity of myosin to denaturation because the rapid glycolysis results in a rapid rigor (Van Laack and Lane, 2000).

8.2 Chemical composition of meat

Chemical composition of meat is important for its nutritional value, technological, and sensory quality. Food composition data are important for a spectrum of users ranging from international organizations and private individuals, to food assistance programs, epidemiologists correlate patterns of disease with dietary components and nutritional assessment of individual intake and dietetic counselling. Applying food safety standards on a product is very important because it relates closely to human's health (Mohammed, 2013).

Poultry meat is composed mainly of water and dry matter, which is mainly represented by proteins, lipids, vitamins, and minerals. Various types of poultry meat have similar approximate chemical compositions (Table 8.1) (Soriano-Santos, 2010).

Table 8.1 Approximate composition of poultry meat (g/100 g) of different species.

Component	Broiler	Turkey	Duck	Quail
Water	74.6	72.5	70.8	74.3
Ash	1.0	0.8	1.2	1.1
Protein	12.1	13.7	12.8	13.1
Lipid	11.1	11.9	13.8	11.1
Fiber	-	-	-	-
Carbohydrates	1.2	1.1	1.4	1.4

Source: Soriano-Santos, 2010.

The content of the different components in poultry meat is comparable to the composition of the meat obtained from other slaughter animals, although content of particular components varies in quite wide ranges. The chemical composition of poultry meat varies depending on the species, breed, line, hybrid, muscular type, etc.; as an example, meat from chicken and turkey breast have highly protein amount than the other parts from carcass with large fat deposits.

The quality of animal fat and the amount of nutrients, largely depend on the animal's diet or its genetic pattern. Recently, also specific farming techniques (organic, free range) have been shown to affect some compositional aspects of meat (specifically, poultry meat).

The energetic value of poultry meats can vary considering the presence of skin (due to its fat content) increases the caloric value by around 25-30% (Marangoni et al., 2015).

8.2.1 Protein content

Considering all macronutrients, proteins are the minor contributors to the daily caloric intake. Moreover, it is also important to highlight that proteins are the only macronutrients for which, as in case of micronutrients, has been established a precise recommended intake. According to the European Food Safety Authority (2012), as an example, the average recommended daily intake of protein (minimum consumption level required to satisfy the recommended intake for 50% of healthy subjects for adults (both men andwomen)) is 0.66 g protein/kg body weight per day based on nitrogen balance data, and reaches up to 1.12 g per kg of body weight for infants. The recommended protein intake also increases for men and women over 65 years of age in order to prevent sarcopenia, which occurs frequently in the elderly.

The nutritional value of proteins is determined first of all by their content of essential amino acids, and their digestibility. The amino acid content of a protein is determined by chemical analysis, which is compared with that of a reference amino acid pattern. The score obtained from this comparison is corrected for protein digestibility. Animal foods in general are considered to be foods with high protein qualities. Red meats, poultry, fish, eggs, milk, and milk products contain complete protein. More than 20% of these foods energy content is protein. Americans, on average, obtain about 63% of their protein intake from animal foods (14% of protein from poultry) (Soriano-Santos, 2010). The protein content of most meat (including poultry meat) ranges between 15 and 35%, depending on the water and fat content of the product. Cooking also causes an increase in protein concentration, which reaches up to 60% in weight for skinless turkey drumstick and skinless chicken drumstick (Marangoni et al., 2015).

Meat proteins are characterized by their content of essential amino acids. The human body needs 20 different amino acids, nine of which are called essential because these amino acids cannot be synthesized by the body, and therefore, they must be introduced through the diet. Essential amino acids for adults are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Additionally, children need arginine. Food proteins that supply all the indispensable amino acids in the proportions needed by the organism, are called complete. Animal foods are considered to have high protein qualities, although their qualities are not always similar because of differences in essential amino acids (Soriano-Santos, 2010).

Poultry meat is a highly appreciated source of aminoacids (Table 8.2) necessary for the synthesis of proteins. The nutritional value of each food can be determined by the quantity and the quality of the several amino acids present or absent. If a certain food supplies enough of seven of the eight essential amino acids, the lacking amino acid is defined as the "limiting amino acid". In addition to its richness, meat proteins are characterized because of their content in all the essential amino acids with no limiting amino acids (reviewed inde Castro Cardoso Pereira and dos Reis Baltazar Vicente, 2013).

Table 8.2 Amino acid composition of breast and thigh muscles from broiler chickens aged 42 days (mg/g of protein)[1].

Amino acid	Breast muscle	Thigh muscle
Thr	36	27
Val	45	33
Met	20	14
Ile	42	30
Leu	68	51
tyr	35	19
Phe	24	23
His	44	24
Lys	77	58

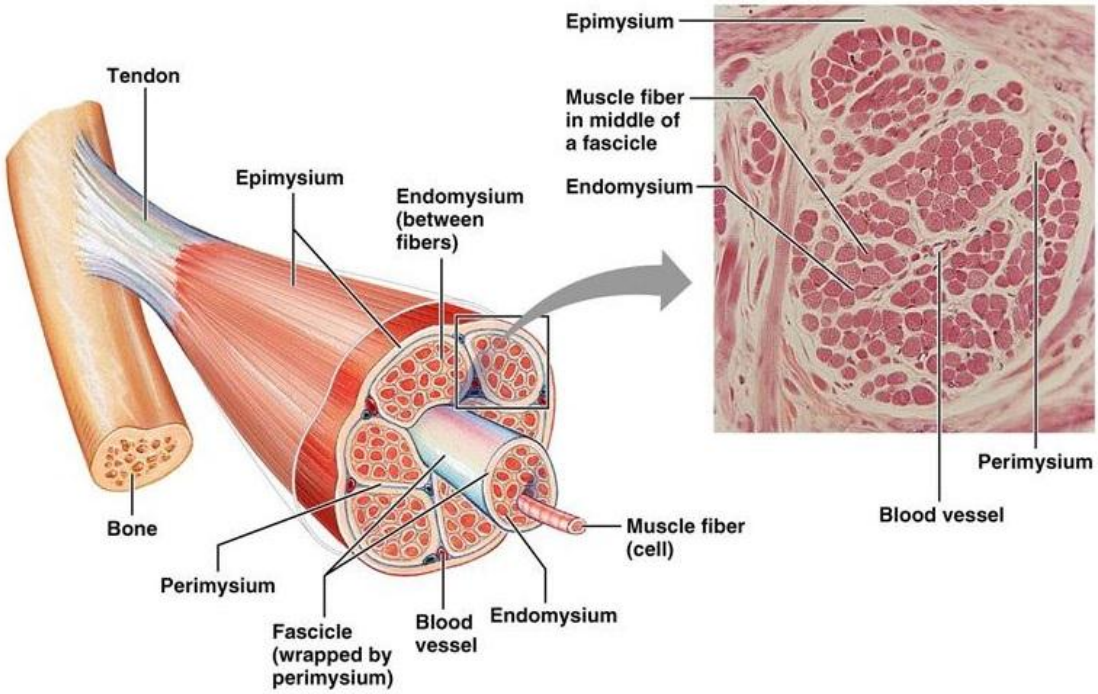
[1]Results related to 100% of dry matter.
Source: Soriano-Santos, 2010.

8.2.1.1 Collagen

Connective tissue consists of proteins, complex polysaccharides and water as different mixtures depending on the type of tissue. The main protein in intramuscular connective tissues is collagen (Mobini, 2015). It is the most abundant protein in the organism and it is present in skin, bone, and tendon (Etherington and Sims, 1981; Maiorano et al., 1995). The term "collagen" is derived from the Greek word for glue and was initially used to describe that constituent of connective tissue which yields gelatin on boiling (Bhattacharjee and Bansal, 2005). Collagen is an essential molecule in vertebrates, because it plays the pivotal role in maintaining the structure of tissues. However, collagen and collagen-like proteins have many other important roles, such as cell adhesion, chemotaxis, cell migration, and the regulation of tissue remodeling during cell growth, differentiation, morphogenesis, and wound healing (Boudko et al., 2008). It is found in muscle as 1-9% of the dry, fat-free mass where it exists as networks of fibers. In the living muscle these fibers resist over-extension which may cause damage to the tissue. Three collagenous structures can be distinguished morphologically (Etherington and Sims, 1981) (Figure 8.2):

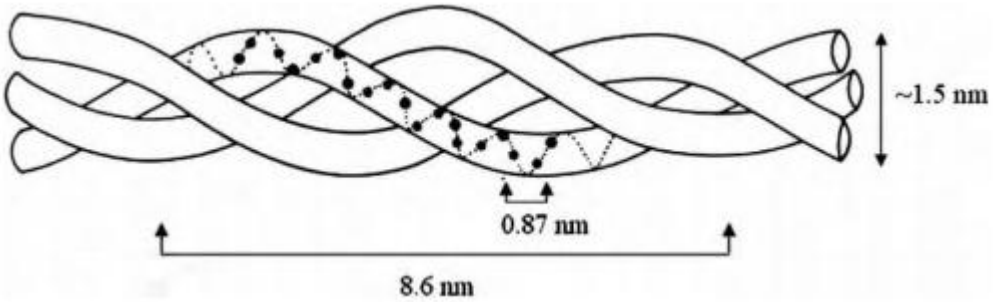
- ✓ Endomysium: is the thinner portion of the intramuscular connective tissue and it is found directly in contact with the sarcolemma and thus with every single muscle fiber. It represents the 0.47-1.2% of the dry weight of every single muscle. The endomysium penetrates between the muscle fibers, forming a network in which the fibers lie adjacent in hexagonal shaped cells. Moreover, the endomysium is the only intramuscular element that, within the same fascicle, contacts the elements of the same motor unit even through muscular fibers. The endomysium extends itself without interruption in the perimysium's collagen (Turrina et al., 2013).
- ✓ Perimysium: this part of the connective tissue does not present a solution of continuity with the epimysium. Perimysium represents the 0.43-4.6% of the dry weight of the muscles (Turrina et al., 2013). Exists the evidence that the perimysium of striated muscle can play a role in muscle force transmission, but there is also a clear role that perimysial boundaries between adjacent muscle fiber bundles to accommodate shear deformations generated when muscle contract. Differences in shear strains generated in anatomically different muscles appear to be the major explanation of why the division of muscles into fiber bundles by perimysium varies so much from muscle to muscle (Purslow, 2008). It has been shown that perimysium could be divided into two different types: primary perimysium surrounding the muscle fiber bundles and secondary perimysium surrounding the muscle fiber bundles in larger scale (Nakamura et al., 2003).
- ✓ Epimysium: this layer is thicker than the above mentioned elements of the intramuscular tissue and is formed by collagen fibers with a larger diameter. The epimysium covers all the muscle bellies, forming a lamina that clearly defines the volume of each muscle. Additionally, the epimysium takes on a role of: (i) containment, limiting the expansion of the muscle with the disposition in concentric layers of the collagen; (ii) transmission of forces, that are received from the perimysium and from the direct insertion of the fibers into some parts of the muscle; (iii) sliding surface of the muscle in respect to the surrounding structures and vice versa (Turrina et al., 2013).

Figure 8.2 Composition of muscle (*Source*: http://keywordsuggest.org/gallery/405343.html).



The main structural unit of collagen is tropocollagen molecule, with a length of ≈ 300 nm and a diameter of about 1.5 nm, which are arranged in a staggered configuration (Gautieri et al., 2011). Tropocollagen consists of three polypeptide chains (1000 amino acids residues each of them) (Bezkorovainy and Rafelson, 1996). Collagen has a very unique amino acid sequence in which a glycine residue appears every third position, forming the triplet repeat Gly-X-Y. Collagen also has a high imino acid content and typically contains proline and (2S,4R)-4-hydroxyproline (4(R)Hyp) in the X and Y positions, respectively. This characteristic sequence constraints lead to the formation of a triple helical structure (Figure 8.3). Specifically, each of the three polypeptide chains forms a polyproline II-like left-handed structure, and these structures twist around each other to form a loose right-handed superhelix (Schumacher et al., 2005). The number of Gly-Pro-Hyp repeats is the main factor in determining collagen thermostability. Approximately 90% of collagen tripeptide units contain at least one non-imino acid residue in the X and/or Y position, and these residues probably play a role in collagen structure, stability, and function (Boudko et al., 2008).

Figure 8.3 Triple helix structure of collagen (*Source*: Meyers et al., 2008).



The family of collagen types can be divided into fiber forming collagens (I, II, III, V and XI), network forming collagens (IV, VIII and X), fiber associated collagens with interrupted triple helix (IX, XII, XIV, XIX and XXI), filamentous collagen (VI), anchoring fibers forming collagen (VII), collagens with transmembrane domain (XIII and XVII) and the collagen types that have been only partly described (reviewed inVoutila, 2009). Specifically, type I collagen, the predominant genetic type in the collagen family, being the major component of tendons, bones, ligaments and muscle (Maiorano et al., 1993). This collagen type contains one-third of Glycine, contains no tryptophan or cysteine, and is very low in tyrosine and histidine. The next one-type II collagen, is the main component of a nose cartilage, the outside of the ears, the knees and parts of larynx and trachea. Type III collagen is present in skin, blood vessels and muscle (Maiorano et al., 1993), while type IV collagen is present in the basement membrane. Type VI is microfibrillar collagen and type VII is anchoring fibril collagen. Types IX, XI, XII and XIV are fibril associated collagens with small chains (Gorgieva and Kokol, 2011).

Collagen fibrils and the fibrous matrices are stabilized by covalent crosslinks. The development of these crosslinks is an age-related process and it is the increase in the number of the heat-stable covalent attachments that makes meat from older individuals much tougher during cooking. Thus, in evaluating the quality of meat it is important to consider both the quantity and the maturity of the connective tissue component (Etherington and Sims, 1981). The initial step in crosslink formation is the conversion of the epsilon amino group of selected lysine or hydroxylysine residues to the corresponding aldehyde. Cross-links then form by spontaneous reaction of an allysine or hydroxyallysine with lysine or hydroxylysine residue. Two pathways of crosslink formation for fibrillar collagens have been described. First crosslinks formed on either pathway are difunctional and are described as reducible crosslinks because they possess Schiff base double bonds (McCormick and Thomas, 1998).

8.2.2 Lipid content and fatty acids composition

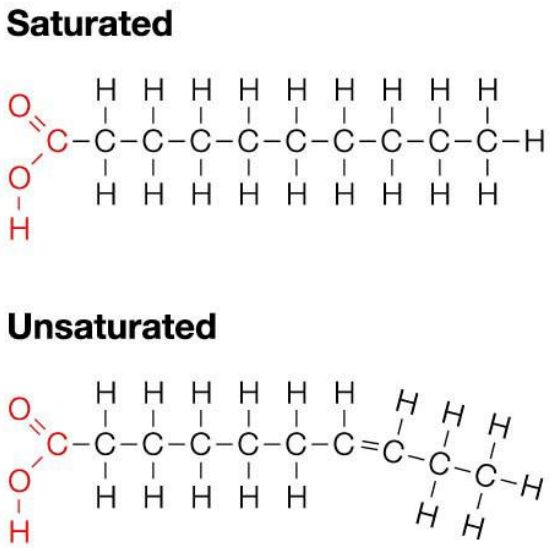
Meat contributes to fats, especially saturated ones; its consumption, is thus potentially associated with a high intake of these nutrients and the corresponding negative health consequences. However, the suggested dietary target for fats in the general healthy population ranges from 25 to 35% of total energy, so that a typical average intake of 2.000 kcal results in 70 or more grams of these nutrients per day. In addition, when fats are consumed in the context of a healthy and well balanced diet, they plays several important roles, such as providing essential fatty acids (linoleic and α-linolenic acids) and fat-soluble vitamins (A, D, E, and K). They represent the major source of energy, promotes a sense of satiety due to slowing effects on gastric emptying, reducing, for the same reason, the bioavailability of carbohydrates (and, thus, the glycemic response). Moreover, they enhance the taste, smell, and texture of foods (Marangoni et al., 2015).

Total lipid content of meat and meat products varies around 3-25 g/100 g of food. The fat content varies widely depending on the animal species, age and the part of carcass used. Moreover, the lipid content and the fatty acids composition can be affected by animal feeding, an aspect that is exploited for modification of fatty acids composition of meat, with interesting results in monogastric animals such as poultry (Valsta et al., 2005).

Fatty acids (FA) are the constituent elements of fats. These molecules are characterized by a backbone of carbon atoms with a methyl group (CH_3) at one and (the omega or n-end) and a carboxyl group (COOH) at the other (the delta end). Hydrogen atoms are joined to the string of carbon atoms, forming a hydrocarbon chain. The carbon chain can have different lengths ranging from 2 to 80 carbon atoms, but in food, usually, FA are present as 14, 16, 18, 20, and 22 atom chains. Short chain FA are usually made up of less than six carbon atoms; long chain FA regularly contain 12 or more carbon atoms.

If all of carbon atoms in the FA chain are linked by single bonds (the FA contains all the hydrogen atoms that it can hold), the FA is said to be a saturated fatty acid (SFA). If one or more double bonds are present in the FA chain (the FA does not hold its potential full complement of hydrogen atoms), the FA is considered to be an unsaturated fatty acid (Figure 8.4). If there is only one double bond present in an unsaturated fatty acid, it is said to be a monounsaturated fatty acid (MUFA). If there is more than one double bond present, the FA is said to be a polyunsaturated fatty acid (PUFA).

Figure 8.4 The chemical structure of saturated and unsaturated FA (*Source*: http://www.precisionnutrition.com/all-about-healthy-fats).



Moreover, PUFA can be classified as n-3 (omega 3) or n-6 (omega 6) PUFA; this depends on the position of the first double bond in the FA chain. All the n-6 PUFA contain the first double bond between the sixth and seventh carbon atoms from the terminal methyl group, while all the n-3 PUFA have the first double bond between the third and fourth carbon atoms (Lunn and Theobald, 2006).

FA in meat lipids are mainly SFA and MUFA. The most ubiquitous FA are oleic (C18:1), palmitic (C16:0), and stearic (C18:0) acids. Poultry and pork contain a higher amount of unsaturated fatty acids (10-15% of total FA) than beef and lamb, and also a significant amount of PUFA. Linoleic acid (C18:2) is the predominant PUFA, followed by α-linolenic acid (up to 0.5%) (Valsta et al., 2005).

Fatty acids are required by the organism for several other functions than simply as an energy source, and there is an increasing awareness of the potential health benefits of specific types of fatty acids. Most of these compounds can be synthesised in the body, but humans lack the enzymes required to produce two fatty acids. These are called the essential fatty acids (EFA) and must be acquired from the diet. In humans, the essential fatty acids are the n-3 PUFA linolenic acid and the n-6 PUFA linoleic acid. Although humans can elongate dietary α-linolenic acid to the long chain n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the rate of synthesis may not be sufficient to meet requirements, and it is therefore recommended that good sources of these fatty acids are included in the diet (Lunn and Theobald, 2006). Linoleic acid (LA) and α-linolenic acid (ALA) and their long chain derivatives are important components of animal and plant cell

membranes. These two classes of EFA are not interconvertible, are metabolically and functionally distinct, and often have important opposing physiological functions. Their balance is important for good health and normal development (Simopoulos, 2002). The ratio of n-6/n-3 PUFA is known to be a risk factor in cancers and coronary heart diseases, especially the formation of blood clots leading to a heart attack (Wood et al., 2003). This ratio is important because an excess of one family of these fatty acids can interfere with the metabolism of the other, reducing its incorporation into tissue lipids, and altering their total biological effects (Ruxton et al., 2004). Replacing SFA with MUFA or n-6 PUFA reduces low density lipoprotein (LDL) cholesterol. Unsaturated fatty acids, such as LA or MUFA, also slightly raise high density lipoprotein (HDL) cholesterol, which assists in the removal of triacylglycerols from the bloodstream (Lunn and Theobald, 2006). Interest in the health effects of the long chain n-3 PUFA is also increasing. There is strong supportive, but not yet conclusive evidence that these fatty acids protect against fatal heart disease. Several chronic disorders have been suggested to be linked with lack of n-3 PUFA include hypertension, inflammatory and immune disorders, depression and neurological dysfunction. The recognition of the importance of DHA in neuronal development in the foetus and the newborn has also highlighted the key role of this class of fatty acids in infant as well as adult nutrition (Williams, 2000; Lunn and Theobald, 2006).

Lipids contained in poultry meat are more valuable than those from other large slaughter animals, due to the significant content of unsaturated FA, especially PUFA, such as linoleic acid, linolenic acid and arachidonic acid (Table 8.3). Moreover, the lipid composition of broiler meat can be modified by adding to the diet linoleic and linolenic acids, vegetable oils, and fish oils (Lopez-Ferrer et al., 2001). FA of the animal tissue have a double origin: endogenous, from *de novo* synthesis, and exogenous, provided by the diet. PUFA deposition depends almost exclusively on dietary supplementation. In monogastric animals, especially in chickens, it is well established that the FA profile of feed directly affects to the FA composition of fat depots and therefore on meat quality. However, SFA are less modifiable than unsaturated FA, and MUFA vary in an inverse way to PUFA (Barroeta, 2007). By feeding broiler chickens with small amounts of a supplement rich in α-linolenic acid, such as flax seed, the n-3 PUFA in thigh meat can be increased from 86 mg to 283 mg/100g, and that in the minced carcass from 93 to 400 mg/100g (Farrell, 2013). Diets supplemented with PUFA-rich oils compared with diets supplemented at the same level with SFA-rich animal fats, lead to a lower body fat deposition in broilers. Several authors observed that a higher PUFA content of chicken diets caused a lower fat deposition in separable fat depots, principally subcutaneous and intramuscular fat (reviewed in Barroeta, 2007).

