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PhD thesis

Galactooligosaccharides delivered *in ovo*: effect on performance and meat quality traits of slow-growing broiler chickens exposed to heat stress. Survey on the quality characteristics of chicken breast meat from intensive farming.

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ABSTRACT

In recent decades, the consumers have become more health conscious paying more attention to nutrition and health claims, choosing niche products such as organic or products that do not include the use of GMOs in animal's diet, paying particular attention to animal welfare. Regarding animal welfare, the intestinal microbiota plays a key role in the physiology of the animal. In recent years, *in ovo* technology has enabled the administration of probiotics, prebiotics and synbiotics to the embryo at an early stage of development, influencing the structure of microbiota in newly hatched chicks, allowing a greater protection against the risk of gastrointestinal infections, and improving productive performance and meat quality traits of treated chickens. The aim of this thesis was twofold: a) to study the effects of commercial prebiotic's (galactooligosaccharides, GOS) *in ovo* injected on performance and meat quality in slow-growing chickens exposed to heat stress; b) evaluate the chickens' meat qualitative characteristics from two different production lines of the Amadori company, Vegetale® and Campese® lines, respectively consisting of fast-growing commercial hybrids reared with intensive techniques, and slow-growing hybrids reared semi-extensively, both fed with a plant-based diet without GMOs. The evaluation was carried out through considering different factors, such as genotype, sex and slaughter age.

Male chickens (Hubbard RedBro x Hubbard JA57) were used for the first study. On the 12th day of incubation, after candling, 3.000 eggs with viable embryos were randomly divided into 3 experimental groups: prebiotic group (GOS) injected with a single dose of 3.5 mg of GOS/egg in 0.2 mL of physiological solution; saline group (S) injected with

0.2 mL of physiological solution (0.9% NaCl); control group (C) not injected. After hatching, 900 male chicks (300 chicks/treatment) were reared in free-range pens under thermoneutral conditions (TN; 6 pens/group, 25 birds/pen) or under heat stress conditions (HS, 30 °C from 36 at 50 days; 6 pens/group, 25 birds/pen). *In vivo* performances (live weight and food intake per box, mortality) were recorded, and the food conversion ratio calculated. At 50 days, 15 carcasses/treatment/temperature were randomly selected and weighed from all slaughtered chickens. The pectoral muscle was taken from each carcass and weighted, the physico-chemical analyses were carried out on the pectoral muscle. *In ovo* injection with GOS has a mild effect on *in vivo* performance and meat quality in slow-growing broilers exposed to heat stress. As expected, thermal challenge applied for the last 14 days of finisher feeding phase, had a dampening effect on growth performance. However, considering that it is the first study carried out with the aforementioned genotype, further investigations are necessary to better understand the activity of these substances on animal metabolism.

In the second part of the thesis, the meat qualitative characteristics (pectoral muscle) of slow-growing commercial hybrids of different sexes (Test 1, Red 75-Campese®), and slow-growing (Test 2, Red 75-Campese®) and fast-growing (Test 3, Ross 308-Vegetale®) of different slaughter ages was assessed. The results showed that the meat of both Campese® and Vegetale® line, despite being different from a physico-chemical and nutritional point of view between males and females and between animals of different ages, was of good quality, reflecting the data reported in literature.