Table 8.3 Content of n-6 and n-3 polyunsaturated fatty acids (mg/100 g) in selected meats.

	n-6		n-3			
	LA 18:2 n-6	AA 20:4 n-6	ALA 18:3 n-3	EPA 20:5 n-3	DPA 22:5 n-3	DHA 22:6 n-3
Poultry meat	1.443	98	73	5	18	25
Chicken[1]	2.880	80	140	10	10	30
Chicken[2]	550	80	20	10	20	30
Turkey[1]	1.700	110	110	0	20	20
Turkey[2]	640	120	20	0	20	20
Pork	831	68	53	3	7	2
Bovine meat	277	24	105	5	8	4
Lamb	369	84	54	5	7	10

[1]With skin; [2]Without skin.
Source: Marangoni et al., 2015.

Additionally, poultry meat can be considered as a "functional food", which provide bioactive substances with favourable effects on human health, like conjugated linoleic acids (CLA), and a balanced n-6/n-3 PUFA ratio (Cavani et al., 2009). CLA have 2 double bonds, one in *cis* and one in *trans* configuration. It is well documented that these type of FA acts as anticarcinogenic, prohibits arteriosclerosis, improve the immune system, and reduce plasma cholesterol and fatness (reviewed in Grashorn, 2007).

Another interesting aspect, relates the effects of FA on several technological aspects of meat quality. FA have very different melting points, and the differences in FA composition have important influence on hardness or softness of meat fat. Additionally, FA have several other important effects on tenderness, juiciness, color, flavour and shelf life of meat (Wood et al., 2003).

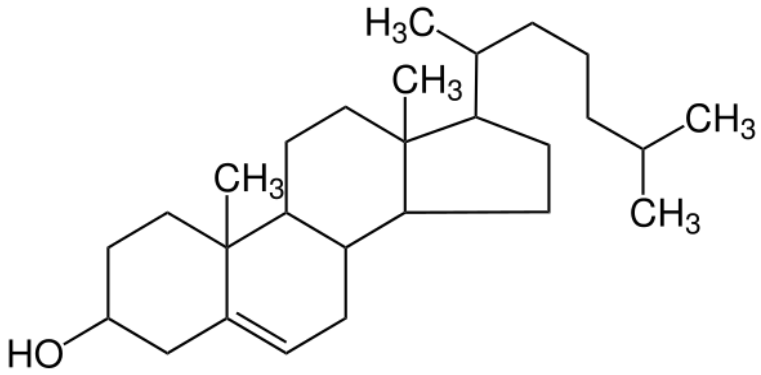
8.2.2.1 Cholesterol

The nutritional guidelines of the World Health Organization (2003) suggest that dietary fat should provide between 15 and 30% of total calories and that saturated fats should be limited to no more than 10% of caloric intake. Additionally, it is also reported that cholesterol intake should not exceed 300 mg per day. Therefore, the knowledge about cholesterol content in foods is important, especially in poultry and fish meat, because consumption of these foods is currently increasing based on the recommendations of healthy nutrition (Komprda et al., 2003). Limitations in fat and cholesterol intakes are considered important measures to prevent obesity and hypercholesterolemia, conditions that are considered to predispose to several chronic diseases of the circulatory system. Additionally, relationships appear to exist between a high-fat intake, especially saturated fat, and an

increased risk of some types of cancers, particularly cancers of colon, breast and prostate (Reddy, 1995).

Cholesterol is the main sterol (Figure 8.5) in higher animals. It is synthesized by animal liver and also supplied in diet through animal products such as meats, poultry, fish and dairy products (Hongbao, 2004). Despite dysfunction in cholesterol metabolism can lead to hypercholesterolemia which is a major factor in the development of atherosclerosis, cholesterol is an essential component of cell membrane, as a precursor for the synthesis of steroid hormones, vitamin D, and bile acids that aid in digestion and cellular signal transduction (Thomas et al., 2012).

Figure 8.5 The chemical structure of cholesterol (*Source*: www.wikipedia.org).



Cholesterol is transported in the blood in combination with specialized proteins. The two major blood cholesterol carriers are low density lipoprotein (LDL) and high density lipoprotein (HDL). LDL is known as “bad cholesterol”, and delivers blood cholesterol throughout the organism, depositing it as “plaque” on artery walls. HDL cholesterol is known as “good cholesterol”, and functions as a vehicle in the blood to remove waste from the organism (Bellows and Moore, 2012).

Dietary cholesterol is strictly linked with foods of animal origin as all of them contain it, since cholesterol is an essential constituent of animal cells (Chizzolini et al., 1999). Cholesterol is a nutritional important component of meats. Its content (Table 8.4) varies between about 30 and 120 mg/100g of food, being even higher in offals (Valsta et al., 2005). Chicken or turkey breast meat content is 53 mg/100g; turkey thigh meat (61.5 mg/100g) and chicken thigh meat is 82.9 mg/100 g (Soriano-santos, 2010).

Table 8.4 Average cholesterol content (mg/100 g) and calorific value (Kcal/100 g) of some types of meat and fat.

Type of meat	Cholesterol	Energy value
Beef (muscles)	60.00	115
Veal (muscles)	70.00	101
Pork (muscles)	65.00	114
Mutton (fillet)	70.00	122
Chicken (average)	81.00	144
Turkey (average)	74.00	231
Lamb (intermuscular fat)	75.00	673
Beef (intermuscular fat)	99.00	710
Pork (intermuscular fat)	93.00	749

Source: Chizzolini et al., 1999.

Cholesterol content of meat can differ considering the different species and type of muscle, although the magnitude of these factors appear to be generally low (Chizzolini et al., 1999). Significant and interesting differences, instead, have been observed in cholesterol content between muscle fibers types. As an example, a study conducted by Smith et al. (1993) made a comparison between pectoralis muscles fibres of duckling (16% white fibres) and chicken (100% white fibres). Duckling pectoralis muscle fibers have been found to have more cholesterol (99.11 mg/100g muscle) than broiler pectoralis (47.41 mg/100g muscle). Additionally, it is known that cholesterol content in animal tissues can be influenced by dietary treatment, despite the regulatory mechanisms on the level of synthesis and absorption, which supposedly maintain cholesterol concentration in these tissues (reviewed in Komprda et al., 2003).

Blood cholesterol concentration can be affected by the FA profile of lipids included in the diet. More specifically, it appears that SFA of 12-16 carbon atoms increase blood total, LDL and HDL cholesterol concentration and the LDL:HDL ratio (Wiseman, 1997). Polyunsaturated n-6 FA tend to decrease LDL cholesterol levels, while PUFA of the n-3 series have not been shown to have consistent effects on blood cholesterol (Harris, 1997), although long chain n-3 PUFA are effective in lowering of blood triacylglycerols levels, a risk factor for cardiovascular disease (Austin, 1997).

8.2.3 Vitamins, macro- and microelements

The contribution of poultry meat to provide several kinds of vitamins (Table 8.5), is equally significant. The importance of meat as an essential source of some micronutrients is due to the fact that it is either their only source, or they have a higher bioavailability (Olaoye,

2011). Vegans are at risk of deficiency of micronutrients which are found exclusively in animal-derived food, such as vitamin B12, riboflavin and selenium, and even supplementation with B12 and selenium is sometimes not sufficient (Boelsma et al., 2001).

Table 8.5 Vitamin composition of chicken and turkey meat (value per 100 g).

	Chicken[1]	**Turkey**[2]
Vitamin C, total ascorbic acid (mg)	2.3	-
Thiamin (mg)	0.07	0.05
Riboflavin (mg)	0.14	0.19
Niacin (mg)	8.23	8.10
Vitamin B6 (mg)	0.43	0.65
Folate, DFE (µg)	7	7
Vitamin B12 (µg)	0.37	1.24
Vitamin A, RAE (µg)	16	9
Vitamin A (IU)	52	30
Vitamin E, α-tocopherol (mg)	0.21	0.09
Vitamin D (D2 + D3) (µg)	0.1	0.2
Vitamin D (IU)	5	8
Vitamin K, phylloquinone (µg)	1.8	-

[1]Chicken, broilers or fryers, meat only, raw.
[2]Turkey, whole, meat only, raw.
Source: https://ndb.nal.usda.gov/ndb/search/list.

Poultry meat is a valuable source of the most of hydrophilic vitamins, and it is the ideal dietary source of vitamin B12. It is found only in animal products and can hardly be compensated by plant-derived food (Biesalski, 2005; Marangoni et al., 2015). The low dietary intake is probably the main cause of vitamin B12 deficiency. However, it can also be due to absorptive process impairments; strict vegetarianism is also associated with low vitamin B12 intake. The deficiency of this vitamin is the main cause of megaloblastic anaemia and it is also associated with high levels of blood homocysteine, which is a cardiovascular disease risk factor (reviewed in de Castro Cardoso Pereira and dos Reis Baltazar Vicente, 2013). Moreover, vitamin B12 has a key role in central nervous system functions at all ages (Reynolds, 2006) and its deficiency is associated with neuropathy, which is particularly common in the elderly (Weir and Scott, 1999). The amounts of B-group vitamins such as niacin, vitamin B6, and pantothenic acid in poultry, are very similar to those of other meats and do not significantly reduce during cooking. While red meat is the most abundant in terms

of vitamin B12, poultry supplies an important amount of niacin. Lipophilic vitamins are less abundant in meat compared to plant-derived foods (Marangoni et al., 2015).

Meat and meat products are also an important source of other micronutrients. Among others, folic acid and vitamin A play an essential role in human health. Together with vitamin B12, folic acid is crucial for fetal development (Zeisel, 2009). It is mainly found in liver, especially from calf (supplying 87% of daily requirement), while pig liver supply 81% of daily requirement (de Castro Cardoso Pereira and dos Reis Baltazar Vicente, 2013). Folic acid deficiency is of major concern especially in almost all developing countries and has been shown to lead to neural tube defects. For pregnant women and women planning a pregnancy, an intake of > 400µg/day is considered protective against neural tube defects. Vitamin A is an essential micronutrient for growth and development. Except offals, meat is not an important source of vitamin A. Liver is a good source, considering that 100 g supply more than 338% of the dietary recommended value (de Castro Cardoso Pereira and dos Reis Baltazar Vicente, 2013). It is essential for the growth and development of various cells and tissues, such as the embryonic lung development (Biesalski, 2005).

Poultry meat also provide several minerals (Table 8.6), such as iron, zinc and selenium. Minerals are essential for bone health, muscle and nerve function, regulation of blood sugar levels and therefore are important in diseases such as hypertension, cardiovascular diseases, osteoporosis and diabetes (reviewed in Decker and Park, 2010).

Table 8.6 Mineral composition of chicken and turkey meat (value per 100 g).

	Chicken[1]	**Turkey**[2]
Calcium, Ca (mg)	12	11
Iron, Fe (mg)	0.89	0.86
Magnesium, Mg (mg)	25	27
Phosphorus, P (mg)	173	190
Potassium, K (mg)	229	235
Sodium, Na (mg)	77	118
Zinc, Zn (mg)	1.54	1.84

[1]Chicken, broilers or fryers, meat only, raw.
[2]Turkey, whole, meat only, raw.
Source: https://ndb.nal.usda.gov/ndb/search/list.

Iron is vital for almost all living organisms, participating in a wide variety of metabolic processes. Disorders of iron metabolism are among the most common diseases of humans, encompassing a broad spectrum of pathological conditions, ranging from anaemia to

neurodegenerative conditions (Gurzau et al., 2003). Dietary iron is available in two forms: heme and non-heme. The most important sources of heme iron are hemoglobin and myoglobin derived from consumption of meat, poultry, and fish, while non-heme iron is abundant in cereals, legumes, fruits, and vegetables (FAO/WHO, 2001). Heme iron is highly bioavailable (15-35%) and dietary factors have little effect on its absorption, while non-heme iron absorption is much lower (2-20%) and strongly influenced by the presence of other food components (Abbaspour et al., 2014). Heme constitutes 95% of functional iron in the human body. Its deficiency can cause serious diseases in humans, including anaemia and porphyria. Moreover, heme is involved in regulation of several neuronal genes, and alterations in its metabolism are associated with Alzheimer's disease (Hooda et al., 2014). Meat and meat products can contribute up to 18% of iron daily requirements (de Castro Cardoso Pereira and dos Reis Baltazar Vicente, 2013). Poultry meat is a good source of this mineral (100 g of chicken thighs provide 1.4 mg of iron, compared to 1.3 mg for an equal amount of rump steak from an adult bovine) (Marangoni et al., 2015).

Selenium is a trace element of essential importance in human biology. As selenocysteine, the 21st aminoacid, selenium is a component of selenoproteins, some of which have important enzymatic functions. Additionally, selenium has important health effects related to the immune response and cancer prevention (Rayman, 2000). The main source of selenium intake is the diet. The total amount of selenium in the diet depends on the food type and composition. The most important foods which provide selenium are bread and cereals, meat, fish, eggs, and milk/dairy products (Roman et al., 2013). The selenium content of meat depends on many factors. Offals contains relatively high levels of selenium, in particular liver and kidneys (Fairweather-Tait et al., 2011). Poultry meat is an excellent source of this microelement and contains one of the highest average levels of selenium (0.14 mg/kg) (Sneddon, 2012).

In the same way, zinc is another trace element of essential importance in human health. It has critical effect in homeostasis, in immune function, in oxidative stress, in apoptosis, and in aging, and significant disorders of great public health interest are associated with zinc deficiency (Chasapis et al., 2012). In developed countries, meat provides 40-60% of the dietary zinc. On the contrary, in developing countries, pulses and cereals represent the major source of this element (Nriagu, 2007).

Meat has also an important role in promoting the bioavailability of nutrients found in other foods when consumed concurrently. As an example, the absorption of non-heme iron contained in other foods is increased when they are consumed with meat (Marangoni et al., 2015).

8.3 Technological properties

8.3.1 pH

The pH measurement of meat is widely used to assess the shelf life and quality of the carcass in meat inspection and in meat industry (Mäki-Petäys et al., 1991). pH is one of the most important qualitative attribute of meat that has a key role to determine the protein behavior in fresh and processed meat, and is defined as the negative log of the hydrogen ions concentration (Lonergan, 2008). pH affects important meat characteristics, especially tenderness, water holding capacity and color (Poulanne et al., 2002).

Muscle pH values are mainly affected by the amount of glycogen stored in the muscle at the moment of slaughter (Haslinger et al., 2007). Glycogen is a polysaccharide of glucose that serves as a form of energy storage both in humans and animals. Quantitatively, the liver and muscle, account for most of the body's glycogen stores. As a store of carbohydrate, glycogen plays an important biochemical role for the homeostatic control of blood glucose and energy balance of animals (Pethick et al., 1995). In resting muscle, the content of glycogen is 100 mmol/kg wet weight or more, expressed as glucose. The content varies greatly depending on animal species, muscle type, feeding, and levels of stress and exercise (Poulanne et al., 2002).

The glycogen concentration in muscle is a multifactorial situation that is difficult to predict. It is highly variable and depends on several factors, such as the type of muscle fibers, the species, the sex of animal, genetics, nutritional state and the level of stress of the animal in the period prior to slaughter. Additionally, there are also post-slaughter factors that influence final pH, such as the packaging and the freezing of meat (reviewed in Hargreaves et al., 2009). Metabolism of muscle glycogen plays the primary role in the conversion of muscle to meat and the expression of different quality attributes (Lonergan, 2008). After death, the circulation is stopped and demands for the oxidative metabolic pathways can no longer be met. ATP necessary for normal physiological processes can then only be generated by anaerobic glycolysis. Because of the unavailability of oxygen, the breakdown of glycogen results in lactic acid accumulation and consequently in declining pH, in a process called muscle acidification (Paredi et al., 2012). In poultry, to determine the intensity of acidification fall, pH is measured at 24 hours after death of animals and this is called ultimate pH (pH_u). However pH_u can be already measured in breast meat after 6-8 hours *post mortem* under commercial chilling conditions (Petracci and Baéza, 2009).

The *post mortem* decline in muscle pH is due to an accumulation of lactic acid as a result of glycolysis (Owens and Sams, 2000). The conversion process of glycogen to lactic

acid takes about 48 hours for completion. If the level of glycogen is adequate (about 40-50 mmol glucose/kg muscle), the pH_u of muscle reaches 5.5 after 48 hours (Pethick et al., 1995). Poor nutrition and stress can reduce muscle glycogen content at slaughter, resulting in elevated pH_u (slow pH decline), which, when higher than pH 5.8-5.9, can result in dark, firm and dry meat (DFD) (Gardner et al., 2005). When the pH of meat is above the isoelectric point of myofibrillar proteins, water molecules are tightly bound, causing more light to be absorbed by the muscle, and meat appears darker in color (Saláková et al., 2009).

When the pH decline is rapid, the combination of high temperatures and low pH can result in denaturation of proteins, that are responsible for binding water, influencing texture and confer color. The result is a very light color, soft texture and poor water holding capacity. This condition is known as pale, soft and exudative meat (PSE) (Lonergan, 2008). Low pH_u reduces the importance of myoglobin in selectively absorbing green light, resulting in meat that appears less red and more yellow (Saláková et al., 2009). Higher muscle pH is further from the isoelectric point of the contractile proteins, therefore the proteins are more functional, resulting in lower cook losses (Owens and Sams, 2000).

As a consequence of slaughter, glycogen level in the muscle decreases, and thus, also the available energy to keep the muscle in a relaxed form; additionally, the release of calcium consequent to acidification of muscle, leads to cross-bridges being formed between myosin and actin filaments. The combination of these factors, resulting in the emergence of *rigor mortis,* which determines significant alterations of the energy metabolism, that lead to a further pH decline and a simultaneous decrease in muscle flexibility (Paredi et al., 2012).

The residual glycogen improves meat quality in several ways. It can improve microbiological quality, since the bacterial population utilises glycogen as an energy source rather than protein. Moreover, the residual glycogen is thought to undergo browning reactions with protein during the cooking process, and so it could contribute to flavour (Pethick et al., 1995).

8.3.2 Color

Color is an important quality attribute that strongly influences consumer acceptance of different food products, including poultry meat (Qiao et al., 2001). Meat purchasing decisions are influenced by color more than any other quality factor because consumers use discoloration as an indicator of freshness and healthiness (Mancini and Hunt, 2005).

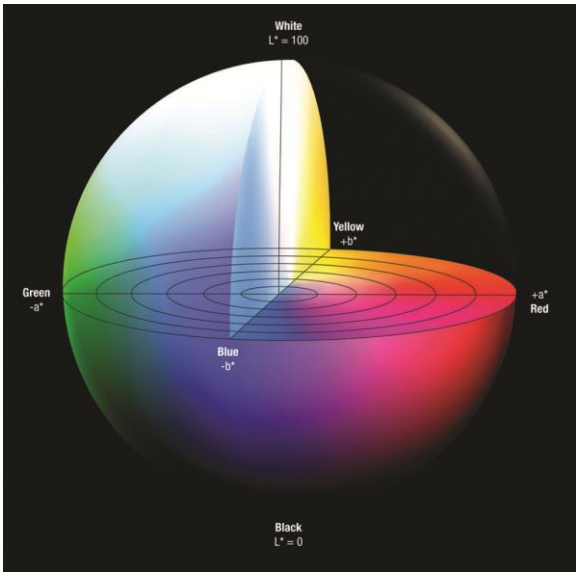
Meat color measurement is very important for many reasons: to acquire information on customers' evaluation, to better understand preferences and reactions to different shades of color, to investigate chromatic changes, and to determine its causes and evolution over time.

Moreover, the possibility of using color measurements to predict the functional properties of poultry meat has been suggested by several studies (Guidi and Castigliego, 2010).

Meat color can be measured subjectively or objectively. Subjective measures are generally taken by people trained to do it. The main problems of this kind of measures arise from the fact that the methods utilized sometimes differ from country to country, and, additionally, the color perceived by the human eye, is a sensorial experience, and therefore, for this reason, the evaluation of this parameter may be subject to bias.

Concerning objective measurements, actually, the most commonly used colorimetric scale is the CIEL L^*, a^*, b^* (International Commission on Illumination, 1986), nevertheless other color scales can be used, such as the Hunter L,a,b and YXZ space.The CIELAB color scale is organized in a cube form, a sort of tridimensional space in which the color can be collocated and relies on three color parameters, expressed as L^*, a^*, b^* (Figure 8.6).

Figure 8.6 Representation of color solid for CIE L^* a^* b^* color space (*Source*: Hunt and King, 2012).

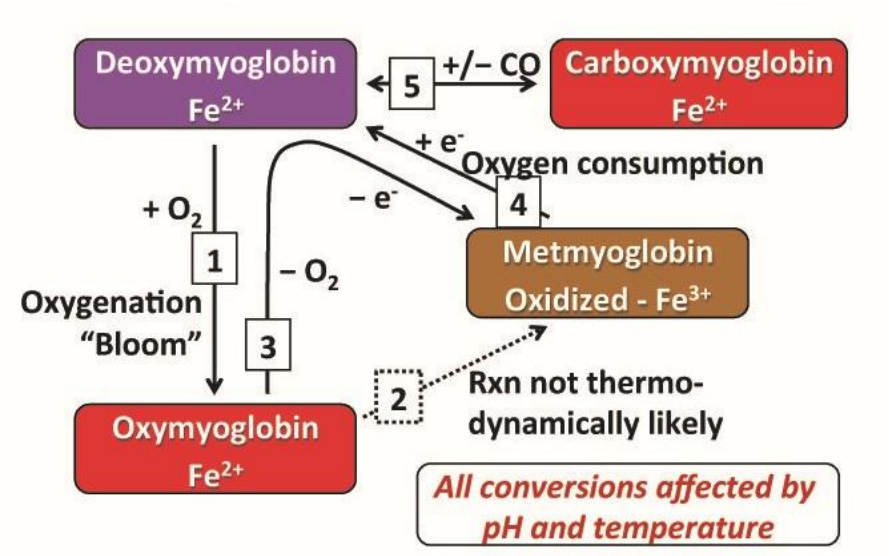


L^* expresses the lightness, with 0 associated with black (complete absorption of light) and 100 with white (complete reflection). a^*indicates the level of redness (or greenness, since the two colors are complementary), ranging from -60 (pure green) to 60 (pure red). Finally, b^*indicates yellowness (or blueness), also ranging from -60 (pure blue) and 60 (pure yellow). Hue angle is the arctangent of b^*/a^*, determined by rotation about the $a^*$and b^*axes and defines the color. The color intensity (chroma) is defined as $(a^{*2}+b^{*2})^{-2}$ (Guidi and Castigliego, 2010). Moreover, is important to underline that currently, several instruments (colorimeters and spectrophotometers) are available to determine the objective measure of meat color (Hunt et al., 2012).

According to the studies carried out on broiler chicken meat quality, ideal values of lightness (L*) should be between 46 and 53 (Barbut, 1997; Zhang and Barbut, 2005). Redness (a*) values for broiler chicken breast meat ranging between − 0.96 and 4.50, and yellowness (b*) values were in the range of 6.7 to 13.5 (reviewed in Narinc et al., 2013). According with Guidi and Castigliego (2010), the color of meat depend primarily on: (i) the type and quantity of pigments present in the muscle; (ii) the types of fibers composing the muscle and their spatial relationships, which determine the scattering grade of light and thus its deepness of penetration; (iii) the intramuscular fats and surface dehydration, which confer different degrees of glossiness and thus affect light scattering and reflection.

Myoglobin is the main responsible for meat color, although other heme proteins such as hemoglobin and cytochrome C may also play a role in beef, lamb, pork and poultry color (Mancini and Hunt, 2005). Myoglobin is a water-soluble protein containing 8 α-helices (A–H) linked by short non-helical sections. Within the 8 α-helices of myoglobin, a prosthetic heme group containing a centrally located iron atom, which is positioned in the protein's hydrophobic core. Of the six bonds associated with this iron atom, four connect iron to the heme ring, the 5th attaches to the proximal histidine-93, and the 6th site is available to reversibly bind ligands including diatomic oxygen, carbon monoxide, water, and nitric oxide. The ligand present at the 6th coordination site and the valence state of iron, determine meat color via four chemical forms of myoglobin: deoxymyoglobin (DMb), oxymyoglobin (OMb), carboxymyoglobin (COMb), and metmyoglobin (MMb) (Figure 8.7).

Figure 8.7 Schematic representation of the chemistry of myoglobin (*Source*: Hunt et al., 2012).



Deoxymyoglobin results in a dark purplish-red or purplish-pink color typical of the interior color of fresh meat and that in vacuum packages. Deoxymyoglobin contains ferrous

(Fe2+) iron with a vacant (no ligand attached) 6^{th} coordination site. To maintain DMb, very low oxygen tension (< 1.4 mmHg) within vacuum packages or the interior of muscle is necessary. Oxygenation of DMb forms a bright-red color via the formation of OMb, which has diatomic oxygen attached to the 6^{th} coordination site of ferrous iron (Fe2+). The oxygen ligand also interacts with the distal histidine-64, producing a more compact protein structure than DMb, which has no ligand present to link iron to the distal histidine. Carboxymyoglobin formation occurs when carbon monoxide attaches to the vacant 6^{th} position of DMb, producing a stable bright-red color when the environment is devoid of oxygen. Atmospheres containing oxygen (albeit concentration dependent) will result in the conversion of carboxymyoglobin to either OMb or MMb. Metmyoglobin is the oxidized tan to brown colored form of myoglobin and it contains ferric iron (Fe3+). Typically, MMb forms easily at low concentrations of oxygen (< 7 mmHg or about 1 to 2% oxygen). Water is the ligand at the 6^{th} position of the iron in MMb (Hunt et al., 2012).

The level of myoglobin within a muscle is influenced by species, muscle function, and age of the animal. The state of iron and what compound is bound to the myoglobin ligand is mainly affected by storage conditions of meat. As myoglobin content increases, color intensity of meat increases from white or pink, to very dark red. The higher myoglobin content in beef, differentiates it from the lighter color of pork or poultry meat that has a lower myoglobin content. Moreover, muscles within a species and a carcass can also vary in color. Muscles vary in myoglobin content based on the physiological role of the muscle. High use muscles, such as the leg muscle in chicken and other species, have higher myoglobin content due to the need for this protein to store and deliver oxygen in the muscle. Myoglobin content also increases as animals increase in age, so that meat from older animals is darker than meat from younger animals (Miller, 2002).

Other important factors that influence poultry meat color include the sex and age of the bird, and the strain (Fletcher et al., 2000). Additionally, *ante mortem* stress, many *post mortem* conditions (method of immobilization, several cooler parameters affecting rate of chilling, carcass spacing and alignment, scalding and singing, criteria for carcass electrical stimulation, and application of antimicrobial interventions), *postmortem* processing and packaging methods can influence meat color (Hunt et al., 2012). Moreover, has been reported that meat thickness as well as the color measurement position on the fillet have also been shown to affect meat color values (Bianchi et al., 2005). Color it is also the next parameter often used as an indicator of PSE and DFD meat (Owens and Sams, 2000). Barbut (1997) reported correlations between color and most of the physical measurements. This observations suggested that a rapid color measurement method could be used to recognise meat defects or

identify flocks with a high incidence of PSE and DFD meat. Lightness (L^*) of breast muscle can be used in the technological evaluation of meat, to detect pre-slaughter or processing effects with a good reliability with different genetic strain (Saláková et al., 2009). Some authors divided chicken breast meat in three groups according to the level of lightness: darker than normal ($L^* < 46$), normal ($48 < L^* < 53$), and lighter than normal ($L^* > 53$), and showed that each group was associated with a precise pH, which averaged around 6.23, 5.96, and 5.81, respectively. It has been demonstrated that excessively fast lowering of pH, associated with inadequate temperatures, determines the premature precipitation of muscle proteins, which tend to aggregate, causing water loss and the detachment of myofibrils from the cell membranes (reviewed in Guidi and Castigliego, 2010).

8.3.3 Water holding capacity (WHC)

The average water content of the muscle is approximately 75%. Water is a dipolar molecule and therefore is attracted to charged species like proteins. In muscle, water can be found in three different forms: 1) bound water, (2) entrapped water, and (3) free water. By definition, bound water is water that exists in the vicinity of non-aqueous constituents (like proteins) and has reduced mobility (e.g., does not easily move to other compartments). This water is very resistant to freezing and to being driven off by conventional heating. Another fraction of water that can be found in muscles and in meat is termed entrapped (also referred to as immobilized) water. This water is held within the structure of the muscle but is not bound per se to proteins. In early *post mortem* tissue, this water does not flow freely from the tissue, yet it can be removed by drying and can be easily converted to ice during freezing. Entrapped or immobilized water is most affected by the rigor process and the conversion of muscle to meat. Free water is water whose flow from the tissue is unimpeded. Weak surface forces mainly hold this fraction of water in meat. Free water is not readily seen in pre-*rigor* meat, but can develop as conditions change that allow the entrapped water to move from the structures where it is found (Huff-Lonergan, 2010).

Fluid lost from fresh meat through passive exudation is referred to as muscle exudate or drip. The amount of muscle exudate is an indicator of water holding capacity (WHC), which refers to the ability of the uncooked meat to retain its inherent or added water through *post mortem* processing and storage (Honikel and Hamm, 1994). WHC is an important meat quality attribute which has a strong influence on product yield, which in turn has economic implications (Cheng and Sun, 2008). WHC of uncooked poultry is also very important because it influences sensory characteristics of the cooked product (Lesiak et al., 1996). Has been reported that WHC is significantly correlated with muscle pH, indicating that lower cook

losses are associated with higher muscle pH and better proteins functionality. WHC is one of factors used for evaluating PSE meat (Woelfel et al., 2002).

According with den Hertog-Meischke et al. (1997), the WHC of meat is influenced by a wide range of factors, both *pre-* and *post mortem*. These include physiological factors, such as species, breed, sex and age; rearing conditions, especially feeding patterns, administration of growth promoters, and activity prior to slaughter. In the immediate pre-slaughter period, stresses on the animal such as fasting and different stunning methods are likely to influence WHC (Cheng and Sun, 2008). Moreover, other factors regarding further processing, such as chilling rate, packaging, temperature of *post rigor* storage, freezing and thawing, can have an influence on WHC (den Hertog-Meischke et al., 1997).

Determination of WHC is generally subjected to very large variations, which is either due to methodological error or to the existence of large intra-muscular variations (Botka-Petrak et al., 2005). One method used to determine WHC is expressible moisture (Woelfel et al., 2002). The most widely employed method for its determination is the filter-paper press technique first employed by Grau and Hamm (1953).

Drip loss is another method to measure WHC. Drip loss that occurs in fresh poultry could be water from the muscle cell or water that was picked up during water immersion chilling (Lesiak et al., 1996). A third parameter used to evaluate WHC is cook loss, which is evaluated by measuring percentage weight loss during cooking. Cook loss is a very complex parameter that may be affected by many factors with the relative importance of L* value and pH changing from 3 to 24 hours *post mortem*. Has been reported, that when L* value increased, cook loss also increased (Woelfel et al., 2002).

8.4 Sensory attributes of poultry meat

Sensory analysis is unequivocally assigning in the scientific methods. It is one of the oldest way of quality control, but in principle is an essential part of the mandatory assessment of food quality (Haščík et al., 2011). Sensory panels provide complementary information to instrumental method, and neither can be replaced. For instance, instruments do not account for the moisture-related characteristics (e.g., juiciness) that evaluators may perceive while chewing. Moreover, between sensory panel evaluations and instrumental measurements might exist relationships. Has been established a range of instrumental shear force values corresponding to different portions of the consumer texture scale (Liu et al., 2004).

Many authors reported that the sensory analysis, allowing producers to identify, understand and respond to consumer preferences more effectively (Haščík et al., 2011). Additionally, the identification of sensory characteristics and consumer preference helps

industry producers to segment their market and to increase their competition strengths. For example, with the increasing popularity of the consumption of meat from animals raised in organic farms, more and more research is aimed at examining the differences between birds from the intensive and extensive system. In one of this kind of study, the sensory attributes analysis showed that there were great differences between the modern chicken and traditional chicken. The sensory analysis showed that the traditional live village chicken was characterized by sensory attributes such as yellow colour, hard meat, oily meat, and sweet, while the ready-to-cook broiler (imported) was characterized by white color, chewy meat, intense odour, and smooth (Sow and Grongnet, 2010). But results of another experiment indicate that chicken breast meat from an organic source does not "taste better" than chicken breast meat from a conventional source. These findings may be of particular interest to consumers who pay premiums of 120-180% over conventional prices for organic chicken meat (Lawlor et al., 2003).

The two most important sensory quality attributes for poultry meat are appearance and texture. Appearance is critical for both the consumers' initial selection of the product as well as for the final product satisfaction. Appearance quality attributes include skin and meat color, cooked meat pinkness, and appearance defects such as bruises and haemorrhages. Since appearance is so critical for consumer selection, poultry producers go to great lengths to produce products with the appropriate color for a particular market and to avoid appearance defects which will affect negatively product selection or price. Meanwhile texture is the single most important sensory property affecting final quality assessment (Fletcher, 2002). Texture is a sensory property and thus, only a human being (or an animal in the case of animal food) can perceive and describe it. Texture it is a multi-parameter attribute, not just tenderness or chewiness, but a range of characteristics (Surmacka Szczesniak, 2002). Meat texture sensation is dictated by the presence of several factors including the amount of intramuscular fat (Nishimura el al., 1999), WHC and actomyosin complex. Moreover, Wattanachant (2008), reported that age and genetic strain are also two inherent factors that affect texture. Moreover, texture is one of the tactile senses influencing the perception of tenderness, which is also a major quality determinant and has been described as the most important sensory characteristic of meat (Cavitt et al., 2004). Tenderness depends upon a number of factors that can be controlled pre- and post-slaughter. In addition to these factors, there is evidence that tenderness can also be affected by variation in muscle structure (e.g., myofibril and connective tissue architecture, sarcomere length, muscle anatomical location) (Chang et al., 2010). Instrumental determination of texture, or tenderness, is usually evaluated on intact pieces or cores large enough to ensure representative sampling of the muscle so that treatment

effects can be accurately measured. The majority of the instrumental data used to determine tenderness in cooked intact poultry meat have been generated on the Warner-Bratzler, Tensile tests or the Kramer Shear Press (Petracci and Baéza, 2009).

8.5 Safety aspects of poultry meat

The safety aspects and quality of poultry meat are equally important to producers, retailers and consumers, and both involve microbial contaminations on the processed product (Mead, 2004). The terms foodborne diseases or foodborne illnesses, or even food poisoning, are used to describe gastrointestinal complications that occur after recent consumption of a particular food or drink (Dhama et al., 2013).

Poultry meat can be contaminated by a variety of foodborne pathogens that may cause human illnesses following ingestion, due to the handling of raw meat, undercooking or mishandling of the cooked product. In terms of specific pathogens that contribute substantially to foodborne illnesses, *Campylobacter* and *Salmonella* species are prevalent in poultry, cattle, swine, and sheep, whereas enterohemorrhagic *Escherichia coli* O157:H7 is a major concern in cattle and sheep and *Yersinia enterocolitica* in swine (Doyle and Erickson, 2006). Others pathogens, include the more recently reported *Arcobacter* and *Helicobacter* spp. and, occasionally, verotoxigenic *Escherichia coli* (Mbata, 2005). Numerically, the most important agents are *Salmonella* and *Campylobacter* spp. Data from European Union show that in 2001, there were 157.822 reported cases of human salmonellosis and 156.232 cases of *Campylobacter enteritis*. Contamination of poultry carcasses and parts with these organisms is well documented, and data are available from many parts of the world, but inter-country comparisons are not usually possible, because of differences in sampling and methods of testing (Mead, 2004).

8.5.1 Salmonella

The genus *Salmonella* belongs to the family *Enterobacteriaceae*. *Salmonellae* are facultative anaerobic, Gram-negative, oxidase-negative, rod-shaped bacteria (Plym Forshell and Wierup, 2006). These microorganisms are one of the major causes of food borne illnesses in humans, and there are more than 2450 serotypes of *Salmonella*. Food borne salmonellosis, also known as non-typhoidal salmonellosis or enterocolitis, results in gastroenteritis, which is caused by ingestion of more than 150 serotypes, but *S. Typhimurium* and *S. Enteritidis* are the more common (Dhama et al., 2013). Some serotypes of *Salmonella*, such as *S. Enteritidis*, have specific animal reservoirs and are mainly transmitted by specific foods (Altekruse et al.,

1997). Transmission occurs by ingestion of water and food contaminated with animal feces and also from contaminated food processing equipments. *Salmonellae* are widely distributed in nature. The main reservoir of these bacteria is the intestinal tract of men and warm- and cold-blooded animals. Among warm-blooded animals, chickens, geese, turkeys and ducks are the most important reservoirs (Castiglioni Tessari et al., 2012).

It was concluded that the main food categories that possibly pose the greatest hazard to public health include: (i) raw meat and some meat products intended to be eaten raw; (ii) raw or undercooked poultry meat products; (iii) eggs and products containing raw eggs; (iv) unpasteurised milk and some milk products (Plym Forshell and Wierup, 2006). Poultry and poultry products are considered as a considerable source of these bacteria (Dhama et al., 2013). Epidemiological investigations in Hungary, United Kingdom, United States and Germany confirmed that the food most associated with increased illness in people was the egg (reviewed in Guard-Petter, 2001).

In animals, the serovars that were initially observed to cause diseases, were found to be adapted to specific animal species, such as: (i) sheep (*S. abortus ovis*); (ii) pigs (*S. cholerae suis*); (iii) poultry (*S. gallinarum*); (iv) horses (*S. abortus equi*); (v) cattle (*S. dublin*). These serovars cause disease in the species to which they are adapted and are considered less pathogenic to people (Plym Forshell and Wierup, 2006).

Birds can became infected through fecal-oral transmission. In case of newly hatched chicks, colonization can also take place via the nose or cloaca. Vertical transmission of *Salmonella* has been reported in infected ovaries, oviducts, or infected eggs, and these kind of infections may be asymptomatic in adult birds (reviewed in Foley et al., 2011). Concerning poultry, skin and feathers contaminated by feces, serve as the main sources of contamination. During processing, the loss of digesta and feces from the crop or cloaca can contaminate the surrounding tissues, and therefore the whole carcass. During production, carriage can also represent a problem considering the fecal shedding (Doyle and Erickson, 2006).

Salmonellosis is an important socioeconomic problem in many countries, especially in developing countries, where this etiological agent is reported as the main responsible for foodborne disease outbreaks. It is one of the most problematic zoonosis in terms of public health worldwide because of the high endemicity, but mainly because of the difficulty in controlling it, and the significant morbidity and mortality rates (Castiglioni Tessari et al., 2012). There are several factors which contributing to the emergence of food borne diseases such as salmonellosis. As an example, changes in industry and technology, changes in travel and commerce, and the microbial adaptation to environmental conditions, have contributed to the increase of outbreaks (Altekruse et al., 1997). A special mention, deserve issues related to

the development of antibiotic resistance. The massive use of antimicrobial agents in food animal production has contributed to the occurrence of resistant bacteria in animals, including zoonotic pathogens, which can be transmitted to humans via the food chain (Antunes et al., 2003). The increasing incidence of resistance among bacteria, represent a critical problem which has serious consequences for the treatment and prevention of infectious diseases both in humans and animals (Carramiñana et al., 2004).

Prevention and control programs for infections caused by *Salmonellae* mainly aiming to protect the health of the birds, ensure the safety of the consumers, and strengthen the reliability of the poultry production chain (Castiglioni Tessari et al., 2012). In production, control of *Salmonella* within broiler flocks relies on knowledge of the source of infection. Possible sources include water, feed, litter, farm staff and the environment, both inside and outside the broiler house (FAO/WHO, 2009). The prevention of foodborne diseases depends on careful food production, handling of raw products, and preparation of finished foods. Hazards can be introduced at any point of the production chain (Altekruse et al., 1997). Generally, to minimize the spread of infective agent, optimal hygiene and management routines are of major importance. Improvements in hygiene and management procedures must be continually implemented as a natural part of *Salmonella* control (Plym Forshell and Wierup, 2006). Moreover, the control of bedding material and adopting techniques aimed to eliminate pathogens from water are also important measures (Doyle and Erickson, 2006). The use of competitive exclusion, in which the normal intestinal flora protects the host against invading pathogens, is a valuable part of *Salmonella* control in poultry farming (Plym Forshell and Wierup, 2006). Several studies have shown the usefulness of probiotics and prebiotics in reducing the incidence of *salmonella* infections (reviewed in Doyle and Erickson, 2006).

8.5.2 Campylobacter

Campylobacters belong to *Campylobacteriaceae* family. This family comprises small (0.2-0.9 µm wide and 0.2-5.0 µm long) spiral formed Gram-negative bacteria with 18 species, 6 sub-species and 2 biovars. Compare to other pathogens associated with foodborne illnesses, these bacteria are essentially microaerophilic, with an optimal growth rate in an atmosphere with about 10% CO_2 and approximately 5% O_2. The pathogenic species for humans have a quite strictly temperature range for growth (maximum temperature of about 46°C and a minimum of 30°C) (Humphrey et al., 2007). *Campylobacter* is one of the most important cause of zoonotic enteric infections both in developed and developing countries. The acute infection can have serious long-term consequences, including peripheral neuropathies,

Guillain-Barré syndrome and Miller Fisher syndrome, and functional bowel diseases, such as irritable bowel syndrome. In many countries, the organism is isolated 3-4 times more frequently from patients with alimentary tract infections than other bacterial enteropathogens, such as *Salmonella* or *Escherichia coli* (WHO, 2013). The major routes of transmission in humans are consumption of contaminated or undercooked meat (especially poultry), unpasteurized milk or dairy products, and untreated water. People can also be infected by contact with infected animals or feces (Leedom Larson and Spickler, 2013). Food borne campylobacteriosis is often associated with *C. jejuni* (frequently isolated from chickens) and, to a lesser extent, with *C. coli* (often found in pigs). The main reservoir of pathogenic *Campylobacter* spp. is the alimentary tract of wild and domesticated mammals and birds. *Campylobacter* is commonly found in broilers, cattle, pigs, sheep, wild animals and birds (Schlundt et al., 2004). *Campylobacter* shed in faeces from the GIT are able to survive for considerable periods in the environment, but are not known to grow under those conditions. Survival is enhanced by cool moist and dark environments. As many mammals and birds are known hosts for *Campylobacter*, which can be asymptomatically excreted in significant numbers, then the environment (soil, water, pasture, etc.) must be frequently contaminated with this organism (FAO/WHO, 2009).

Chicken is an important source of human infection by campylobacters. The intestines of poultry are easily colonized with *C. jejuni*. One day old chicks can be colonized with as few as 35 organisms. Most chickens in commercial operations are colonized by 4 weeks. Vertical transmission from parents to progeny has been suggested, but is not widely accepted (reviewed in Altekruse et al., 1999). Birds can became infected through different routes. Among these, the external environment (e.g., the presence of other domestic and/or wild animals), contaminated water, transport, and carry-over from a previous flock, are important factors (Humphrey et al., 2007).