RIASSUNTO

Negli ultimi decenni i consumatori sono sempre più attenti all'alimentazione, scegliendo prodotti di nicchia come quelli biologici o prodotti che non prevedono l'utilizzo di OGM nella dieta degli animali, ponendo particolare attenzione al benessere animale. Relativamente al benessere animale, il microbiota intestinale svolge un ruolo chiave nella fisiologia dell'animale, ed è ormai acclarato che assicurare la salute intestinale è la chiave per garantire la salute e il benessere dell'animale. Negli ultimi anni, la tecnologia di iniezione *in ovo* ha permesso la somministrazione di probiotici, prebiotici e simbiotici, all'embrione in una fase iniziale di sviluppo, influenzando il microbiota nei pulcini appena nati, consentendo una maggiore protezione contro il rischio di infezioni gastrointestinali e il miglioramento delle prestazioni produttive e delle caratteristiche qualitative della carne dei polli trattati. Lo scopo della presente tesi è stato duplice: a) studiare gli effetti dell'iniezione *in ovo* di un prebiotico commerciale (galatto-oligosaccaridi, GOS) sulle performance e sulla qualità della carne in polli a lenta crescita esposti a stress da calore; b) valutare le caratteristiche qualitative della carne di polli di due diverse linee produttive dell'azienda Amadori, linea Vegetale® e linea Campese®, costituite rispettivamente da ibridi commerciali a rapido accrescimento, allevati con tecniche intensive, e da ibridi a lenta crescita, allevati semi-estensivamente, entrambi alimentati con una dieta a base vegetale senza OGM. La valutazione è stata effettuata considerando il genotipo, sesso ed età di macellazione.

Per il primo studio sono stati utilizzati polli maschi (Hubbard RedBro x Hubbard JA57). Al 12° giorno di incubazione, dopo la speratura, 3.000 uova con embrioni vitali sono

state divise casualmente in 3 gruppi sperimentali: gruppo prebiotico (GOS) iniettato con una singola dose di 3,5 mg di GOS/uovo in 0,2 mL di soluzione fisiologica; gruppo salino (S) iniettato con 0,2 mL di soluzione fisiologica (0,9% NaCl); gruppo di controllo (C) non iniettato. Dopo la schiusa, 900 pulcini maschi (300 pulcini/trattamento) sono stati allevati in recinti a terra in condizioni termoneutrali (TN; 6 box/gruppo, 25 animali/box) o in condizioni di stress termico (HS, 30°C da 36 a 50 giorni; 6 box/gruppo, 25 animali/box). Sono state registrate le performance *in vivo* (peso vivo e assunzione di cibo per box, mortalità) e calcolato l'indice di conversione alimentare. A 50 giorni, tra tutti i polli macellati sono state scelte random e pesate 15 carcasse/trattamento/temperatura. Da ogni carcassa è stato prelevato il muscolo pettorale e pesato, le analisi chimico-fisiche sono state effettuate sul muscolo pettorale. Il GOS ha avuto un lieve effetto sulle prestazioni *in vivo* e sulla qualità della carne nei polli esposti a stress da calore. Lo stress da calore ha avuto un effetto negativo sulle prestazioni di crescita degli animali. Tuttavia, considerando che si tratta del primo studio effettuato con il suddetto genotipo, sono necessarie ulteriori indagini per comprendere meglio l'attività del prebiotico sul metabolismo animale.

Nella seconda parte della tesi sono state studiate le caratteristiche qualitative della carne (muscolo pettorale) di ibridi commerciali a crescita lenta di sesso diverso (Prova 1, Red 75-Campese®), e a crescita lenta (Prova 2, Red 75-Campese®) e rapida (Prova 3, Ross 308-Vegetale®) di diverse età di macellazione. I risultati hanno evidenziato che la carne della linea Campese® e Vegetale®, pur essendo differente dal punto di vista chimico-fisico e nutrizionale tra maschi e femmine e tra animali di età diverse, è risultata di buona qualità rispecchiando i dati riportati in letteratura.

1. INTRODUCTION

The global population, 7.8 billion today, is expected to surpass 9 billion by 2050. The Food and Agriculture Organization (FAO) has forecast that in 2050, 70% more food will be needed to fulfil the demand of the growing population, which is a great challenge due to resource and arable land limitations. Recently, FAO (2011) predicted a considerable increase in the consumption of food of animal origin by 2050 (+ 58% for milk and dairy products, + 73% for meat and derived products, compared to 2010 levels), while the consumption of food of plant origin should remain stable (Maiorano, 2016).

Meat is a concentrated nutrient source, considered essential to optimal human growth and development (Higgs, 2000). Meat and its derived products provide relevant quantities of essential nutrients at higher concentrations compared with other foods. Poultry meat, like other meats, or other foods (milk and eggs) has a protein component usually defined as "high quality", conversely, plant-derived foods, which - despite containing a relevant quantity of protein - have a less favourable protein profile.