Considering that this microorganism is ubiquitous, it is difficult to prevent colonization and infection in poultry. Generally, the implementation of strict biosecurity measures is recommended (Leedom Larson and Spickler, 2013). Biosecurity in pre-harvest interventions includes: (i) access control to minimize access by unauthorized personnel, birds, rodents etc; (ii) farm worker control (e.g., hygiene barriers that require footwear changes before entering poultry house); (iii) drinking-water sanitation (e.g., chlorination or organic acids); (iv) bedding material/floor litter source, change between flocks, treatment (type, reuse, etc.); (v) wildlife and rodent control; (vi) cleaning and disinfection of entire house and equipment between flocks (WHO, 2013). Cross-contamination can be reduced by cleaning and decontamination of transport crates and other equipment used at slaughter. Because the

surface of carcasses can become contaminated during the slaughter process, methods to decrease the contamination of meat post-slaughter are also very important (Leedom Larson and Spickler, 2013). Other specific interventions that have been successful used in research, but have not yet been shown to be effective in commercial contexts as a single therapy include the use of bacteriocins, bacteriophages, competitive exclusion, organic and inorganic acids in feed or drinking-water, essential oils in the drinking water prior to processing, vaccination, and breeding for genetic resistance (WHO, 2013).

Chapter 9
Defects and myopathies of poultry meat

In comparison with beef and pork, chicken meat is characterized by a lower concentration of fat and cholesterol. Additionally, it contains a high degree of unsaturated fatty acids, with a balanced n-6 to n-3 polyunsaturated fatty acid ratio (reviewed in Zheng et al., 2015). As a consequence of the healthy image of this product, the growing demand from consumers for poultry meat has resulted in pressure on breeders, nutritionists and growers to increase the growth rate of animals, feed efficiency, and size of breast muscle (Petracci and Cavani, 2012). Nowadays, chickens are marketed in about half the time and at about twice the body weight compared to 50 years ago (Barbut et al., 2008). The achievement of a high body weight within a short period of time can cause various meat quality problems (reviewed in Tijare et al., 2016). These defects are usually caused by changes in the biochemistry and morphology of muscles, as well as by *post mortem* events. Among the main meat defects, Pale, Soft and Exudative (PSE) and Dark, Firm and Dry (DFD) meats, are two of the major quality problems facing the meat industry. These defects reduce consumer acceptability, shelf life and yield of meat, thus, affecting profits tremendously. Among the main predisposing factors contributing to PSE and DFD in meats are breed, sex, species, pre-slaughter and post-slaughter handling of animals (Adzitey and Nurul, 2011). In recent years, it has been shown that different species of birds, including waterfowl, may be characterized by DFD and PSE type meat (Okruszek et al., 2008). Several studies, have shown that the fast growing meat-type hybrids exhibit a high incidence of different myopathies (e.g., deep pectoral muscle disease) and meat quality problems associated with toughness and poor cohesiveness (reviewed in Petracci and cavani, 2012). Moreover, because of the rapid growth rates, emerging myopathies such as white striping and wooden breast, are the cause of a serious worsening of the visual appearance of meat and remarkable economic losses for the poultry industry (Kuttappan et al., 2012a, b; Petracci et al., 2014).

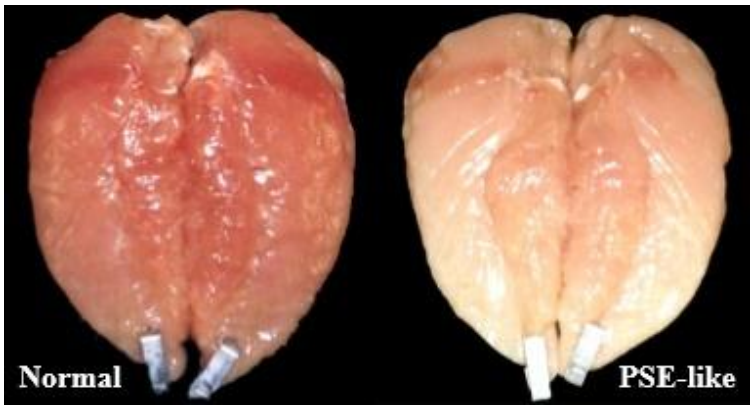
9.1 PSE-like broiler breast meat

The intense genetic selection for rapid muscle growth in poultry industry, has resulted in the appearance of some defects, such as poorer WHC of breast muscle during processing and storage. The loss in functionality of the poultry breast meat is often associated with an

increase of the meat paleness so that this condition is often referred as pale, soft, and exudative (PSE)-like (Petracci et al., 2009).

The PSE meat defect is one of the most frequent challenges to the meat industry associated with the intensive selection (Petracci and Cavani, 2012). PSE syndrome has been described for the first time mainly in pork, since the beginning of the 60's. In the 90's, more recently, PSE syndrome has been described in poultry, essentially in turkeys and chickens (Remignon et al., 2007). Researchers working with poultry meat have described breast meat that appears lighter than normal (Figure 9.1) with lower ultimate pH and water holding capacity, obviously similar to what has been described in swine. Those observations led to adoption of the PSE term for this particular poultry muscle defect (Smith and Northcutt, 2009).

Figure 9.1 Normal and PSE-like broiler breast meat (*Source*: Petracci and Cavani, 2012).



Incidence of PSE in poultry breast muscles may be a consequence of an accelerated rate of muscle glycolytic metabolism before slaughtering due to stress-related genetic factors. In pale turkey muscles, the rate of glycolysis is nearly two times higher than in normal muscles (Lesiow and Kijowski, 2003). Consequently, a low muscle pH value will be encountered, while muscle temperature is still high, leading to a final combination of low pH and high temperature values (Galobart and Moran, 2004; Remignon et al., 2007). The very serious defect of PSE meat is the drip loss. In this type of meat, water is not closely bound to proteins, and cell membranes are very permeable. The cause of drip loss is the protein denaturation (Garcia et al., 2010). Moreover in meat with PSE defect, the reduction of the normal red color, is the effect that at high scattering the amount of absorbed light is low and the haem pigments selectively absorbed green light thus reducing the normal red color. This makes PSE meat less red and more yellow (Adzitey and Nurul, 2011).

Indirect and direct causes of defect PSE have been the subject of many studies, mainly in Europe. They can be divided into two categories: 1) studies focusing on the role of

intensive genetic selection of poultry; 2) studies taking into account primarily environmental factors. As for genetics, it has been shown that selection for body weight or muscle development has induced histological and biochemical modifications of the muscle tissue, which can be related with PSE-like condition (Petracci et al., 2009). French researchers studied the genetic correlation between the quality of meat and broiler utilization traits, reported that the selection of birds due to the development of muscle and body weight did not change the pH of the meat, but it affects the intensity of meat color (Le Bihan-Duval et al., 2001). According to scientists, the impact of genetic selection is not clearly defined and still exist need for further research in order to verify the impact of changes in birds genotypes on meat quality (Petracci et al., 2009).

Among the environmental factors affecting the formation of PSE meat defect, the most significant is the stress *ante mortem*. Exposing animals to acute stress just before slaughtering leads to PSE. Acute or short term stress that can lead to PSE include the use of electric goads, fighting among animals just before sticking, beating of animals prior to slaughter and overcrowding in the lairage (Adzitey and Nurul, 2011). Moreover, among environmental factors to induce PSE-like meat occurrence, heat stress during the end of the growing phase or pre-slaughter period seems to play the major role (Petracci et al., 2009). The studies which focused on the influence of the season on the occurrence of meat quality defects, including PSE (McCurdy et al., 1996; Bianchi et al. 2007), show that this kind of meat defect is more common in warm than cold season. Similar results were obtained in a study conducted on turkeys (Bianchi et al., 2004). The meat from turkeys which were slaughtered during summer months was characterized by a lower pH at 15 minutes and 24 hours after slaughter and by a lower WHC compared to birds slaughtered during winter. In addition, there were a significant seasonal differences in the distribution of water in the muscles and the interactions between proteins and water.

9.2 Dark, firm and dry meat

Stress, can be responsible also in the occurrence of dark, firm and dry (DFD) meat defect. When animals are exposed to chronic or long term stress before slaughter this defect of meat can occur. Chronic stress prior to slaughter leads to the depletion of stored glycogen, thus, the less amount of glycogen available *post mortem*, affect the normal process of acidification, leaving a high value of pH. DFD meat is characterized by a high ultimate pH (>6.0) and deficiencies in glucose and glycolytic intermediates (Newton and Gill, 1981). Due to the high pH, lean surfaces act similarly to a dry sponge resulting in increased water binding capacity within the muscle. The muscle appears dark because of higher intracellular water,

which reflects less light. In addition, the high pH actively holds iron in the reduced (ferrous) state. The muscle is firm due to the high WHC, and the surface feels dry as the water is tightly held within the muscle (Miller, 2007).

Research has shown that dark appearance of turkey and chicken muscle is indicative of DFD meat, which in other species is believed to be caused by long-term *ante mortem* stress leading to glycogen depletion in muscle (Mallia et al., 2000). Moreover, DFD meat is of significantly lower quality, having a reduced shelf life and a greater capacity to support microbial growth. The higher pH and the increased WHC are both conducive to microbial growth. Decreased levels of muscle glycogen lead to overall limited amounts of glycogen in the meat that can be converted to lactic acid. Lactic acid bacteria normally grow on meat and compete with spoilage causing bacteria (Miller, 2007). This is directly connected with microbial resistance of meat which under conditions of higher pH is lower. Low pH is not conducive to the development of microbes in muscles, therefore prolonging the shelf life of the product (Allen et al., 1997). The limited amounts of glycogen, allows the anaerobic flora to produce spoilage odours at an early stage (Newton and Gill, 1981).

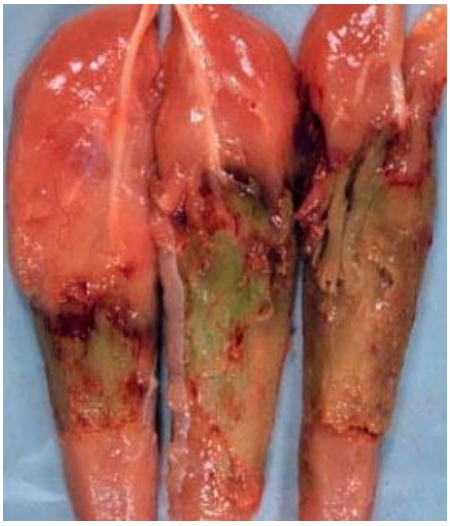
9.3 Deep pectoral myopathy

With the increase in growth rate and muscle size, there has been an increase in incidence of pectoral myopathies (Petracci and Cavani, 2012). As known, in 1950, the fattening period required for acquiring a slaughter weight of 1.8 to 2.0 kg in chickens, was 12 weeks. After just over than four decades, the duration of the fattening period required to achieve the same weight is even less than 6 weeks (Bogosavljević-Bosković et al., 2006). It is assumed that this rapid growth results in histological and biochemical changes in the muscle structure of birds.

Deep Pectoral Myopathy (DPM), also called as the Green Muscle Disease (GMD), or the Oregon disease, was first described in 1968 as “degenerative myopathy” in turkeys, and it was subsequently studied at the Oregon State University. Despite this condition was first recognized in adult meat-type turkey and chicken breeders, it has become more and more common in meat-type growing birds (Bianchi et al., 2006).

This condition 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 (Petracci and Cavani, 2012). The lesions can vary in color, progressing from a pinkish haemorrhagic appearance to a gray-greenish discoloration (Figure 9.2) (Bilgili and Hess, 2008).

Figure 9.2 Deep pectoral myopathy (*Source*: Bilgili and Hess, 2008).



In case of acute lesions, the entire Supracoracoid muscle appears pale and swollen and covered by a fibrinuous, sometimes haemorrhage, membrane. The necrotic tissue is white or salmon pink in color, with only outside edges turning green. Lesions are usually limited to the middle portion of the Supracoracoid muscles. In chronic cases, the middle portion of the muscle is yellow-green in color. When cut, the muscle surface is dry and friable (Bilgili and Hess, 2002). Symptoms characteristic of the necrosis, such as changes in color and texture of pectoral muscles, may be seen by consumers as signs of spoilage and the basis for complaint. Consequently, consumers who purchased a carcass affected by myopathy will probably no longer trust the producer and will refrain from buying their products in the future (Kjowski et al., 2014).

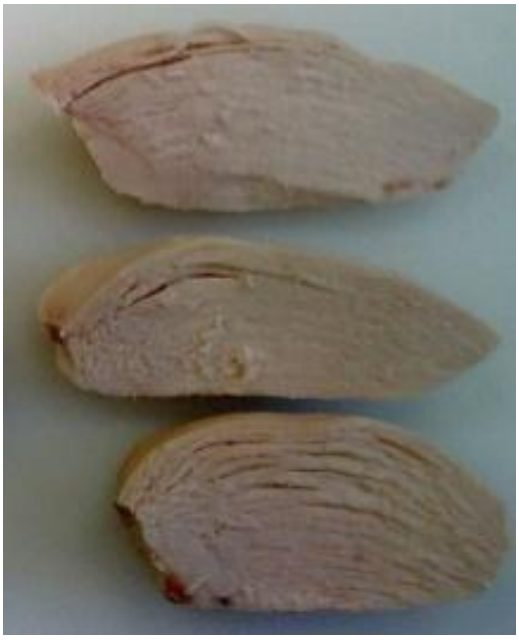
Changes resulting from DPM, proceeds in several stages. Traits of muscles with DPM symptoms in stage I include reddening of muscles with haemorrhages or blood extravasations. In the stage II, the color of muscle is from red turns to green, and in the III stage the color is whitish-grey (Kijowski et al., 2009). Because of this fact, exist possibility that limiting the bird's activity during the growing period may be the cause of this phenomenon in fast growing turkeys and chickens. In the formation of the anomaly, an important role is played by the location of the smaller pectoral muscle in the enclosed space, with limited relaxation potential. This muscle lies in a rigid compartment, defined by the keel on two sides and by a tough inelastic muscle cover (fascia) (Bilgili and Hess, 2002). DPM is due to an ischaemic necrosis of muscle because the fascia does not allows the muscle to expand, thereby resulting in a stoppage of arterial blood flow (Grunder, 1983). Has been reported that the most important parameter assessing muscle color with DPM symptoms is the a^* value. This parameter may take negative values, corresponding to green color; if it is zero, this means grey colour, although a positive value refers to red color (Kijowski et al., 2009). The most

important factors affecting the incidence of DPM, include rearing conditions (welfare of animals), body weight, age, sex and genetic background. DPM is a degeneration caused by human interference. The prevention consists in the limitation of sudden, unnecessary and excessive mobility of the flock by reducing stress factors, and may be achieved by appropriate farm management (Kjowski et al., 2014).

9.4 Poor cohesiveness

The continuing shift in the market from whole birds to further processed products, has highlighted an increasing in meat quality problems associated with the occurrence of meat defects (Dransfield and Sosnicki, 1999) like PSE, DFD, and DPM, but also with intramuscular connective tissue defects, such as poor cohesiveness. This anomaly is an important quality issue in poultry, and is due to the immaturity of intramuscular connective tissue (IMCT) in relation to the very early slaughter age of modern chicken and turkey strains. Structure of mentioned intramuscular connective tissue influence the integrity of muscle fibers.

Figure 9.3 Broiler breast meat with poor cohesiveness (*Source*: Petracci and Cavani, 2012).



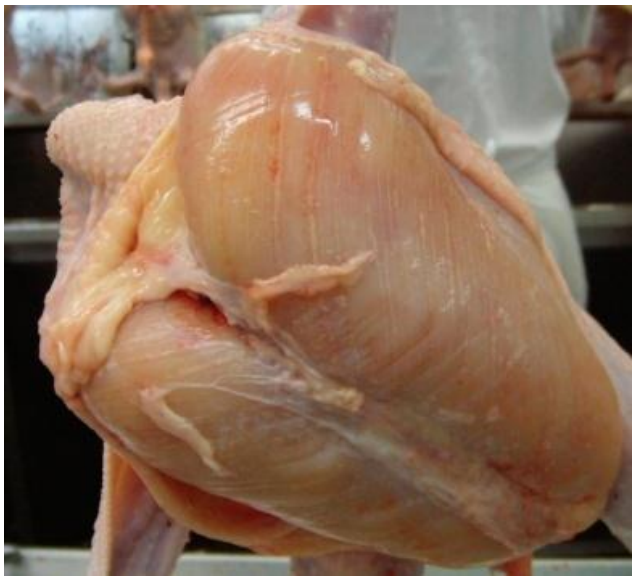
IMCT consist of three layers: endomysium, perymisium and epimysium (Petracci and Cavani, 2012). The structure of IMCT obviously changes as animals grow older and it seems that the increasing thickness of endomysium and perymisium are related to the increasing meat toughness (Voutila, 2009). Moreover, researchers have found that solubility of collagen, the major component of the intramuscular connective tissue, decreases with animal age and varies between muscles and animal species. This is due to the maturation of collagen cross-

links in muscles. Also different proportions of collagen types have an effect on thermal stability of collagen (Voutila et al., 2006). The collagen cross-links determine the physical strength and heat stability of IMCT. The collagen content of lean meat is 0.2-0.4%. In fast-growing birds the collagen is immature resulting in low heat stability. Consequently, poultry meat is tender, but may turn fragile, and mushy. Cooked chicken breast meat (Figure 9.3) results generally fragmented (soft), and raw turkey breast meat is so loose in structure that it is possible to pull the muscle fiber bundles away with the fingers (reviewed in Petracci and Cavani, 2012).

9.5 White striping

An emerging concern regarding chicken breast meat quality, associated with the faster growth rates of the modern meat-type hybrids, is the white striping (WS) defect. WS is described by the appearance of white striation (Figure 9.4) parallel to muscle fiber on the surface of *Pectoralis major* muscles (Petracci et al., 2012).

Figure 9.4 Broiler breast muscle with a severe degree of white striping (*Source*: Ferreira et al., 2014).



A recent study conducted by Petracci and coworkers (2013) has estimated that the incidence of white-striped breast fillets was around 12%. Based on the visual scoring of the severity of condition, fillets can be classified as normal or no striping (NORM), moderate (MOD), or severe (SEV) fillets (Kuttappan et al., 2012a). As known, visual appearance is the most important attribute used by the consumer to assess the quality of meat products. Because WS can be easily identified on the surface of raw chicken breast, the condition can affect the

visual appearance, therefore, reducing the leaning of consumers to buy this type of meat (Kuttappan et al., 2012b).

This defect is represented by a muscular dystrophy which often starts in the cranial part of the breast, near the wing attachment. Although the etiology is unknown, this condition has been directly associated with broiler age, as well as body weight (Ferreira et al., 2014). Other important factors are genotype, sex, diet, and slaughter weight (Petracci et al., 2015). Results obtained by Kuttappan et al. (2012a) suggest that an increased growth rate had a consequent increased occurrence of higher degrees of WS in broiler breast fillets. Histological evaluations (Kuttappan et al., 2013a) shown an increased incidence of histopathological changes such as degenerative or necrotic lesions, fibrosis, and lipidosis as the degree of WS increased from NORM to SEV. Some authors suggest that it is likely that fat and connective tissue infiltrate the areas where fiber degeneration occurs (reviewed in Ferreira et al., 2014). Moreover, the effects of WS can be involved in changes of chemical composition (Kuttappan et al., 2012a; Kuttappan et al., 2013a; Mudalal et al., 2014) and in a worsening of technological properties of meat. Kuttappan et al. (2013b) observed an increased b^* value (yellowness) of meat, corresponding to the occurrence of SEV degrees of WS, that can be attributed to the higher percentage of fat detectable in case of severe WS. Petracci et al. (2013) observed a higher pH values in case of SEV degrees of this condition, and a negative impact on water holding and binding capacity of breast meat.

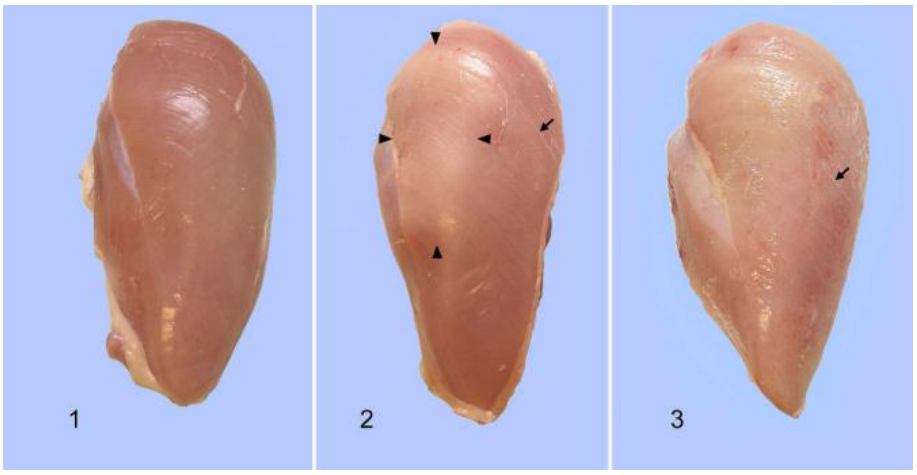
The changes in chemical composition due to the occurrence of WS, could have consequences on nutritional properties of chicken meat. Results obtained by Petracci et al. (2014) shown a remarkable worsening of nutritional value of meat. In fact, both SEV and MOD white-striped breasts were characterized by a higher fat content, lower protein level, and a decreased quality of protein due to the higher collagen content. Additionally, breasts with SEV degree of WS, shown a higher energy content compare to normal meat.

As can be expected, all these aspects may contribute to a decreasing of the consumer preference towards chicken meat, with consequent serious problems for the poultry meat market.