Marangoni *et al*., (2015) reported that the consumption of poultry meat, as part of a vegetable-rich diet, is associated with a risk reduction of developing overweight and obesity, cardiovascular diseases, and type 2 diabetes mellitus. In addition, white meat (and poultry in particular) is considered moderately protective or neutral on cancer risk. The FAO recognized the relevance of poultry meat for humans, who considers this widely available, relatively inexpensive food to be particularly useful in developing countries (where it can help to meet shortfalls in essential nutrients), considering also the absence of religious restrictions. Moreover, poultry meat consumption contributes to the

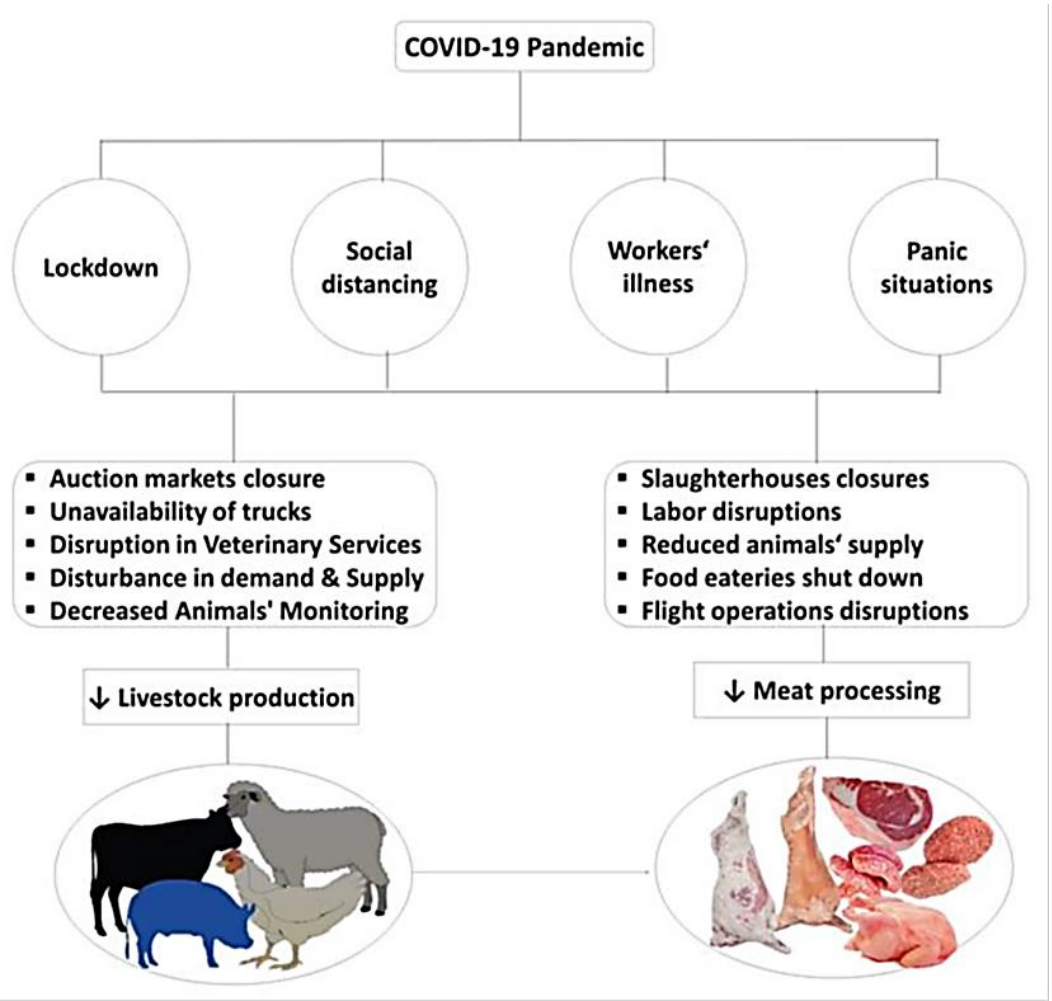
overall quality of the diet in specific ages and conditions (prior to conception, during pregnancy up to the end of breastfeeding, during growth, and in the geriatric age) and is suitable for those who have an increased need for calorie and protein compared to the general population (Marangoni *et al*., 2015).