9.6 Wooden breast

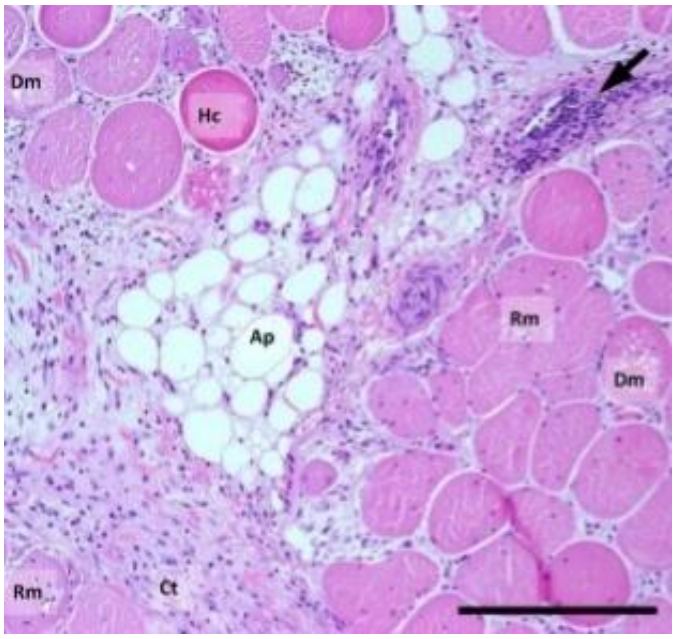
Wooden breast (WB) myodegeneration is an emerging issue macroscopically characterized by a remarkable palpatory hardness of the *Pectoralis major* muscle, often accompanied with pale color and WS (Figure 9.5). Moreover, the hardened areas are consistently out-bulging, and the surface is often covered with clear or slightly turbid viscous fluid and/or petechiae or multifocally distributed small hemorrhages (Sihvo et al., 2014).

Figure 9.5 Wooden breast (WB) myopathy on right *Pectoralis major* muscle. **1.** Unaffected muscle of normal color and consistency. **2.** Focal manifestation of WB myodegeneration. Hardened consistency and pale color affect the cranial area of the muscle (lined by arrowheads) which is surrounded by unaffected muscle. White stripes follow the muscle fiber orientation (arrow). **3.** Diffuse WB myodegeneration. The muscle is diffusely hardened and pale. White stripes of variable width are present (arrow) (*Source*: Sihvo et al., 2017).



Considering the causes, as in case of WS, it can be assumed that the faster growth rate, especially of breast muscle, involves an unsustainable pressure on muscle metabolism which leads to the beginning of degenerative characteristics. Additionally, the increased free radical production and accumulation of intracellular calcium may promote alteration of fiber membrane integrity and degradation of proteins due to activation of proteases and lipases. This can lead to fiber necrosis that overtakes the regenerative capacity of muscle (Petracci et al., 2015). Histological analysis of the muscle have shown an active degeneration and regeneration of muscle fibers, and infiltration of immune cells with increased deposition of adipose and connective tissue (Figure 9.6), therefore indicating that WB can be characterized as a myodegeneration with fibrosis and regeneration (reviewed in Bailey et al., 2015).

Figure 9.6 Histopathological changes of breast muscle affected by wooden breast. Features of the muscle include degenerating muscle fibers (**Dm**), regenerating fibers (**Rm**), adipose tissue (**Ap**), hypercontracted fibers (**Hc**), increased connective tissue (**Ct**) and cellular infiltration (**arrow**). Black bar shows scale (100 μm) (*Source*: Bailey et al., 2015).



As in case of WS, WB defect damage the visual appearance of breast meat with a consequent reduction of consumer acceptability. Thus, this type of meat is usually downgrades, especially in severe cases, and used for the transformation in processed meat products, leading to serious economic losses for the poultry industry (Petracci et al., 2015).

Moreover, it was demonstrated that WB myopathy can affect the chemical composition of meat, maybe changing the nutritional value. Results obtained by Soglia et al. (2016) shown a higher moisture, fat, and collagen content coupled with lower amounts of protein and ash. The higher collagen content results in a reduction of the nutritional value due to the low digestibility of collagen and deficiency of some essential amino acids (Mudalal et al., 2014; Petracci et al., 2014). Additionally, it was also reported that this anomaly is associated with a serious worsening of the technological properties of breast meat. Soglia et al. (2016) observed a higher pH_u and lower water holding and water binding capacity in pectoral muscles affected by WB. Trocino et al. (2015) found a higher cooking losses and shear force.

Regarding the high pH values of meat affected by WB, it is also important to consider the implications for the microbiological quality and the shelf life. As well known, the growth of microorganisms is strongly affected by meat pH. Breast meat characterized by high pH values is more exposed to the contamination by different types of harmful microorganisms that impair taste, flavour and appearance (reviewed in Petracci et al., 2015).

PART 2. RESEARCH WORKS

Chapter 10

Research N. 1:

Probiotic and synbiotic supplementation in broiler chicken feed: economic impact, growth performance, carcass traits and meat quality

10.1 AIM

In recent years, the key role of gut microbiota in several aspects of animal physiology, such as nutrition, immune function, and health status, is increasingly evident. Antibiotics have been used for a long time in the poultry industry, both as therapeutic agents to treat diseases, and also as growth promoters (AGPs) in order to enhance health, growth and performance of birds without therapeutic aims. However, this approach has undesired effects, such as the development of antibiotic resistance (Ghosh and LaPara, 2007), and the presence of antibiotic residues in poultry meat and poultry products (reviewed in Er et al., 2013). In this context the use of AGPs was banned by the European Union from January 1, 2006 (EC regulation No 1831/2003). The ban on the use of these compounds at sub-therapeutic level has contributed to increased incidence of enteric diseases (e.g., salmonellosis and campylobacteriosis), resulting in higher mortality rates and lower productivity, as well as increased health risks for the operators in poultry farming, and increased relevance of contamination of poultry meat and poultry products for human consumption, causing serious economic damage to the poultry industry. Since the ban of AGPs in European Union, several feed additives (e.g., phytogenic substances, biomolecules from microalgae, organic acids) have been tested in broiler chickens in order to improve health and performance. Among others, in the post-antibiotics era, probiotics, prebiotics and synbiotics (combination of pro- and prebiotics) offer a natural and safe solution to replace AGPs through the modulation of the activity of the gastrointestinal microbiota, and therefore, are considered beneficial to the host animal. These bioactives have been investigated in several studies with promising results regarding their effects on health status and the productive performance of chickens, as well as meat quality.

Therefore, the aim of this research was to assess the effects of a probiotic preparation and a synbiotic combination supplemented in feed on economic impact, growth performance, carcass traits and meat quality in broiler chickens.

This research work was carried out by the University of Science and Technology in Bydgoszcz (Poland) in cooperation with the Department of Agricultural, Environmental and Food Sciences of the University of Molise in Campobasso.

10.2 MATERIALS AND METHODS

10.2.1 Animals and experimental design

Three hundred sixty one-day-old female chicks (Ross 308) were randomly allotted to 3 dietary treatments: basal diet (Control, **C**); basal diet with 1% of Lavipan® (JHJ Sp. z o. o., Gizałki, Poland) (**L**) consisting of *L. lactis* IBB500, *C. divergens* S1, *L. casei* ŁOCK 0915, *L. plantarum* ŁOCK 0862 and *S. cerevisiae* ŁOCK 0141; basal diet with a combination of Lavipan® (1%) with RFO (raffinose family oligosaccharides) (0.8%) (**LR**). RFO were isolated and purified from seeds of lupin *Lupinus luteus* L. *cv*. Lord, according to the method described by Gulewicz et al. (2000). Both formulations (L and LR) were supplemented for the first 7 days of chick's life. Animals were reared in a commercial poultry house (PiastPasze Sp. z.o.o., Olszowa, Poland) that provided good husbandry conditions (e.g., stocking density, litter, ventilation). Temperature was gradually decreased from 33°C on d 0 to 18°C on d 41 and was kept constant thereafter. 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. Chickens were raised in 30 floor pens (10 replicate pens/treatment, 12 chicks/pen). To provide commercial conditions, the poultry house was filled with 9000 as-hatched chicks. Animals were fed *ad libitum* with commercial diets (Table 10.1) according to their age and had free access to water.

Body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR), at 10, 21 and 40 days of age, were calculated on pen basis. Mortality for the overall experimental period was calculated for each pen replicate. The European broiler index (EBI) was calculated for each pen replicate, according to the following formula:

$$\text{EBI} = \text{liveability (\%)} \times \text{live weight (kg)} \times 100/\text{age (d)} \times \text{FCR}$$

Table 10.1 Composition and nutritive value of the diets.

	Period		
Item (% unless noted)	1 to 10 d	11 to 21 d	22 to 40 d
Ingredients			
Wheat meal	45.70	47.80	50.20
Soybean meal	25.20	21.90	19.30
Maize meal	10.00	10.00	10.00
Rapeseed meal	10.00	10.00	10.00
Soybean oil	5.20	7.30	7.70
Calcium phosphate	2.26	1.50	1.00
Alimet liquid	0.33	0.27	0.24
Vitamin-mineral premix	0.30	0.30	0.30
L-lysine	0.29	0.20	0.25
Limestone	0.28	0.37	0.53
NaCl	0.17	0.21	0.19
Sodium carbonate	0.16	0.10	0.12
L-threonine	0.15	0.14	0.18
Calculated nutritional value			
ME, kcal/kg of diet	3073	3269	3329
Crude protein	21.50	20.10	19.20
Crude Lipid	6.80	8.90	9.30
Crude Fiber	3.40	3.40	3.30
Lysine	1.30	1.10	1.10
Methionine	0.60	0.50	0.50
Cysteine	0.40	0.40	0.40
Methionine+cysteine	1.00	0.90	0.80
Threonine	0.90	0.90	0.90
Tryptophan	0.30	0.30	0.20
Arginine	1.30	1.20	1.20
Phosfor retainable, g/kg	4.70	3.60	2.90
Calcium	0.90	0.70	0.70
Sodium	0.15	0.14	0.14
Potassium	0.90	0.80	0.80

10.2.2 Slaughter surveys and sampling

At 41 days of age, 10 randomly chosen birds per treatment, between more big, identified by numbered permanent wing bands, were individually weighed and slaughtered. At slaughter, hot carcass weight was recorded and carcass yield was calculated. The right pectoral muscle (PM) was removed from all carcasses (n = 30) and weighed; its percentage was calculated based on hot carcass weight. In the same way, weight and yield of legs were calculated as well.

pH, color and water holding capacity (WHC) were recorded on the right PM at 24 hours *post mortem*. pH was measured using a portable pH meter (FiveGo^{TM}, Mettler-Toledo, Switzerland) equipped with a penetrating glass electrode. Tri-stimulus color coordinates (lightness, L*; redness, a*; yellowness, b*) were detected on the right PM muscle using a Chroma Meter CR-300 (Italia s.r.l., Milano) (Figure 10.1). Reflectance measurements were performed after the samples had oxygenated in air for at least 30 minutes by which time measurements were stable (Škrlep and Čandek-Potokar, 2007), taking three readings for each sample. Moreover, water holding capacity (WHC) was measured using filter paper (Whatman No.1) press method (Grau and Hamm, 1957) (Figure 10.2) and was expressed as free water in meat.

Figure 10.1 Color measurement.

Figure 10.2 WHC measurement.

The left PM was vacuum-packaged and stored frozen (-20°C) until chemical analysis for total lipids and fatty acids profile.

10.2.3 Total lipids and fatty acids analysis

Lipid extraction from breast muscle was performed using the method described by Folch et al. (1957). The extracted lipids were esterificated and then analyzed by gas chromatography (GC). Analysis was performed using a GC Trace 2000 (ThermoQuest EC Instruments) equipped with a flame ionization detector (260°C) and a fused silica capillary Column (Omegawax 320, Phenomenex, Torrance, CA, USA) 30 m x 0.32 mm x 0.25 μm film thickness. The carrier gas was helium (25cm/sec). The oven temperature was maintained constant at 200°C. The individual fatty acid peaks were identified by comparison of retention times with those of known mixtures of standard fatty acids (PUFA-2, Supelco, Bellofonte, PA, USA) run under the same operating conditions. Results were expressed as percentage of the total fatty acids identified. To assess the nutritional implications, the n-6/n-3 fatty acids and the PUFA/SFA ratios were calculated. Moreover, in order to evaluate the risk of atherosclerosis and the potential aggregation of blood platelets, respectively the atherogenic index (AI) and the thrombogenic index (TI) were calculated, according to the formulae suggested by Ulbricht and Southgate (1991) as follows:

$AI = C12:0 + 4 \times C14:0 + C16:0/\Sigma MUFA + \Sigma PUFA(n-6) \text{ and } (n-3)$

$TI = C14:0 + C16:0 + C18:0/0.5\Sigma MUFA + 0.5\Sigma PUFA(n-6) + 3\Sigma PUFA(n-3) + (n-3)/(n-6).$

10.2.4 *Statistical analysis*

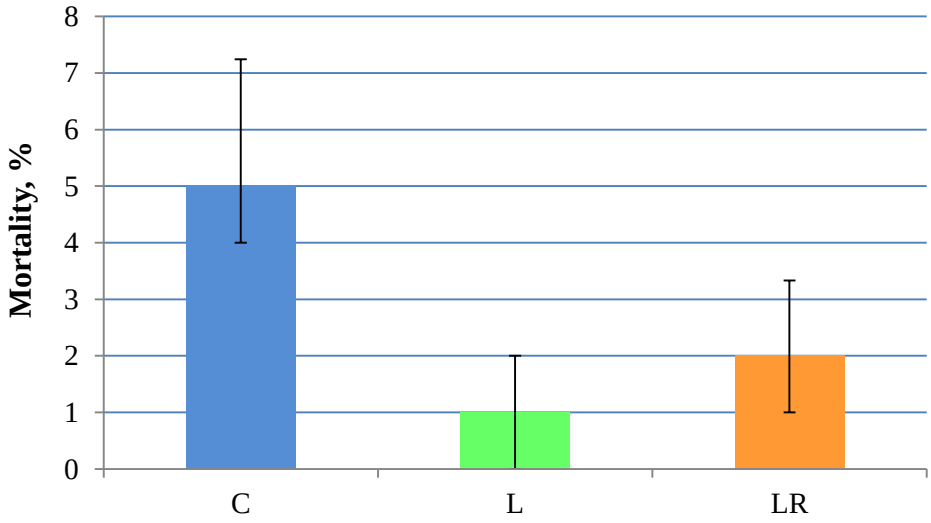
Data were analyzed by one way analysis of variance (ANOVA) (SPSS Inc., 2010). Scheffé's test was applied to compare the differences among means.

10.3 RESULTS AND DISCUSSION

10.3.1 Productive performance and carcass traits

The mortality was lower in groups fed with supplementation of probiotic (1 %) and synbiotic (2.27 %) compared with C group (5 %), however, the differences were not significant (P = 0.207) (Figure 10.3).

Figure 10.3 Mortality rate values of Ross broiler chickens fed supplemented with different bioactives.



Results regarding the effects of dietary supplementation of probiotics and synbiotic on body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) are presented in Table 10.2.

In recent years, the interest in the use of natural feed additives such as probiotics and synbiotics has grown considerably, and several studies have investigated their effects on health, well-being and productive performance of poultry and other animal species (reviewed in Gaggìa et al., 2010). In the current research, the BWG within the first 10 days of life was affected by dietary probiotics and synbiotic (P = 0.037). In general, both L and LR groups were characterized by a better BWG values in comparison with C (207.89, 208.99 and 197.63

g/bird, respectively). This finding could be due to the fact that the administration of feed additives such as probiotics, had more pronounced effect in young growing animals (reviewed in Anjum et al., 2005). No significant differences in the BWG were found for the rest of the rearing period, even if the chickens from treated groups showed a slightly higher ($P > 0.05$) BWG compared to those of group C. In fact, treatment with L and LR was associated with modest increases in total BWG (+3.1% and +1.3%, respectively) as compared with C group. Although these increases may seem minor, if we consider a high number of chickens reared, the economic implications could be interesting. Our results are in line with those reported by Abdel-Raheem and Abd-Allah (2011) who found that the weight gain of chickens fed probiotics or synbiotics was significantly increased during the first 3 weeks compared with the control. Also Yeo and Kim (1997) found that average daily weight gain of chickens fed probiotics was significantly increased during the first 3 weeks but not during the 4-6th weeks of growth. Conversely, Ghasemi et al. (2010), did not observe significant differences in BWG at 10 days of life in chickens fed supplemented with a synbiotic preparation (*Enterococcus faecium* + inulin) but a significant increase in BWG was observed at 28 days and whole experimental period in broilers fed the diet containing 0.1% and 0.15% synbiotic, but not 0.5% synbiotic. Saiyed et al. (2015), who evaluated the effects of probiotics and synbiotics in feed and in different doses, during the starter phase (0-4 weeks) and finisher phase (5-6 weeks), did not found significant effect on final live weight compared to the control. Differently, Anjum et al. (2005) found a higher average live weight gain of broiler chicks fed supplemented with a multistrain commercial probiotic (Protexin) during starter (0-4 week) period. This finding is in agreement with several reports demonstrating that probiotic supplemented in feed (Alkhalf et al., 2010; Dizaji et al., 2012; Hatab et al 2016) or water (Timmerman et al., 2006) to the birds improve the body weight and daily weight gain. Moreover, an interesting work reported that probiotics and synbiotics improved slightly the body weight gain and reduced the mortality of the birds in heat stress condition compared with control group in heat stress condition (Sohail et al.,2012).

Table 10.2 Mean values and SEM for body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of Ross broiler chickens fed supplemented with different bioactives.

Groups[1]	C	L	LR	SEM	*P - value*
BWG, g/bird					
1-10 d	197.63	207.89	208.99	2.04	*0.037*
11-21 d	528.88	543.44	531.10	3.77	*0.244*
22-40 d	1469.19	1494.83	1473.84	15.99	*0.796*
1-40 d	2250.50	2320.65	2279.87	18.74	*0.319*
FI, g/d/bird					
1-10 d	258.00[b]	267.42[a]	262.30	1.48	*0.027*
11-21 d	800.79	817.80	811.11	5.29	*0.432*
22-40 d	2651.27	2690.28	2655.98	19.13	*0.677*
1-40 d	3682.13	3768.49	3719.75	22.95	*0.316*
FCR, kg/kg					
1-10 d	1.31	1.29	1.26	0.01	*0.134*
11-21 d	1.52	1.51	1.53	0.01	*0.443*
22-40 d	1.81	1.80	1.81	0.01	*0.983*
1-40 d	1.64	1.63	1.63	0.01	*0.785*

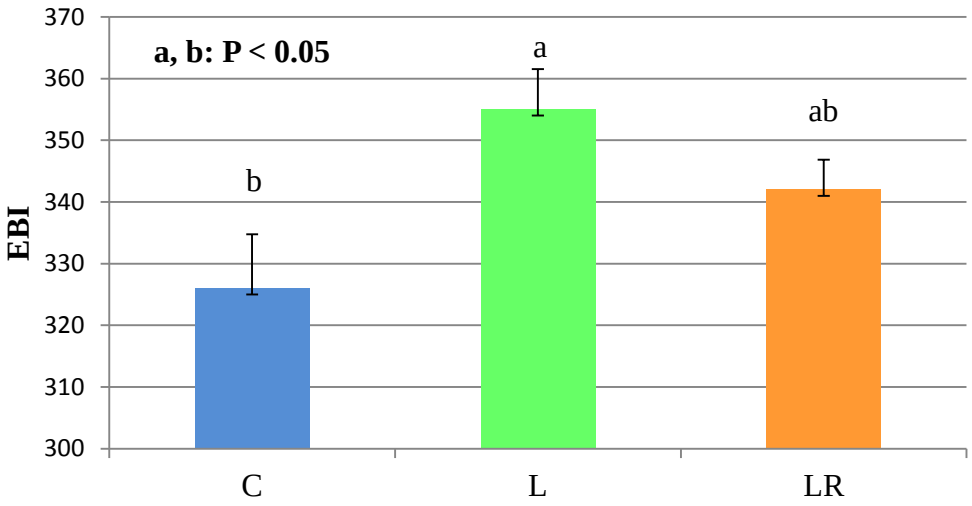
[1]Groups: C = Control, basal diet; L = basal diet + Lavipan; LR = basal diet + Lavipan + RFO.
SEM = standard error mean. a-b: $P < 0.05$.

In general, FI within the first 10 days of life was affected by supplementation of probiotics and synbiotic ($P = 0.027$); it was higher ($P < 0.05$) for chickens of L group compared to C. The total amount of FI was also slightly higher in L and LR groups compared to C, but the differences were not significant ($P > 0.05$). Contrasting findings are reported in literature. Paryad and Mahmoudi (2008) found a higher cumulative FI in broiler chickens fed 1.5% of *Saccharomyces cerevisiae*. Abdel-Raheem and Abd-Allah (2011) found a higher cumulative FI in broiler chickens fed with probiotic or synbiotic. On the contrary, Hatab et al. (2016) supplemented in feed layer chicks with a probiotic mixture (*B. subtilis* and *E. faecium*) with 1 and 2 gm/kg, until 10 weeks of age, observed a decrease of FI and a lower FCR, as well as better growth performance. Authors suggested that this trend may be attributed to the total effect of supplementation of probiotic mixture on the maintenance of beneficial microbial population that improving feed intake, digestion and the uptake of nutrients (fatty acids and glucose) and increasing digestive enzyme activity. In the present research, FCR was similar between experimental groups for the whole rearing period ($P > 0.05$). Conversely, taking into account the effects of probiotics, Falaki et al. (2010) found a better FCR in birds under treatment with a commercial probiotic (Primalac 900 g ton^{-1}) during the first 3 weeks; while no effects were observed with supplementation of synbiotics (mixture of Fermacto and Primalac). Differently, Alkhalf et al. (2010) have noticed that the probiotic treatment groups

had less total FCR than control group; however, they did not found significant differences in the means of FCR between probiotic groups and control group at 1 and 2 weeks of age.

EBI is a parameter widely used to describe the efficiency of broiler production. In the current experiment, EBI was better in both L and LR compared with C group; however, treatment with L was associated with higher ($P < 0.05$) value (+2.6%) compared to the C group (Figure 10.4). The higher EBI value means higher average body weight, good liveability and higher feed efficiency in stipulated number of days thus give overall economics of the birds considering various important traits. Hence, both probiotic and synbiotic groups are more economical than C group when EBI is considered.

Figure 10.4 European broiler index (EBI) values of Ross broiler chickens fed supplemented with different bioactives.



Results regarding the effects of dietary probiotic and synbiotic supplementation on slaughter traits of Ross broiler chickens are reported in Table 10.3. In the present study, no differences ($P > 0.05$) were found between control and experimental groups in case of final body weight (BW), ranged from 2559.58 to 2642.92 g.

Table 10.3 Mean values and SEM for slaughter traits of Ross broiler chickens fed supplemented with different bioactives.

Groups[1]	C	L	LR	SEM	*P - value*
Final BW, g	2642.92	2559.58	2619.58	26.78	*0.436*
Carcass weight, g	1714.27	1718.35	1718.22	23.62	*0.997*
Carcass yield, %	64.84	67.08	65.55	0.51	*0.197*
PM weight, g	526.62	512.37	529.35	10.06	*0.771*
PM yield, %	30.73	29.78	30.90	0.45	*0.565*
Legs weight, g	453.68	438.74	443.99	5.21	*0.508*
Legs yield, %	26.58	25.57	25.91	0.28	*0.329*

[1]Groups: C = Control, basal diet; L = basal diet + Lavipan; LR = basal diet + Lavipan + RFO.
BW = Body weight. PM = Pectoral muscle.
SEM = standard error mean.

In the current experiment, the dietary probiotic and synbiotic supplementation had no effects ($P > 0.05$) on carcass weight (ranging from 1714.27 to 1718.35 g) and carcass yield (ranging from 64.84 to 67.08 %), as well as on pectoral muscle (PM) weight (ranging from 512.37 to 529.35 g), PM yield (ranging from 29.78 to 30.90 %), and leg weight (ranging from 443.99 to 453.68 g) and legs yield (ranging from 25.57 to 26.58 %). Midilli et al. (2008), who assessed the effects of dietary probiotic (Bio-Plus 2B®), prebiotic (Bio-Mos®) and their combination did not found any significant effect on carcass weight and carcass yield of broiler chickens at 42 days of age. Conversely, Falaki et al. (2010) found a higher carcass weight and breast muscle yield in broiler chickens slaughtered at 42 days of age fed supplemented with a basal diet containing a commercial probiotic or synbiotic formulation. However, the same authors, did not found any significant effects on carcass yield and legs yield of broiler chickens. On the contrary, Saiyed et al. (2015) reported no effect on carcass weight, while found favourable impact of probiotics and synbiotics on carcass yield. Conversely, considering the effects of probiotics, Zheng et al. (2015) found a higher PM and legs weight in 42 days old broilers fed supplemented with *Enterococcus faecium*.

10.3.2 pH, color and WHC

Effects of dietary probiotic and synbiotic supplementation on pH and color of PM after 24 hours *post mortem*, and water holding capacity (WHC) are presented in Table 10.4. As well known, pH is one of the most important qualitative attribute of meat that has a central role in determining the protein behavior both in fresh and processed meat products (Lonergan, 2008) and it is also an important contributing factor to meat quality expressed as tenderness, color, and storage life (Van Laack et al., 2000). pH_{24} (ranging from 5.79 to 5.84) was not significantly affected ($P > 0.05$) by both dietary probiotic and synbiotic supplementation.

After slaughter, the internal supply of oxygen diminishes and aerobic respiration ceases after cessation of blood circulation; the glycolisis becomes therefore the only pathway in muscle to generate energy (Zheng et al., 2015). The *post mortem* glycolysis bring to an increasing accumulation of lactic acid in the muscle, resulting in a decline of pH (Lopez et al., 2011). The rate and the extent of pH decline have a large impact on meat quality properties (Eleroğlu et al., 2013). Generally, since the pH of meat from chickens slaughtered under stress conditions can change during *rigor mortis*, the pH measured after 24 hours *post mortem* is the best prediction index for the quality evaluation of meat (Park and Park, 2011). The normal pH_{24} value of chicken meat is about 5.7-5.9 (reviewed in Haščík et al., 2015). Thus, it can be concluded that the values of pH recorded in our study are in line with the acceptable range for commercial meats. In case of probiotics, in the study described by Zheng et al. (2015) the influence of *E. faecium* on meat quality traits was observed. In their study, at 45 minutes and 24 hours *post mortem* the pH values of the PM of the treated groups (6.11 ± 0.13) were higher ($P < 0.05$) compare to control (5.77 ± 0.10). In case of synbiotics, Maiorano et al. (2012) found similar values of pH_{45}, pH_{12}, and pH_{24} among control and synbiotics groups.

Table 10.4 Mean values and SEM for physico-chemical properties of breast muscle from Ross broiler chickens fed supplemented with different bioactives.

Groups[1]	C	L	LR	SEM	*P - value*
pH_{24}	5.79	5.83	5.84	0.02	*0.424*
Color 24h					
L*	50.97	49.19	50.24	0.80	*0.670*
a*	5.09	4.45	4.62	0.16	*0.260*
b*	2.90	2.83	2.54	0.22	*0.787*
WHC, %	17.21	16.00	16.83	0.27	*0.177*

[1]Groups: C = Control, basal diet; L = basal diet + Lavipan; LR = basal diet + Lavipan + RFO.
SEM = standard error mean.

Another important parameter taken into account in our research was the meat color. Among the quality attributes that affect the technological properties of meat, color influences more than any other factor the consumer acceptance of meat, since it is associated with the freshness and healthiness of the product (Mancini and Hunt, 2005). In the current study, the lightness of meat (L*) measured in PM at 24 hours *post mortem* was similar between experimental groups (P> 0.05), ranging from 49.19 to 50.97. The L* value indicates the degree of paleness and is associated with poor meat quality; pale, soft, and exudative meat is an increasing problem in the poultry industry (Eleroğlu et al., 2013). According to several authors (reviewed in Guidi and Castigliego, 2010), normal L* values of chicken breast meat

should be between 48 and 53, thus, it can be concluded that the recorded values can be considered acceptable for commercial poultry meat. Considering the effects of probiotics, our results are in line with those found by Zheng et al. (2015), who did not found significant differences in this parameter measured at 24 hours *post mortem* in PM of broiler chickens fed supplemented with *E. faecium*. On the contrary, taking into account prebiotics, Park and Park (2011) found a significantly higher L* value in breast meat of broiler chickens treated with inuloprebiotics. Redness (a*) and yellowness (b*) of meat measured in PM at 24 hours *post mortem* were similar among groups ($P > 0.05$). On the contrary, the results obtained by Park and Park (2011) showed a higher b* value in PM of chickens fed supplemented with inuloprebiotics; while, Zheng et al. (2015) found a lower b* value in breast muscle of chickens treated with *E. faecium*.

Among technological properties considered in the presented research has been the water holding capacity (WHC). WHC of raw poultry meat is an important meat quality attribute which strongly affect product yield, which in turn has economic implications; moreover, WHC is important also because of its effects on sensory characteristics of the cooked product (Lesiak et al., 1996; Cheng and Sun, 2008). In our study, dietary probiotic and synbiotic treatment did not affect WHC of breast muscle ($P > 0.05$). Our results are consistent with those of Pelicano et al. (2003), who did not found differences in the WHC of PM of chickens fed supplemented with different probiotic strains. Zheng et al. (2015) found a significantly lower drip loss and cooking loss in PM of broilers fed *E. faecium*. On the contrary, taking into account prebiotics, Park and Park (2011) observed a significantly higher WHC in breast muscle of chickens treated with inuloprebiotics.

10.3.3 Lipid content and fatty acids composition

Results concerning the effects of dietary probiotic and synbiotic supplementation on total lipid content, fatty acids composition (% of total fatty acids) and nutritional ratios in PM of chickens, are reported in Table 10.5.

The total lipid content, ranged from 1.45 to 1.52 g/100 g, was not affected by dietary probiotic and synbiotic supplementation. Meat contributes remarkably to fat intake, especially in terms of saturated fatty acids. Despite a high intake of these nutrients is associated with negative health consequences, when fats are consumed in the context of a healthy and well balanced diet, they plays several central roles, such as providing essential fatty acids (linoleic and α-linolenic acids) and fat-soluble vitamins (A, D, E, and K) (Marangoni et al., 2015). Compared to meat of other species, such as beef and pork, chicken meat is characterized by a lower concentration of fat. The average lipid content of meat and meat products is around 3-

25 g/100 g of food. The fat content varies widely depending on the animal species, age and the part of carcass used (Valsta et al., 2005). The results concerning this trait are in contrast with those obtained by Ponte et al. (2008), Alina et al. (2012) and Milićević et al. (2015). In fact, the obtained values of total lipid content in their experiments were higher compared with those observed in the present study. In monogastric animals, especially in poultry, the lipid content and the fatty acids composition can be affected also by animal feeding (Valsta et al., 2005).

Broiler fat is characterized by a significant amount of monounsaturated fatty acids, and, in comparison with red meat, substantial amounts of polyunsaturated fats, especially the omega-6 linoleic acid and arachidonic acid. Moreover, it may represent an important source of long-chain omega-3 fatty acids (reviewed in Attia et al., 2017). Taking into account fatty acids profile (Table 10.5), total polyunsaturated fatty acids (PUFA) were the most abundant fatty acids (ranging from 42.69 to 43.09 %) followed in descending order by saturated fatty acids (SFA) (ranging from 27.95 to 29.85 %) and monounsaturated fatty acids (MUFA) (ranging from 27.45 to 28.96 %). In the current research, the total SFA, MUFA and PUFA content was not affected significantly by the probiotic and synbiotic treatment, as well as the individual fatty acids ($P > 0.05$). These results are consistent with those reported by Kalavathy et al. (2006), which found no significant differences in the total amount of SFA, MUFA and PUFA as well as considering the individual fatty acids, except for oleic acid (C18:1) that was higher in breast muscle of broiler chickens fed supplemented diet with *Lactobacillus* cultures compared with the control group. Likewise, Yang et al. (2010) did not found significant differences on the total SFA, MUFA and PUFA content in breast meat of chickens fed a supplemented diet with *C. Butyricum*; however, *C. butyricum*-supplemented diet significantly increased C 20:5 n-3 (EPA). Our results are inconsistent with those of Zhou et al. (2009), who found a greater concentration of total MUFA and lower amount of SFA in breast muscle of broiler chickens fed a diet supplemented with chitooligosaccharide (GOS, oligosaccharide obtained by chemical and enzymatic hydrolysis of polychitosan) supplementation. Among the individual SFA, the most abundant was the palmitic acid (C16:0; ranging from 19.62 to 21.09 %), followed by stearic acid (C18:0; ranging from 8.12 to 8.76). Palmitic acid is thought to increase cholesterol level together with lauric and myristic acid, while stearic acid has a little or no effect (Zock et al., 1994). Several nutritional studies underlined the relationship between a high intake of SFA and the risk of cardiovascular diseases. The restriction of SFA in the diet is more effective than limiting total fat consumption (reviewed in Zock et al., 1994). Among the individual MUFA, the most abundant was the oleic acid (C18:1; ranging from 25.58 to 27.21 %; $P > 0.05$). This finding is in agreement with levels found by other authors in chicken

meat (Zhou et al., 2009; Milićević et al., 2014). As well known, from the nutritional point of view, oleic acid plays a key role in human diet as involved in lipaemia decreasing, both LDL cholesterol and triglycerides, reducing the risk of stroke (D'Alessandro et al., 2012).

Fatty acids are not a simple energy source, and they are required by the organism for several other functions. There is an increasing interest of the potential health benefits of specific types of fatty acids. PUFA are essential components of biological membranes and are precursors of a wide range of lipid regulators of cellular metabolism (Gurr, 1999). Among the individual PUFA, the most abundant was linoleic acid (C18:2 n-6; ranging from 33.65 to 34.12%), followed by arachidonic (C 20:4 n-6; ranging from 3.60 to 4.05%) and α-linolenic acid (ALA, C18:3 n-3; ranging from 2.69 to 2.78%). There are different results in literature concerning the effects of these bioactives on the total amount of PUFA. Salma et al. (2007), who evaluated the effect of dietary *Rhodobacter capsulatus* on the fatty acids composition of breast muscle, found an increased total PUFA concentration in case of chickens fed for 6 weeks with 0.04% of *R. Capsulatus*; in addition *R. Capsulatus* increased total MUFA and decreased total SFA. On the contrary, Hossain et al. (2012) observed a lower PUFA content in breast meat from broiler chickens fed diets supplemented with herbal extract (*A. canaliculatum*) combined with different strains of probiotic microorganisms, but no effect on total amount of SFA and MUFA.

Dietary probiotic and synbiotic supplementation had no effects on all calculated nutritional ratios considered in the current research as well (Table 10.5). The n-6/n-3 PUFA ratio, ranging from 8.76 to 8.92, resulted similar among groups ($P > 0.05$). Recent epidemiological studies state that the most important risk factor for atherosclerosis and coronary heart diseases is a high n-6/n-3 ratio rather than a high intake of cholesterol (reviewed in Milićević et al., 2014), and it is recommended that this ratio should be less than 4.0 (Department of Health, 1994). Therefore, it could be concluded that the values of n-6/n-3 ratio obtained in this research are higher compared to those recommended.

The PUFA/SFA ratio is used for assessment of lipids on the basis of the proportions of the different FA groups. In the present study, the PUFA/SFA ratio, known to be a measure of the propensity of the diet to influence the occurrence of coronary disease, was not affected by probiotic and synbiotic dietary supplementation ($P > 0.05$). Dietary intake of unsaturated fatty acids has been shown to reduce the risk of cardiovascular disease and possibly the incidence of some cancers, asthma and diabetes among other conditions (Milićević et al., 2014). At the same time, the recommended ratio of PUFA/SFA should be above 0.4. The values of PUFA/SFA ratio obtained in the current research (ranging from 1.46 to 1.55), were higher

compared to those described by Milićević et al. (2014; ranging between 0.39 to 0.97 in breast muscle).

The atherogenic (AI) and thrombogenic (TI) indices represent a criteria for evaluating the likelihood of fatty acids to have atherogenic or thrombogenic properties, respectively and both are recommended for a healthy diet (reviewed in Maiorano et al., 2016). In particular, these indices take into account the different effects that single fatty acids might have on human health and in particular on the probability of increasing the incidence of pathogenic phenomena, such as atheroma and/or thrombus formation (Garaffo et al., 2011). In the current research AI (ranging from 0.29 to 0.32) and TI (ranging from 0.60 to 0.66) (Table 10.5) were similar between experimental groups ($P > 0.05$). These values can be considered as low, in agreement with Ulbritch and Southgate (1991), and not comparable with the results reported by Castellini et al. (2006), in Ross chickens organically reared (AI: 0.49, TI: 1.54 ± 0.88), and in the meat of ruminants (AI: 1.29 ± 0.26, TI: 1.54 ± 0.179, D'Alessandro et al., 2012), but similar to poultry meat (AI: 0.56 ± 0.13, TI: 0.55 ± 0.14, Laudadio and Tufarelli, 2010; AI: 0.43 ± 0.02, He et al., 2015). Low atherogenic and thrombogenic indices indicate pro-health status of a meat in terms of fatty acids composition and the antioxidant capacity (Attia et al. , 2017). Moreover, a high negative relation was found between hypocholesterolemic index and atherogenic and thrombogenic indices (Attia et al. , 2017).

Table 10.5 Mean values and SEM for total lipids (g/100g), fatty acids composition (% of total fatty acids) and nutritional ratios of breast muscle from Ross broiler chickens fed supplemented with different bioactives.

Item[2]	Groups[1]			SEM	*P - value*
	C	L	LR		
Total lipids (g/100g)	1.48	1.45	1.52	0.08	*0.942*
Fatty acids					
C 14:0	0.21	0.22	0.22	0.01	*0.984*
C 16:0	19.62	20.25	21.09	0.45	*0.420*
C 16:1	1.61	1.73	1.75	0.08	*0.717*
C 18:0	8.12	8.76	8.55	0.19	*0.366*
C 18:1	27.21	25.86	25.58	0.44	*0.260*
C 18:2 n-6	34.12	33.65	33.65	0.53	*0.921*
C 18:3 n-3	2.78	2.71	2.69	0.11	*0.953*
C 18:3 n-6	0.11	0.12	0.10	0.01	*0.540*
C 20:1 n-9	0.15	0.13	0.13	0.00	*0.183*
C 20:3 n-3	0.50	0.55	0.58	0.03	*0.415*
C 20:4 n-6	3.60	4.05	3.81	0.22	*0.716*
C 20:5 n-3	0.14	0.14	0.13	0.01	*0.833*
C 22:4 n-6	0.86	0.82	0.80	0.05	*0.889*
C 22:5 n-3	0.10	0.09	0.08	0.01	*0.554*
C 22:6 n-3	0.89	0.91	0.84	0.05	*0.823*
Partial sum					
ΣSFA	27.95	29.22	29.85	0.58	*0.396*
ΣMUFA	28.96	27.72	27.45	0.48	*0.400*
ΣPUFA	43.09	43.05	42.69	0.51	*0.947*
Σn-6	38.69	38.64	38.37	0.45	*0.957*
Σn-3	4.39	4.41	4.32	0.07	*0.888*
Nutritional ratios					
n-6/n-3	8.84	8.76	8.92	0.10	*0.833*
PUFA/SFA	1.55	1.48	1.46	0.04	*0.651*
Atherogenic index	0.29	0.30	0.32	0.01	*0.410*
Thrombogenic index	0.60	0.63	0.66	0.02	*0.399*

[1]Groups: C = Control, basal diet; L = basal diet + Lavipan; LR = basal diet + Lavipan + RFO.

[2]SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

SEM = standard error mean.

10.4 CONCLUSIONS

In summary, the results of this research indicate that the probiotic preparation and the synbiotic combination supplemented in feed for the first 7 days of chick's life, had slight but interesting effects on growth performance of birds. In fact, the BWG within the first 10 days of life was affected by the administration of bioactives, and was better both in L and LR groups in comparison with C. Although no significant differences in the BWG were found for the rest of the rearing period, treatment with L and LR was associated with modest increases in total BWG (3.1 % and 1.3%, respectively) as compared with C group. Similarly, FI within the first 10 days of life, was affected by the treatment and was higher in L group compared to C. The total amount of FI was also slightly higher in L and LR groups compared to C, but the differences were not significant. The FCR was similar between experimental groups for the whole rearing period. The mortality was lower in groups fed with supplementation of probiotic (1 %) and synbiotic (2.27 %) compared with C group (5 %), however, the differences were not significant. Additionally, EBI was better in both L and LR compared with C group; however, treatment with L was associated with higher value (+2.6%) compared to C group. All values of slaughter traits, physicochemical properties, total lipids and fatty acids composition, were similar between experimental groups.

Therefore, in conclusion, it can be assumed that, taking into account the effects on growth performance, the economic impact could be relevant if considered to feed a high number of animals reared in commercial conditions with basal diet with 1% of Lavipan® (consisting of *L. lactis* IBB500, *C. divergens* S1, *L. casei* ŁOCK 0915, *L. plantarum* ŁOCK 0862 and *S. cerevisiae* ŁOCK 0141) and basal diet with a combination of Lavipan® (1%) with 0.8% RFO (raffinose family oligosaccharides).

Chapter 11

Research N. 2:

Effect of *in ovo* administration of different synbiotics on growth performance, carcass traits and meat quality in broiler chickens

11.1 AIM

After the withdrawal of antibiotics as growth promoters (AGPs) in European Union because of the development of antibiotic resistance, it was necessary to find alternative and safe solutions to replace AGPs in the poultry industry. In this context, among the several alternatives proposed, probiotics, prebiotics and their combination (synbiotics), offer an interesting way to solve the intestinal problems of birds through the manipulation of the intestinal microbiota. The conventional administration of these bioactives in feed and/or water during the first hours/days post-hatching, could lead to conflicting results due to the environmental conditions, such as the individual feed and water intake, the quality of water (chlorinated), and other factors. Additionally, in order to be effective, these bioactives should be administered as early in life as possible, minimizing the environmental variables that can compromise their efficacy. To reduce the effect of these factors, *in ovo* injection technology of probiotics, prebiotics and synbiotics has been developed. This emergent and innovative method, consists on controlled injection of bioactive substances directly into the egg air chamber during embryogenesis (Gulewicz and Bednarczyk, Polish patent Nb. 19772). The method allows the accurate and precise delivery of the bioactive substance at very low doses to all embryos at early stage of development, minimizing the effect of environmental variables and influencing the microbiome structure in newly hatched chicks.

Therefore, the aim of this research was to evaluate the effect of two different synbiotics administered *in ovo* on performance and meat quality traits in broiler chickens.

This research work was a part of an European research project (ECO-FCE) carried out by the University of Science and Technology in Bydgoszcz (Poland) in cooperation with the Department of Agricultural, Environmental and Food Sciences of the University of Molise in Campobasso.