1.1 World poultry production

Global meat production is projected to expand by nearly 40 Mt by 2029, reaching 366 Mt. Overall, the bulk of meat production growth is attributed to developing regions, which will account for 80% of the additional output. However, in the short term, the supply response of the various meat types remains influenced by African Swine Fever (ASF) outbreaks in Asia, as well as reductions of beef cattle numbers and sheep flock in Australia due to weather conditions. Post-2021, these factors will stabilise and a gradual recovery in the production of meat is expected to follow (OECD-FAO Agricultural Outlook 2020-2029).

World total meat production had a contraction in 2020, depressed by animal diseases especially ASF and Highly Pathogenic Avian Influenza (HPAI), COVID-19-related market disruptions, and the lingering effects of droughts (Figure 1).

Figure 1. Impact of COVID-19 on meat production and supply chain (Source: Ijaz *et al*., 2021).



World meat output fell in 2020 to 333 million tonnes (in carcass weight equivalent), 1.7 percent less than in 2019. As for meat demand, COVID-19 lockdowns, physical distancing, and market closures resulted in substantially reduced food service sales (for example in restaurants, airline catering services, trains, universities, schools, and day care centres), leading to excess supplies of meat, only partially offset by increased retail sales. World trade in meat products increased by 2.4 percent to 37 million tonnes in 2020, a significant slowdown from the 6.8 percent recorded in 2019. The increase observed in

2020 would be entirely account of pig meat, since trade in bovine, poultry, and ovine meats (Table 1) are anticipated to stagnate or decline in 2021. China was the principal engine of trade growth in 2020, with its imports surging by 24 percent. World poultry meat output reached 137 million tonnes in 2020, 2.4 percent more than in 2019. Increases are expected in China, the EU and the UK, Brazil, and Mexico, while production is seen falling in India, Thailand, Turkey, and the USA in 2021. In China, poultry meat output is projected to expand, albeit slowly, supported by relatively firm demand, amid lingering high pig meat prices. Although the detection at the beginning of the year of new cases of HPAI in several European countries led China to prohibit imports of live birds from those origins, the effect on domestic production is likely to be limited, as the measure coincided with the lifting of a 2015 ban on live bird imports from the USA. New investments in processing facilities are expected to boost poultry production in the EU and the UK by 1.2 percent. However, the positive outlook could turn negative if recent price drops linked to COVID-19 continue. In Brazil, poultry meat output is increased in response to high import demand, especially from China, but also from other countries attracted by Brazil's status as an HPAI-free origin and improvements in the country's biosecurity standards. Poultry production is grown in South Africa on high consumer demand, and in Mexico, on competitive feed costs and improved genetics. By contrast, poultry production in India is likely to fall as outmigration of the labour force from cities following the COVID-19 shutdown has reduced the availability of workers in the sector, also depressing consumer demand. Similarly, in Thailand a sharp drop in poultry meat demand by the food retail sector, including street foods, was behind a fall in production. In the USA, tumbling food service sales and labour shortages have led the sector to scale down expansion plans and reduce the production share of large poultry birds preferred by

food services. Requirements for maintaining workspace distances in processing plants are also reported to have reduced meat processing efficiency, contributing to a production decline (Biannual report on global food markets, June 2020, FAO).

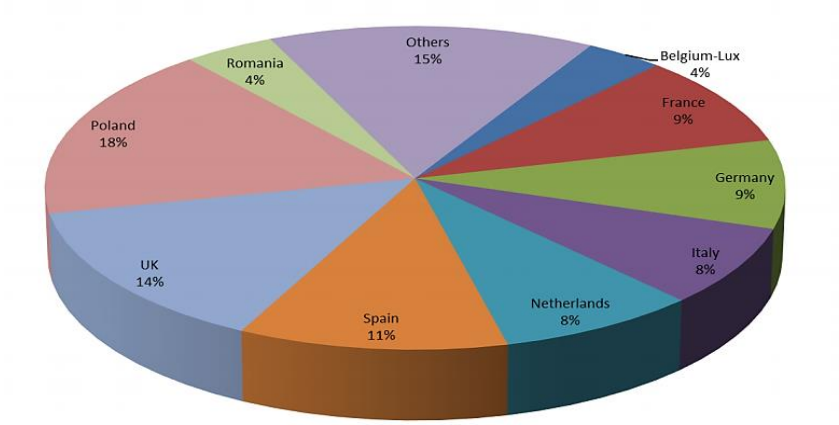
Table 1. World meat production, trade, and percentage year-on-year (Y-O-Y) (readapted to FAO 2020).

	2018	**2019**	**2020**	**CHANGE: 2020 over 2019**
	Million tonnes (carcass weight equivalent) %			
WORLD BALANCE PRODUCTION	342.2	338.9	333.0	-1.7
Bovine meat	71.5	72.6	72.0	-0.8
Poultry meat	127.3	133.6	136.8	2.4
Pigmeat	120.9	109.8	101.0	-8.0
Ovine meat	15.8	16.0	16.2	0.9
Trade	33.8	36.1	37.0	2.4
Bovine meat	10.5	11.2	11.1	-1.0
Poultry meat	13.5	13.9	13.8	-0.3
Pigmeat	8.4	9.5	10.6	11.2
Ovine meat	1.0	1.0	1.0	-2.9

1.2 European poultry production

The EU-27 poultry sector sees more than 70% of the poultry meat is produced in six countries: Poland, United Kingdom (UK), Spain, France, Germany, and Italy (Figure 2). In particular, the above-mentioned countries together produced in 2020 half of the EU's poultry meat (about 12.3 million tons): Poland (18%), followed by the United Kingdom (14%), Spain (11%), France (9%), Germany (9%) and Italy (8%). Poultry meat is mostly from chicken (79.8%) (USDA, 2020).

Figure 2. EU-27 main chicken meat producing countries (USDA, 2020).



After years of growth, EU-27 chicken meat production declined by 1.6% in 2020 due to the impact of the Covid-19 pandemic that led to a lockdown in most EU-27 countries and the temporary closure of hotels, restaurants, and institutional cafeterias (HRI). While, chicken meat demand was less affected than other meats, the loss of meals taken outside the home was not fully replaced by purchases of chicken for at-home consumption. In most EU-27 countries, chicken meat slaughterhouses and processors had to switch from

bulk sales to the HRI sector to sales to the retail sector for household purchases. Most EU-27 countries, therefore, show decreases in production in 2020, except for Germany where at-home consumption remained high, despite pressure on production due to welfare and environmental issues that hamper the instalment of new poultry farms and the enlargement of existing ones (USDA, 2020).

In Poland, the largest EU-27 chicken producer, several cases of HPAI were reported in 2019 and 2020, leading several countries, as South Africa, China, South Korea, Singapore, Japan, Taiwan, the UAE, and the Philippines, to ban the import of Polish poultry and egg products. Based on Poland's regionalisation plan, Polish poultry imports were restricted by other countries (Ukraine, Belarus, Hong Kong, Kazakhstan, Russia, Armenia, Cuba, and Saudi Arabia). Prior to the Covid-19 outbreak, the growth in Polish chicken production was primarily export-driven with close to 50% of its production exported, and most of those exports to other EU-27 member states went to the HRI sector. Additionally, the ban on the export of Polish chicken to many third countries due to its HPAI outbreak led to sharply increased domestic meat stocks, which translated into lower farm-gate prices (USDA, 2020).

While the UK experienced an effective loss of the hospitality sector in March 2020, there has been a significant increase in retail consumption of chicken. Additionally, the take-out/delivery market for restaurants dramatically increased during the pandemic, so much so that the sector scaled up to address demand. The UK increased exports to the Netherlands and Belgium primarily due to the devaluation of the UK pound. Although at slower growth than before the Covid-19 pandemic, it is forecast that the demand for and production of chicken meat will resume in 2021 because of a preference for cheaper

protein sources, such as chicken, due to the unfavourable economic situation across most of Europe (USDA, 2020; OECD-FAO Agricultural Outlook 2020-2029).