11.2 MATERIALS AND METHODS

11.2.1 Animals and experimental design

Five thousand eight hundred fifty broiler chickens eggs from Cobb 500FF hybrid were incubated in a commercial hatchery Drobex (Solec Kujawski, Poland) (Figure 11.1), a Petersime incubator (vision version, Petersime NV, Zulte, Belgium).

Figure 11.1 Drobex Agro commercial hatchery.

On day 12 of incubation, the eggs were randomly divided into three experimental groups treated with different bioactives, *in ovo* injected. Before the injection, the eggs were candled and those unfertilized or with dead embryos were discarded (Figure 11.2).

Figure 11.2 Egg candling.

Eggs were injected into the egg air chamber with the aid of a dedicated automatic system (Bednarczyk et al., 2011) (Figure 11.3).

Figure 11.3 Automatic system for *in ovo* injection (*source*: Bednarczyk et al., 2011).

Eggs were *in ovo* injected with either: 0.2 ml of a physiological saline solution (Control, **C**); 0.2 ml of a synbiotic formulation containing 2 mg/embryo of Bi^{2}tos (Clasado BioSciences Ltd., Sliema, Malta), a nondigestive *trans*-galactooligosaccharides (GOS) from milk lactose digested with *Bifidobacterium bifidum* NCIMB 41171, enriched with 10^5cfu/embryo of *Lactobacillus salivarius* IBB3154 (**SYN1**) or 0.2 ml of a synbiotic formulation containing 2 mg/embryo of raffinose family oligosaccharides (RFO), isolated and purified from seeds of lupin *Lupinus luteus* L. *cv*. Lord, with the same procedure of Research N.1., enriched with 10^5 cfu/embryo of *Lactobacillus plantarum* IBB3036 (**SYN2**). The strains of probiotic microorganisms were both developed in the Institute of Biochemistry and Biophysics in Warsaw (Polish Academy of Sciences). After the injection, the injection hole was covered with a drop of organic glue and the incubation was continued until hatching.

Among the hatched chickens, 2040 males (680 per each group) were randomly chosen and reared in a commercial poultry house (PiastPasze Sp. z.o.o., Olszowa, Poland) that provided good husbandry conditions (e.g., stocking density, litter, ventilation). Temperature was gradually decreased from 33°C on d 0 to 20°C on d 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. 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.

Chickens were raised in pens (n = 75/pen) with 8 pen replicates per treatment for effect on performance. Moreover, separate pens for sampling (n = 10 chickens/pen) with 8 replications per each experimental group were included in the experimental design. Animals were fed *ad libitum* with commercial diets (Table 11.1) according to their age and had free access to water. Feed intake (FI) and feed conversion ratio (FCR) were calculated on a pen

basis. In addition, mortality for the overall experimental period was calculated for each pen replicate.

Table 11.1 Composition and nutritive value of the diets.

	Period		
Item (% unless noted)	1 to 10 d	11 to 21 d	22 to 41 d
Ingredients			
Maize (7.75% CP)	61.16	65.99	67.93
Soybean meal (47.75% CP)	33.09	28.16	26.03
Soybean oil	1.75	2.06	2.77
Limestone flour	1.10	0.98	0.70
NaCl	0.20	0.20	0.23
Dicalcium phosphate	1.605	1.504	1.337
Vitamin-mineral premix 1[1]	1.10	-	-
Vitamin-mineral premix 2[2]	-	1.10	-
Vitamin-mineral premix 3[3]	-	-	1.10
Calculated nutritional value			
ME, MJ/kg of diet	12.72	13.00	13.30
Dry matter	88.87	88.94	88.91
Crude protein	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
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; cholinechloride, 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; cholinechloride, 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; cholinechloride, 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.

11.2.2 Slaughter surveys and sampling

At 42 days of age, two birds per pen (16 per treatment), identified by numbered permanent wing bands, were randomly chosen from the separate pens for sampling and slaughtered. At slaughter, hot carcass weight was recorded and carcass yield was calculated. The right pectoral muscle (PM) was removed from all carcasses (n = 48) and weighed; its percentage was calculated based on hot carcass weight. pH and color on the right PM were recorded at 45 minutes and 24 hours *post mortem*; in addition, water holding capacity (WHC) at 24 hours was measured on the right PM with the same procedure of Research N.1.

The left PM was vacuum packaged and stored frozen (-20°C) until chemical analysis for intramuscular collagen properties, total lipids, fatty acids composition and cholesterol content.

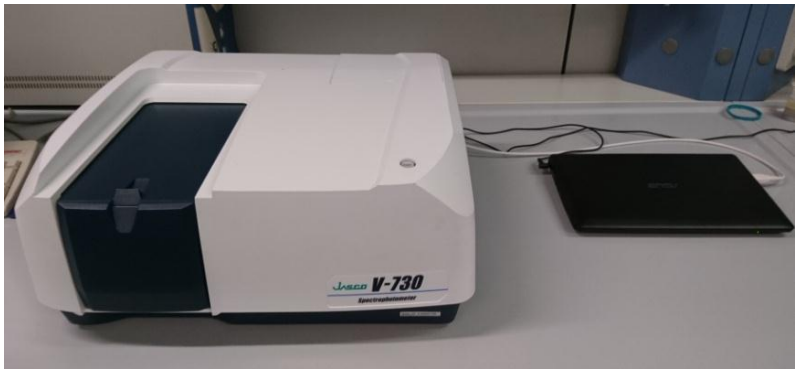
11.2.3 Collagen analysis

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

11.2.3.1 Intramuscular collagen concentration analysis

The 4-hydroxyproline (intramuscular collagen concentration) was quantified using the colorimetric procedure of Woessner et al. (1961). The hydroxyproline was oxidated with chloramine T (sodium p-toluenesulfonchloramide) that was then inactivated by adding perchloric acid. Finally, a solution of p-dimethylaminobenzaldehyde solution was added and the tube was placed in a 60°C water bath for 20 minutes. The absorbance of the solution was then determined using a spectrophotometer (V-730, Jasco Co., Ltd., Tokyo, Japan) (Figure 11.4) at 557 nm. The hydroxyproline concentration was determined directly from the standard curve of L-hydroxyproline. Intramuscular collagen concentration was calculated, assuming that collagen weighed 7.25 times the measured hydroxyproline weight (Eastoe and Leach, 1958) and expressed as µg hydroxyproline/mg of lyophilized tissue.

Figure 11.4 Spectrophotometer, V-730, Jasco.



11.2.3.2 Crosslink concentration analysis

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

11.2.4 Measurement of muscle cholesterol

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

quantitation of muscle cholesterol content was based on the external standard method using a pure cholesterol standard (Sigma, St. Louis, MO).

11.2.5 Total lipids and fatty acids analysis

Total lipids, fatty acids composition and nutritional indices, were calculated with the same procedure of Research N1.

11.2.6 Statistical analysis

Data were analyzed by one way analysis of variance (ANOVA) (SPSS Inc., 2010). Scheffé's test was applied to compare the differences among means.

11.3 RESULTS AND DISCUSSION

11.3.1 Productive performance and carcass traits

Results regarding the effects of *in ovo* injection of synbiotics on mortality rate, performance and carcass traits of broiler chickens are reported in Table 11.2.

Mortality rate of birds was reduced with the synbiotics treatment compared with C group, however the differences were not statistically significant (P = 0.253). Our finding is in agreement with the results obtained on chickens by Abdel-Raheem and Abd-Allah (2011), who evaluated the effects of single or combined dietary supplementation of mannan oligosaccharide and probiotics (*S. cerevisiae*). Similarly, Alkhalf et. al (2010) did not found any significant effect on mortality rate with the administration of a commercial probiotic (Bactocell®) in diets supplemented with 1.6 g, 1 g and 0.8 g of probiotic per kg feed. Moreover, the mortality rate values obtained in the present study with the synbiotics treatment are lower (- 3.2%) than those reported by Maiorano et al. (2017), who assessed the effects of two different commercial prebiotics (DiNovo and Bi^{2}tos) *in ovo* injected. Likewise, a study conducted by Awad et al. (2009), concerning the effects of dietary inclusion of probiotics and synbiotics, showed a higher mortality rates (3.00 - 3.5%). Chickens mortality is an important economic loss for the producers. High mortality is an obvious indicator of poor welfare, and problems should be addressed without delay.

Table 11.2 Mean values and SEM for mortality, growth and slaughter traits of Cobb broiler chickens *in ovo* injected with different synbiotic preparations.

Groups[1]	C	SYN 1	SYN 2	SEM	*P-value*
Mortality, %	1.83	0.83	1.17	0.25	*0.253*
Feed intake, g	4.940	4.930	4.898	24.41	*0.780*
Feed conversion ratio, %	1.59	1.60	1.60	0.01	*0.882*
Final BW, g	3.146	3.127	3.111	9.79	*0.370*
Carcass weight, g	1.951	1.930	1.916	14.87	*0.632*
Carcass yield, %	64.32	63.49	64.15	0.31	*0.527*
Pectoral muscle weight, g	610.4	588.9	587.9	8.12	*0.451*
Pectoral muscle yield, %	31.31	30.46	30.65	0.31	*0.520*

[1]Groups: C = Control, *in ovo* injection of physiological saline; SYN 1 = *Lactobacillus salivarius* + Bi[2]tos; SYN2 = *Lactobacillus plantarum* + Lupin RFO.
SEM = standard error mean.

Feed additives have been efficiently used to enhance certain characteristics of feed and to improve growth rate in monogastric animals (reviewed in Onu et al., 2004). However, in the present study, *in ovo* administration of synbiotics did not affect both FI (P = 0.780) and FCR (P = 0.882). Conversely, Ghasemi et al. (2010) observed that the presence of synbiotics in the diet significantly improved FCR; whereas, Mousavi et al. (2015) found a higher feed consumption in birds fed diets containing synbiotics. Our results are in line with those presented by Maiorano et al. (2017). In their study *in ovo* treatment with prebiotics did not change significantly both FI and FCR. In the current experiment, no differences (P > 0.05) were found between control and experimental groups also in case of final body weight (BW), ranged from 3.111 to 3.146 g. This finding is not in agreement with the results obtained by Abdel-Raheem and Abd-Allah (2011). They observed that at 42 days of age BW increased with the dietary inclusion of probiotics and synbiotics compared to control fed broilers. The present experiment revealed no effects of *in ovo* injection of synbiotics (P > 0.05) also for values of carcass weight and carcass yield, ranged from 1.916 to 1.951 g and from 63.49 to 64.32%, respectively. These results are in line with findings of Maiorano et al. (2012), who observed minimum differences in carcass weight and yield between control and experimental groups that received *in ovo* injection of synbiotics. Additionally, no significant differences were found as well in case of PM weight (ranging from 587.9 to 610.4 g) and PM yield (ranging from 30.46 to 31.31 %) between experimental groups (P > 0.05). Our finding is in agreement with the work conducted on chickens by Maiorano et al. (2012). Conversely, taking into account the effect of prebiotics, Maiorano et al (2017) recorded an increasing effect of these bioactives on breast weight, and lack of marked impact on breast yield.

11.3.2 pH, color and WHC

Effects of *in ovo* administration of synbiotics on pH and color of PM after 45 minutes and 24 hours *post mortem*, and WHC are presented in Table 11.3. pH value measured 45 minutes (ranging from 6.36 to 6.50) and 24 hours *post mortem* (ranging from 5.85 to 5.91) were not significantly affected ($P > 0.05$) by both synbiotic treatments. The pH of muscle/meat is a measurement of the acidity. In chickens, normal ultimate pH (pH_u) values are around 5.8 (Duclos et al., 2007). Thus, it can be concluded that the pH_u values observed in our study are within the pH range accepted for commercial poultry meats. The rate of pH, decline during the *post mortem* period (reviewed in del Puerto et al., 2016). In poultry muscle, glycolysis is faster compared to other species (Addis, 1986). In avian PM a fast pH drop is associated with slaughter conditions due to the glycolytic metabolism and the type of fibers (Type IIb) (reviewed in del Puerto et al., 2016). Compared to pH measured 45 minutes *post mortem*, the pH_u (24 hours after slaughter) values are the most convincing predictor of meat quality. It is connected with a high degree of correlation between pH_{24} and water binding capacity (reviewed in Poznyakovskiy et al., 2015). Our results are partially in agreement with Maiorano et al. (2012) who reported that *in ovo* synbiotic administration affected significantly pH_{45} ($P < 0.05$) and did not have an influence on pH_{24}. Considering the fact that the synbiotic concept combines efficacious probiotic strains with specific prebiotic compounds, is justified to compare our results with studies on effects of pro- and prebiotics on meat quality. In case of probiotics, in the study described by Zheng et al. (2015) the influence of *E. faecium* on meat pH values (at 45 min and 24 hours *post mortem*) was observed; both values were higher in treated groups ($P < 0.05$) compared to control. While, taking into account influence of prebiotics on pH of meat, Park and Park (2011) noted that dietary inulo-prebiotic reduced ($P < 0.05$) pH of chicken meat.

Table 11.3 Mean values and SEM for physico-chemical properties of breast muscle from Cobb broiler chickens *in ovo* injected with different synbiotic preparations.

Groups[1]	C	SYN1	SYN2	SEM	*P-value*
pH_{45}	6.36	6.50	6.36	0.04	*0.228*
pH_{24}	5.85	5.91	5.91	0.02	*0.256*
Color 45 min					
L*	44.82^{AB}	46.76^{A}	44.00^{B}	0.35	*0.003*
a*	4.58	3.58	4.02	0.28	*0.348*
b*	6.91	7.72	6.02	0.36	*0.182*
Color 24 h					
L*	50.81	49.52	49.95	0.42	*0.449*
a*	5.08	4.46	4.77	0.31	*0.737*
b*	11.39	10.85	11.33	0.39	*0.839*
WHC, %	13.35	13.16	13.22	0.28	*0.965*

[1]Groups: C = Control, *in ovo* injection of physiological saline; SYN 1 = *Lactobacillus salivarius*+ Bi[2]tos; SYN2 = *Lactobacillus plantarum* + Lupin RFO.
SEM= standard error mean. A-B: $P < 0.01$.

pH_u influences directly other meat attributes such as color (reviewed in del Puerto et al., 2016), that is an important quality attribute both for the consumer's selection of fresh meat at the retail level, and for the consumer's final evaluation and acceptance of a meat product at time of consumption (Fletcher et al., 2000). In our study, the lightness of meat (L*) measured in PM at 45 minutes *post mortem* was affected only by type of synbiotics injected. The value of this descriptor was higher ($P < 0.01$) for meat from chickens of SYN1 group in comparison with SYN2 (46.76 vs 44.00, respectively); differently, no differences ($P = 0.449$) were detected for L* measured at 24 hours between the three experimental groups. The lightness of breast muscle could be used in the technological evaluation of meat with the standardized threshold value L*, to detect pre-slaughter or processing effects, with a good reliability with different genetic strain (Saláková et al., 2009). Higher values indicate lighter color, indicating that fillets have low pH (Garcia et al., 2010). The optimal lightness range of chicken and turkey fillets is around 49-50 (Barbut, 1997). Thus, it can be assumed that the recorded L* values measured 24 hours *post mortem* are within the acceptable range for commercial meats. Redness (a*) and yellowness (b*) of meat measured 45 minutes and 24 hours *post mortem* were found to be similar ($P > 0.05$) among groups. However, the values observed at 24 hours, when the color is stabilized, are within the acceptable range for commercial meats. Among technological properties of meat considered in the present study, was also WHC. The WHC of

meat products is a very important quality attribute which has an influence on product yield, which in turn has economic implications, but is also relevant in terms of eating quality (Cheng and Sun, 2008). It is important to note that water loss reduces the meat nutritional value because some nutrients may be lost in the exudate, resulting in a meat less tender and worst in flavour (Pelicano et al., 2003), which was not the case observed in this study because the percentage of free water was low (approximately 13 %). However, synbiotic treatment did not affect ($P > 0.05$) WHC of meat.

11.3.3 Intramuscular collagen properties

Data regarding the intramuscular collagen (IMC) properties in chicken meat are reported in Table 11.4. Collagen is an abundant connective tissue protein and is a contributing factor to the variation in meat tenderness and texture. Collagen molecules are bound together through intermolecular crosslinks that help to provide structure and strength. These crosslinks are initially reducible, but over time are replaced by mature, thermally stable, and less soluble crosslinks. These mature crosslinks, rather than the total amount of collagen, are the key factors in collagen-related toughness (Weston et al., 2002). In the current study, collagen concentration (15.98, 15.87 and 15.62 μg/mg in C, SYN1 and SYN2 group, respectively), collagen maturity (0.110, 0.139 and 0.103 mol HLP/mol of collagen in C, SYN1 and SYN2 group, respectively) and HLP concentration (2.64, 2.85 and 2.26 μg HLP/mg in C, SYN1 and SYN2 group, respectively) were not significantly ($P > 0.05$) influenced by *in ovo* synbiotic administration. Maiorano et al. (2012) found a significantly lower collagen content in the PM of broiler chickens (Ross 308) from synbiotic treated groups (18.87 - 21.06 μg/mg) compared with control group (25.27 μg/mg); but no effect of synbiotic was found on muscle HLP concentration (μg/mg) and collagen maturation (mol of HLP/mol of collagen). Compared with our study, Maiorano et al. (2012) found a lower collagen maturity (0.065-0.085 mol/mol) and lower HLP concentration (2.21-2.23 μg/mg). Velleman et al. (1996) found a higher collagen content (25%) in the pectoral muscle of 6-wk-old White Leghorn chickens, but more crosslinked (almost 0.5 mol of HLP/mol of collagen). This marked difference in collagen maturity could be due mainly to the modern chicken strains (Petracci and Cavani, 2012). Infact, in fast-growing birds, collagen is immature, resulting in low heat stability. Consequently, poultry meat is tender, but may turn fragile, even mushy (Puolanne and Voutila, 2009) and cooked chicken breast meat is generally fragmented (soft) (Voutila et al., 2009).

Table 11.4 Mean values and SEM for intramuscular collagen properties of breast muscle of Cobb broiler chickens *in ovo* injected with different synbiotic preparations.

Groups[1]	C	SYN1	SYN2	SEM	*P-value*
Total collagen, µg/mg	15.98	15.87	15.62	0.28	*0.877*
HLP[2], mol/mol	0.110	0.139	0.103	0.007	*0.179*
HLP[2], µg/mg	2.64	2.85	2.26	0.12	*0.262*

[1]Groups: C = Control, *in ovo* injection of physiological saline; SYN 1= *Lactobacillus salivarius*+ Bi[2]tos; SYN2 = *Lactobacillus plantarum* + Lupin RFO.
[2]HLP = hydroxylysylpyridinoline.
SEM= standard error mean.

11.3.4 Total lipids, fatty acids composition and cholesterol content

Table 11.5 shows the effect of *in ovo* administration of synbiotics on total lipid and cholesterol content, fatty acids composition (% of total fatty acids) and nutritional ratios in PM of chickens.

Treatment with synbiotics reduced (P = 0.061) the lipid content compared with control group, markedly (P < 0.05) with synbiotic SYN2. Reduce total lipid level by the use of synbiotics can have positive effects in terms of human health. This is connected with the fact that lowering of fat level reduces the energy density of meat considerably and with a marked reduction of the metabolic consequences on the organism. Lipids are important components in the diet. Although the most of discussions concerning the connection between fats and human health focus on several harmful effects of these nutrients, lipids are fundamental components of the cell membrane (Dowhan and Bogdanov, 2002), and provide essential fatty acids, that are metabolized in substances which possess several important physiological functions (e.g., hormone-like activity) (Kritchevski, 2008). Lipid content in meat is one of the most important factors for consumer acceptance and meat buying decisions. Thus, the knowledge of meat lipid composition is of essential importance. Total lipid content in PM ranged from 1.18 to 1.51 g/100g. Compared to other livestock species, chicken meat is characterized by a lower concentration of total lipids. Our results regarding the value of this trait are not in line with these described by Ponte et al. (2008), Alina et al. (2012), Milićević et al. (2015). In afore-mentioned experiments, the recorded values of lipid contents were higher than these observed in the current study. Changes in body fat deposition between broilers could be due to different dietary fatty acid profiles, may be related to different rates of lipid synthesis or lipid oxidation (Crespo and Esteve Garcia 2001).

In terms of human health, the fatty acids composition of meat products is an important parameter of meat quality. Concerning fatty acids profile (Table 11.5), total monounsaturated fatty acids (MUFA) were the most abundant fatty acids (ranging from 36.87 to 38.06 %)

followed in descending order by polyunsaturated fatty acids (PUFA) (ranging from 29.84 to 34.12 %) and saturated fatty acids (SFA) (ranging from 27.83 to 33.29 %). In the present study, *in ovo* administration of synbiotics affected ($P < 0.001$) the total SFA content. The value of this trait was markedly increased ($P < 0.01$) with SYN1 treatment in comparison with C and SYN2 (33.29% vs 28.40 and 27.83, respectively).