Due to the temporary closure of the HRI sector and loss of tourists, the drop in Spanish chicken exports to the EU-27, and the contraction of household consumption, Spanish chicken production fell by 5% in 2020. This situation, combined with significantly lower prices (down 15% in April 2020) led the Spanish poultry association to request Private Storage Aid from the EU Commission, but it was rejected. However, it is expected that in 2021, Spain chicken production will return to its 2019 level (USDA, 2020).

Over the past 7 years, France's chicken meat production has stalled because of declining exports and competition from other EU-27 suppliers. The lockdown reduced consumer demand for chicken meat, but the French domestic retail market for chicken remained strong, as households prefer purchasing domestically produced chicken (USDA, 2020).

Since the topic of the thesis, which will be presented in the following chapters, concerns Italian chicken meat, more details are given to describe the recent Italian production. Italian poultry meat production in 2019 recorded a slight increase in supply +0.8% compared to 2018. The slight increase concerned the production of chicken meat (+ 1.5%) unchanged the production of turkey meat (301.000 tons) which, together, represent almost all (94%) of the national poultry production. The increasing trend of products with high added value (raw, cooked and breaded preparations) observed in previous years continues. Positive note for the sector is the 4.2 % increase in exports. Consumption in 2019, considering the balance between exports (184.300 tons) and imports (93.800 tons), stood at 1.233.400 tons (1.232.300 in 2018), equal to a per capita consumption of 20.45 kg: + 0.2% compared to the 20.40 kg recorded in 2018. The substantial overall stability of consumption reflects the adjustment of supply to internal demand and to changed

consumption habits. For fresh poultry meat purchases, on an annual basis, was only slightly down (-1%) in 2019, but spending is 0.6% higher. In addition, in 2019 the Italian poultry sector confirmed excellent levels of self-supply, resulting overall self-sufficient at 107.3%. Specifically, 104.8% of the chicken meat consumed in our country is produced in Italy, as well as the 118.3% of the turkey meat (UnaItalia, 2019; ISMEA Mercati, 2019). The Italian poultry industry entered in 2020 with positive expectations and, even as the pandemic struck, the agricultural sector was initially not hit as hard as others. Over the first quarter of 2020, sales of poultry meat through supermarket chains rose 8.9%. Demand for poultry meat over the first quarter was so strong that the producer, at times, were unable to fully respond. In May, the difficulties for COVID-19 that emerged in other EU-27 appeared also in Italy with a reorganization at plant level in the industries with conversion of some production channels to meet changing demanding response to the closure of foodservice (Poultry International, 2020). For example, beef have a far-reaching reorganisation of distribution circuits and supply chains, because the industry is heavily dependent on abroad, while the pig industry has led to a substantial reduction in production, above all due to reduced operation of the slaughterhouses, which had to reorganise their facilities to ensure the safety of their operators (Barcaccia *et al*., 2020).

2. ANTIMICROBIAL RESISTANCE AND STRATEGY TO REDUCE ANTIBIOTICS

2.1 Antibiotics in poultry production

The global poultry sector is characterized by faster growth in consumption and trade than any other major agricultural sector. Such great development of poultry sector is influenced by many factors, such as short fattening duration, excellent space utilization, high reproductive ability of poultry, excellent feed conversion, satisfactory nutritional value of poultry meat and relatively low sales prices. However, this astounding development of poultry sector was also supported by the intensive genetic selection for the growth rate, the improvement in nutrition and management of broilers, but also by the use of antibiotics as growth promoters (AGP). In fact, several factors account for the large quantities of antibiotics used in the past in the poultry industry: the fast growth rate of broilers in intensive rearing systems, leading to health and welfare problems; high stocking densities resulting in health problems and increasing the risk of transmission of diseases; high concentrations of ammonia damaging the chickens' immune systems and increasing vulnerability to respiratory diseases. AGP can be defined as any medicines that destroy or inhibit bacterial growth and are administered at a low subtherapeutic dosage (Hughes and Heritage, 2004). Between 1950 and 2000, the majority of poultry feeds contained AGP used as a tool for the control of pathogenic diseases and for the efficient livestock production (Castanon, 2007). The growth promoting properties of antimicrobial agents in farm animals were discovered in the late 1940s by Moore *et al*. (1946) when