Considering the individual SFA, the amount of palmitic (C 16:0) and stearic (C 18:0) acids was increased ($P < 0.01$) with SYN1 treatment in comparison with C and SYN2. Instead, the amount of myristic (C 14:0) and arachidic (C 20:0) acids was reduced ($P < 0.01$) with SYN1 treatment compared to C and SYN2. These results are in contrast to those reported by Kalavathy et al. (2006), which found no significant differences in the total amount of SFA, as well as in the individual SFA (C 14:0, C 16:0 and C 18:0) values in breast muscle of broiler chickens fed with supplemented diet with *Lactobacillus* cultures. Many nutritional studies have strongly emphasized the relationship between SFA and the risk of cardiovascular diseases, and therefore there is a need to reduce consumption of SFA and increase consumption of PUFA. Quantitatively, palmitic acid was the most concentrated SFA (approximately 20%), followed by stearic acid (approximately 8%). Palmitic acid is known to increase total serum cholesterol despite its effects are lower than those of lauric and myristic acids (Daley et al., 2010); this latter in this study was approximately 0.72% and was significantly reduced ($P < 0.01$) by SYN1 treatment. Stearic acid is considered a "neutral" fatty acid because it has been shown to have no net impact on the plasmatic level of either LDL or HDL cholesterol in humans (Bonamone and Grundy, 1988; Williamson et al., 2005). This effect of stearic acid has been attributed to its reduced digestibility and easy desaturation into oleic acid (Bonamone and Grundy, 1988).

In the current research, the total amount of MUFA was influenced ($P = 0.052$) by *in ovo* synbiotic treatment. In particular, the administration of SYN1 reduced ($P < 0.05$) the total amount of MUFA compared with the SYN2 administration (36.87 vs 38.06%, respectively), but not respect to the C ($P > 0.05$). Different results are reported in literature on the effect of probiotics or prebiotics on total MUFA content. Zhou et al. (2009) detected a greater concentration of total MUFA in breast muscle of broiler chickens fed a diet supplemented with chitooligosaccharide (GOS, oligosaccharide obtained by chemical and enzymatic hydrolysis of polychitosan) supplementation. On the contrary, Yang et al. (2010) did not observed significant effects on the total MUFA content in breast muscle of chickens fed a supplemented diet with *C. butyricum*. Additionally, synbiotics had a significant effect ($P < 0.01$) on myristoleic (C 14:1), palmitoleic (C 16:1) and gadoleic (C 20:1) acids. In particular, both synbiotics used reduced the myristoleic acid compared to C ($0.05 > P < 0.01$) and, in

addition, myristoleic acid value was lower ($P < 0.05$) in SYN1 than in SYN2. Also gadoleic acid was reduced by synbiotics treatment ($P < 0.01$), however the differences were only significant between C and SYN2 ($P < 0.05$). Differently, palmitoleic acid was reduced with SYN1 treatment and increased with the SYN2 compared with C ($P < 0.01$). Among the MUFA, the most abundant was the oleic acid (C 18:1), with similar values in the three experimental chicken groups (ranging from 33.75 to 34.00 %; $P > 0.05$). The highest presence of oleic acid in intramuscular fat was in agreement with levels found by other authors in chicken meat (Zhou et al., 2009; Milićević et al., 2014) and lamb (D'Alessandro et al., 2012; Russo et al., 1999). As well known, considering the nutritional point of view, oleic acid plays a very important role in human diet. It was shown that isocaloric replacement of about 5% of energy from SFA by oleic acid has been estimated to reduce coronary heart disease risk by 20-40% mainly via LDL-cholesterol reduction (reviewed in Lopez-Huertas, 2010). In general, moreover, some epidemiological studies have suggested an inverse relationship between MUFA intake and mortality rates to cardiovascular disease (Hu et al., 1997; Kris-Etherton, 1999).

PUFA are essential for many vital functions in biological membranes and as precursors of a variety of lipid regulators of cellular metabolism (Gurr, 1999). In the current experiment, the total PUFA content was markedly reduced ($P < 0.01$) by the administration of SYN1 compared to C and SYN2; while similar values ($P > 0.05$) were found between C and SYN2. These results are in line with those observed by Hossain et al. (2012), who detected a lower PUFA content in breast meat from broiler chickens fed diets supplemented with herbal extract (*A. canaliculatum*) combined with different strains of probiotic microorganisms. On the contrary, Salma et al. (2007), who evaluated the effect of dietary *Rhodobacter capsulatus* on breast muscle fatty acids composition, found an increased total PUFA concentration when chickens were fed for 6 weeks with 0.04% of *R. Capsulatus*. Differently Zhou et al. (2009) did not found any effect with diet supplemented with prebiotic.

Considering the individual PUFA, the most abundant was linoleic (C 18:2 n-6) acid, its amount was reduced ($P < 0.01$) with SYN1 (21.95%) compared to C (24.19%) and SYN2 (23.88%), following the arachidonic acid (C 20:4 n-6; from 3.15 to 4.62%) that was increased with SYN2 ($P < 0.01$) and decreased with SYN1 ($P < 0.01$) compared to C. α-linolenic acid - ALA (C18:3 n-3) (approximately 2%) was significantly ($P < 0.001$) lowered by both synbiotics compared to C, markedly by SYN1 ($P < 0.01$). Also γ-linolenic (C 18:3 n-6) was reduced ($P < 0.01$) for the administration of both synbiotics compared with C. Moreover, compared with SYN1, SYN2 increased both C 20:2 n-6 ($P < 0.01$) and C 20:3 n-6 ($P < 0.05$), while C group had intermediate value ($P > 0.05$). Differently, Hossain et al. (2012) did not

found significant differences in the concentration of ALA in breast muscle of chickens fed with *Lactobacillus* cultures supplementation, while observed a decrease in arachidonic and docosahexaenoic acids, in total PUFA and n-6 level in meat of chickens fed with supplemented diet with *Alisma canaliculatum* with probiotics, compared with the control group.

In ovo synbiotic treatment was associated also with significant effects on n-3 long chain PUFA derivatives: eicosapentaenoic acid (EPA, C 20:5 n-3), reduced with SYN1 ($P < 0.01$) compare to C and increased with SYN2 ($P < 0.05$) compared with SYN1; docosahexaenoic acid (DHA, C 22:6 n-3), was increased ($P < 0.05$) with SYN1 in comparison with SYN2. Docosapentaenoic acid (C 22:5 n-3) was not affected ($P > 0.05$) by treatment. The α-linolenic and the γ-linolenic acid are two essential fatty acids in human nutrition that serve as precursors of other important compounds (Daley et al., 2010). In particular, α-linolenic acid is converted in the body, albeit at low efficiency, to EPA (below 5% in humans), DPA and DHA (Daley et al., 2010). A favourable preventive effect of EPA and DHA on atherosclerosis, heart attack, depression and cancer has been established (Connor, 2000). SYN1 treatment reduced significantly total n-6 and n-3 PUFA compared with C ($P < 0.01$) and this latter compared also with SYN2 ($P < 0.05$). Both n-6 and n-3 fatty acids are precursors of signaling molecules with opposing effects, that modulate membrane composition, receptor signaling, and gene expression (Schmitz and Ecker, 2008). On the other hand, a diet rich in n-3 EPA is believed to shift the physiological state to one that is less inflammatory than that of a diet containing high amounts of n-6 (Harris et al., 2010). However, it has been shown that the content of n-3 fatty acids in poultry meat, especially as EPA, and DHA can be readily improved by increasing the levels of n-3 PUFA in poultry diets through inclusion of oily fish by-products (Hulan et al., 1988).

In ovo treatment with synbiotics affected also all calculated nutritional ratios considered in the present study. The n-6/n-3 PUFA ratio was influenced by the synbiotic administration ($P = 0.039$), with slightly higher values in SYN1 and SYN2 groups in comparison with C group ($P > 0.05$); this could be due prevalently to the reduction of linolenic acid content, observed with the administration of SYN1 (- 0.78%) and SYN2 (- 0.20%). Nowadays, epidemiological studies suggested that the major risk factor for atherosclerosis and coronary heart diseases was found to be a high n-6/n-3 ratio rather than a high intake of cholesterol and the consequent hypercholesterolemia (reviewed in Milićević et al., 2014), and it is recommended that this ratio should be less than 4.0 (Department of Health, 1994). However, according to the nutritional changes described in the Western diet, this ratio has now increased to be within the range of 10:1 to 20:1 (Patterson et al., 2012).

Therefore, based on this assumption, it could be assumed that the values of n-6/n-3 ratio obtained in this research, are higher compared to those recommended.

The reduction of linoleic acid content with the SYN1 *in ovo* injected reduced the levels of PUFA and consequently PUFA/SFA ratio. In fact, meat from SYN1 had lower value of PUFA/SFA ratio compared with C and SYN2 groups (0.90 vs 1.21 and 1.23 %, respectively; $P < 0.01$). These results suggest that SYN1 *in ovo* injected has a relevant effect on fat metabolism. The PUFA/SFA ratio is known to be a measure of the propensity of the diet to influence the occurrence of coronary disease. Dietary intake of unsaturated fatty acids has been shown to reduce the risk of cardiovascular diseases and possibly the incidence of some cancers, asthma and diabetes among other conditions (Milićević et al., 2014). At the same time, the recommended ratio of PUFA/SFA should be above 0.4. Since some meats naturally have a P/S ratio of around 0.1, meat has been implicated in causing the imbalanced fatty acids intake of today's consumers (Wood et al., 2003). The values of PUFA/SFA ratio obtained in the current research, were higher compared to those described by Milićević et al., 2014 (ranging between 0.39 to 0.97 in breast muscle).

The atherogenic and thrombogenic indices represent a criteria for evaluating the likelihood of fatty acids to have atherogenic or thrombogenic properties, respectively and both are recommended for a healthy diet (reviewed in Maiorano et al., 2016). In particular, these indices take into account the different effects that single fatty acids might have on human health and in particular on the probability of increasing the incidence of pathogenic phenomena, such as atheroma and/or thrombus formation (Garaffo et al., 2011). In the current study, SYN1 administration resulted in an increased ($P < 0.01$) of both atherogenic and thrombogenic indices compared with C and SYN2 (AI: 0.38 *versus* 0.31 and 0.31, respectively; TI: 0.78 *versus* 0.59 and 0.58, respectively) (Table 11.5). The values found in the current study can be considered as low, in agreement with Ulbritch and Southgate (1991), and not comparable with the values reported by Castellini et al. (2006), in two different genotypes of chickens organically reared (AI: 0.49, TI: 1.54 ± 0.88), and in the meat of ruminants (AI: 1.29 ± 0.26, TI: 1.54 ± 0.179, D'Alessandro et al., 2012), but similar to poultry meat (AI: 0.56 ± 0.13, TI: 0.55 ± 0.14, Laudadio and Tufarelli, 2010; AI: 0.43 ± 0.02, He et al., 2015). Low atherogenic and thrombogenic indices indicate pro-health status of a meat in terms of fatty acids composition and the antioxidant capacity (Attia et al., 2017). Moreover, a high negative relation was found between hypocholesterolemic index and atherogenic and thrombogenic indices (Attia et al. , 2017).

Table 11.5 Mean values and SEM for total lipids (g/100g) and cholesterol (mg/100g) content, fatty acids composition (% of total fatty acids) and nutritional ratios in breast muscle of Cobb broiler chickens *in ovo* injected with different synbiotic preparations.

Item[2]	Groups[1]			SEM	*P - value*
	C	SYN1	SYN2		
Total lipids (g/100g)	1.51^{a}	1.38^{ab}	1.18^{b}	0.06	*0.061*
Cholesterol (mg/100g)	41.43	41.88	39.99	1.70	*0.439*
Fatty acids					
C 14:0	0.79^{A}	0.54^{B}	0.83^{A}	0.03	*0.001*
C 14:1	0.58^{Ab}	0.33^{Bb}	0.46^{a}	0.02	*0.001*
C 16:0	18.94^{B}	23.30^{A}	18.84^{B}	0.38	*0.001*
C 16:1	2.54^{B}	1.99^{C}	3.13^{A}	0.09	*0.001*
C 18:0	7.95^{B}	9.19^{A}	7.31^{B}	0.16	*0.001*
C 18:1	33.75	34.00	33.96	0.17	*0.815*
C 18:2 n-6	24.19^{B}	21.95^{A}	23.88^{B}	0.21	*0.001*
C 18:3 n-3	2.25^{Aa}	1.47^{B}	2.05^{Ab}	0.06	*0.001*
C 18:3 n-6	0.34^{A}	0.23^{B}	0.21^{B}	0.01	*0.001*
C 20:0	0.73^{A}	0.26^{B}	0.84^{A}	0.05	*0.001*
C 20:1	0.67^{a}	0.55	0.51^{b}	0.02	*0.009*
C 20:2 n-6	0.62	0.52^{B}	0.67^{A}	0.02	*0.002*
C 20:3 n-6	0.60	0.48^{b}	0.64^{a}	0.03	*0.027*
C 20:4 n-6	3.85^{B}	3.15^{C}	4.62^{A}	0.12	*0.001*
C 20:5 n-3	0.77^{A}	0.56^{Bb}	0.70^{a}	0.02	*0.001*
C 22:5 n-3	0.67	0.73	0.74	0.02	*0.408*
C 22:6 n-3	0.74	0.75^{a}	0.62^{b}	0.02	*0.020*
Partial sum					
ΣSFA	28.40^{B}	33.29^{A}	27.83^{B}	0.44	*0.001*
ΣMUFA	37.54^{ab}	36.87^{b}	38.06^{a}	0.20	*0.052*
ΣPUFA	34.05^{A}	29.84^{B}	34.12^{A}	0.35	*0.001*
Σn-6	29.61^{A}	26.33^{B}	30.02^{A}	0.29	*0.001*
Σn-3	4.44^{A}	3.51^{Bb}	4.10^{a}	0.09	*0.001*
Nutritional ratios					
n-6/n-3	6.75	7.58	7.44	0.14	*0.039*
PUFA/SFA	1.21^{A}	0.90^{B}	1.23^{A}	0.03	*0.001*
Atherogenic index	0.31^{B}	0.38^{A}	0.31^{B}	0.01	*0.001*
Thrombogenic index	0.59^{B}	0.78^{A}	0.58^{B}	0.02	*0.001*

[1]C = Control, *in ovo* injection of physiological saline; SYN1 = *Lactobacillus salivarius* IBB3154 + Bi2tos; SYN2 = *Lactobacillus plantarum* IBB3036+ Lupin RFO.

[2]SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

SEM= standard error mean. a-b: $P < 0.05$; A-C: $P < 0.01$

In the current study the cholesterol level was evaluated as well (Table 11.5). Cholesterol is an important component of meat lipids since high levels of this substance in the diet were been associated with increased incidence of coronary diseases and heart attack (Almeida et al., 1997). The knowledge of cholesterol content in food is important, especially in poultry and fish meat, because the consumption of these foods is currently increasing based on the recommendations of healthy nutrition (Komprda et al., 2003). In the present study, cholesterol content, ranging from 39.99 to 41.88 mg/100g, was similar ($P > 0.05$) among the experimental groups. Similarly, Maiorano et al. (2012) did not find any significant effect of synbiotics and prebiotic *in ovo* injected on cholesterol content of breast muscle in Ross broiler chickens. On the contrary, Salma et al. (2007) found a lower cholesterol concentration in breast muscle of broiler chickens fed with a dietary supplement of *Rhodobacter capsulatus* compared with that from control group. The cholesterol values found in the present study are similar to those reported by Pilarski et al. (2005) in breast muscle of 42-d-old broiler chickens, but lower than that reported by Maiorano et al. (2012; ranging from 70.45 to 78.12 mg/100 g). Cholesterol content in chicken meat can be altered by varying the composition of diet, age, and sex (Wang et al., 2006), as well as the use of different methodologies for cholesterol quantification or for sampling (Bragagnolo and Rodriguez-Amaya, 2002).

11.4 CONCLUSIONS

In summary, the results of the present study indicate that *in ovo* administration of synbiotics on day 12 of incubation, had effects only on some traits taken into account, also depending on the type of synbiotic formulation administered. Mortality rate was slightly higher in C group compared with synbiotics. FI and FCR were similar between experimental groups. In the same way, the treatment did not have significant effects on slaughter weight, weight and yield of carcass, PM weight and its yield percentage. Considering physico-chemical properties, only the lightness (L^*) value after 45 minutes post slaughter was affected by synbiotics, resulting higher in case of SYN1 group in comparison to SYN2. However, all the recorded values were within the acceptable range for commercial poultry meats. Treatment of synbiotics reduced the lipid content in meat compared with control group, markedly with synbiotic SYN2. *In ovo* administration of synbiotics did not affect the cholesterol content of PM. Additionally, synbiotic treatment significantly influenced the fatty acids composition of breast meat. In particular, group SYN1 (*Lactobacillus salivarius* + Bi^{2}tos) displayed a meat with a higher amount of total SFA, lower amount of MUFA, PUFA,

total n-6 and n-3 fatty acids, PUFA/SFA ratio. Furthermore, SYN1 administration resulted in an increased of both atherogenic and thrombogenic indices.

Thus, in conclusion, the results of the present study indicate that *in ovo* administration of synbiotics did not negatively affect productive performance and physico-chemical properties of meat. However, *in ovo* administration of *Lactobacillus salivarius* + Bi^{2}tos (SYN1) affected negatively the fatty acids profile of meat and the values of atherogenic and thrombogenic indices, indicating a higher risk of incidence of pathogenic phenomena. Whereas, SYN2 (*Lactobacillus plantarum* + Lupin RFO) did not affect fatty acids profile and nutritional properties of chicken meat.

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PART 3

CONCLUSIONS

In poultry farming, since the European Union imposed the ban on the use of antibiotics as growth promoters (AGPs) (January 1, 2006) after the development of antibiotic-resistance, in order to safeguard public health and to avoid the presence of antibiotic residues in poultry meat and poultry products, it has become increasingly evident that the gut microbiome plays a pivotal role in the health of the host animal and in many aspects of physiology, such as nutrition, development of the intestine and digestive function, and immunity. The prohibition of AGPs has led to an increased incidence of intestinal problems in birds, with consequent higher mortality rates and serious economic losses for the poultry industry. In this context, probiotics, prebiotics and synbiotics are one of the proposed solutions to prevent enteric diseases in poultry and to decrease the risk of food-borne illnesses in humans.

The aim of this thesis, which involved two different research works, was to assess the effects of different bioactives (probiotics, prebiotics and their combination) administered in feed (Trial 1) or *in ovo* (Trial 2) on productive performance, carcass traits and meat quality in broiler chickens.

The results of the first trial have shown that the probiotic preparation and the synbiotic combination supplemented in feed for the first 7 days of chick's life reduced slightly the mortality. Treated groups showed an improved BWG and FI within the first 10 days of life. Although no significant differences were found for the rest of the rearing period, modest increases in total BWG and FI were recorded both in L and LR groups. The FCR was similar between experimental groups for the whole rearing period. Interestingly, EBI was better in both L and LR groups in comparison with C; however, treatment with L was associated with a significant higher value compared to C. The dietary probiotic and synbiotic supplementation had no effects on carcass weight and carcass yield, as well as on PM weight, PM yield and legs weight and legs yield. In the same way, the treatment did not affect pH, color and WHC of meat. Fatty acids composition and nutritional ratios of meat were similar between experimental groups.

The findings of the second trial indicate that *in ovo* administration of synbiotics on day 12 of incubation had effects only on some traits considered, depending on the type of synbiotic formulation administered. Mortality rate of birds was reduced with the synbiotics

treatments compared with C group, however the differences were not statistically significant. FI and FCR were similar between experimental groups. In the same way, the treatment did not have significant effects on slaughter weight, weight and yield of carcass, PM weight and its yield percentage. Considering physicochemical properties, only the lightness (L^*) value after 45 minutes *post mortem* was affected by synbiotics, resulting higher in case of SYN1 group in comparison to SYN2. Collagen concentration (μg/mg), collagen maturity (mol HLP/mol of collagen) and HLP concentration (μg HLP/mg) were not significantly influenced by *in ovo* synbiotic administration.Treatment of synbiotics reduced the lipid content compared with control group, markedly with synbiotic SYN2. *In ovo* administration of synbiotics did not affect the cholesterol content of PM.Treatment with SYN1 (*Lactobacillus salivarius* + Bi^2tos) displayed a meat with a higher amount of total SFA, lower amount of MUFA, PUFA, total n-6 and n-3 fatty acids and lower PUFA/SFA ratio. Furthermore, SYN1 administration resulted in an increased of both atherogenic and thrombogenic indices. Whereas, synbiotic SYN2 (*Lactobacillus plantarum* + Lupin RFO) did not affect fatty acids profile and nutritional properties of chicken meat.

Thus, in conclusion, the results of the present study indicate that the administration of feed additives, such as probiotics, had more pronounced effect in young growing animals, improving the BWG and the efficiency of broiler production (EBI). Regarding nutritional properties, synbiotics (SYN1 and SNY2) administered *in ovo* reduced the lipid content. *Lactobacillus salivarius* + Bi^2tos (SNY1) affected negatively the fatty acids profile of meat and the values of atherogenic and thrombogenic indices, indicating a higher risk of incidence of pathogenic phenomena.

Therefore, the use of these bioactive substances with different way of administration is definitely a viable option in order to improve the health status and the productive performance of birds, resulting in important economic advantages. On the other hand, *in ovo* administration of synbiotics could represent a valid alternative to the conventional administration in feed. This method allows an accurate and precise delivery of bioactives at very low doses in the early embryonic stage, ensuring a correct development of gut microflora, important in order to avoid infections during the early post-hatching period, and replacing a prolonged and costly in feed supplementation. Additionally, *in ovo* route of synbiotics delivery could have another important economic aspect because of the possibility to test this technology in combination with vaccines or with the administration of other kinds of nutrients. This could open in the near future the opportunity to use *in ovo* technology in industrial scale.

In the light of this, further research is needed to improve knowledge concerning the effect of *in ovo* delivery of synbiotics on performance and meat quality of broiler chickens in

both experimental and field conditions. Moreover, future trials are needed in order to select the right combination of probiotics, prebiotics and synbiotics, and to obtain safe and functional formulations. Additionally, it is necessary to deepen research to better understand the effect of these bioactives on animal metabolism (e.g., lipid and fatty acids metabolic pathways).

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