they observed that chickens fed streptomycin exhibited increased growth response. Since then, the use of growth promoters has been expanded to include a wide range of antibiotics that are applied to several species. The mechanisms of growth promotion are still not exactly known and different hypotheses have been proposed in the last decades. However, with the advent of novel molecular biology and bioinformatics techniques, it is clear that modifications in microbiota composition (structure and diversity), which occur when antibiotics are included in animal diets, may ultimately result in an optimal and balanced microbiota that is less capable of evoking an inflammatory response, increases energy harvest from nutrients, and helps animals perform to their genetic potential (reviewed in Gadde *et al*., 2017). It was reported that the net effect of using in-feed antibiotics in the poultry industry was a 3–5% increase in growth and feed conversion efficiency (Choct, 2001; Dahiya *et al*., 2006), contributing significantly to the economic effectiveness of the poultry production sector. Antibiotic usage has enhanced the health and well-being of poultry by reducing the incidence of disease and has also facilitated the efficient production of poultry, allowing the consumer to purchase, at a reasonable cost, high quality meat and eggs. However, this approach has had significant and unwanted side-effects, such as the spread of drug-resistant pathogens in both livestock and humans, posing a significant public health threat. Due to the emergence of microbes resistant to antibiotics which are used to treat human and animal infections ("anti-microbial resistance", AMR), the European Commission decided to phase out, and ultimately ban (EC Regulation No. 1831/2003), the marketing and use of antibiotics as growth promoters in feed. Since 1 January 2006 the use of antibiotics (other than coccidiostats and histomonostats) as feed additives is forbidden. Antimicrobial agents are therefore administered to chickens and other food animals as preventive or curative treatments. The

European Union's strategy to ban AGPs has been adopted by countries such as Mexico, New Zealand, and South Korea. This is the responsible thing to do, although it may prove to be too radical a step for some countries. The USA, Australia, Japan, or Canada have enacted laws to partially ban antibiotic-derived additives and to exclude some (Krysiak *et al*., 2021).

The use and misuse of antimicrobials in many parts of the world are recognized as key drivers of the emergence and spread of AMR. The AMR is an ancient and naturally occurring phenomenon in bacteria; however, the use of antimicrobial drugs – in health care, agriculture or industrial settings – exerts a selection pressure which can favour the survival of resistant strains (or genes) over susceptible ones, leading to a relative increase in resistant bacteria within microbial communities (FAO, 2016). Antimicrobial resistance is when a microbe evolves to become more or fully resistant to antimicrobials which previously could treat it, with consequent loss of therapies efficacy and serious risks for the human health. Natural resistance may be intrinsic (always expressed in the species), or induced (the genes are naturally occurring in the bacteria, but are only expressed to resistance levels after exposure to an antibiotic). Acquisition of genetic material that confers resistance is possible through all of the main routes by which bacteria acquire any genetic material: transformation, transposition, and conjugation (all termed horizontal gene transfer); plus, the bacteria may experience mutations to its own chromosomal DNA. The acquisition may be temporary or permanent. Plasmid-mediated transmission of resistance genes is the most common route for acquisition of outside genetic material; bacteriophage-borne transmission is fairly rare (Reygaert, 2018). Antimicrobial resistance affects both humans and animals and resistance can also spread from animals to humans through the food chain or direct contact. The emerging and steady increase in the

occurrence of bacteria that are resistant to multiple antibiotics has become a global public health threat due to the lack of therapeutic options to treat certain infections in humans. A well-known example of a bacterium that is resistant to a number of antibiotics is meticillin-resistant *Staphylococcus aureus* (MRSA), which has caused infections that are difficult to treat across the European Union. Infections by multidrug-resistant bacteria are estimated to cause 25,000 deaths in the EU every year. Antimicrobial resistance also places a tremendous burden on healthcare systems and society, with an annual cost due to healthcare expenditures and productivity losses estimated at approximately €1.5 billion in the EU (European Medicines Agency, EMA). Due to the interdependence and interconnectedness of epidemiological pathways between humans, animals and the environment, determining the relative importance of factors influencing AMR emergence and spread in animal production is a significant challenge, and is likely to remain one for some time. Addressing AMR is a cross-sectorial issue, requiring action by different policy areas, from health to agriculture, aquaculture and environment, from research to users, stakeholders and policy makers. It requires urgent multisectoral action in order to achieve the Sustainable Development Goals (SDGs). Combatting the threat of antimicrobial resistance, particularly resistance to antibiotics, is a high priority for the EMA and the European medicines regulatory network. In veterinary medicine, EMA is promoting prudent use of antimicrobials in animals, collecting data on the use of veterinary antimicrobials in the European Union (EU), and providing scientific recommendations on the use of specific antimicrobials in animals. Since 2010, the Agency has been leading the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project, collecting information on how antimicrobials are used in animals across the EU and the European Economic Area. Furthermore, the European

Committee for Medicinal Products for Veterinary Use (CVMP) is focusing on implementing the provisions of the Veterinary Medicines Regulation; in fact, in October 2018, Parliament approved new legislation to ban the prophylactic (preventive) use of antibiotics in farming, which will come into force in 2022. Regulation (EU) 2019/6 of 11 December 2018 on veterinary medicinal products which will continue to strengthen the EU's fight against antimicrobial resistance. The latest ESVAC report, published in October 2020, shows that sales of antibiotics for use in animals in Europe fell by more than 34% between 2011 and 2018. Of particular importance, the veterinary sales of antibiotics considered critically important in human medicine present a decreasing trend. Between 2011 and 2018, sales have reduced by: 24% for third and fourth generation cephalosporins; 70% for polymyxins; 4% for fluoroquinolones; 74% for other quinolones (ESVAC, 2018). The situation across Europe remains contrasting. Out of the 25 countries that provided data covering 2011-2018, 18 countries observed a decline in sales of veterinary antibiotics overall sales have dropped by more than 5%. However, 5 countries recorded an increase of more than 5% and two other countries noted a minor decrease (below 2%) in overall sales. The substantial decline in some countries indicate that there is also a potential for a decrease in other countries.

According to the National Residual Plan (PNR) drawn up by the Italian Ministry of Health, only 81 out of about 33,000 samples analyzed were positive. Poultry is probably the livestock sector where more progress has been made towards a rational use of antimicrobials and reduction of antimicrobial resistance. From 2011 to 2018, for example, through adherence to a voluntary reduction plan**,** the consumption of antibiotics in the poultry sector decreased by 82%, proving the commitment of the whole farming sector (Unaitalia, 2021).

2.2 Alternatives to antibiotic

Reducing the dependence on the use of antimicrobials in animal production is one of the main EU challenges. In line with the EU animal health strategy, "prevention is better than cure", alternative strategies to antimicrobials need be developed. In addition, the current public opinion is forcing livestock sector to develop alternatives, or at least substantially reduce the amount of antibiotics used to maintain production efficiency and produce safe meat and egg products. In the recent years, after the AGP removal, the poultry industry has been suffering from unsatisfactory production efficiency, bacterial overgrowth in the small intestines, nutrient malabsorption, and associated food contamination (Cervantes, 2015). One disease syndrome that is clearly emerging in the EU broiler industry simultaneously with the ban of growth promoting antibiotics is often referred to as 'dysbacteriosis'. This is a poorly described condition of the gut and may be synonymous with conditions such as 'wet litter', 'small intestinal bacterial overgrowth', 'malabsorption', and 'feed passage syndrome' (Huyghebaert *et al*., 2011). To overcome these emerging problems, substantial scientific progress has been made to find non-antibiotic alternatives which are mainly focused on the regulation of the intestinal microbiota to improve animal health and resilience. Alternatives to antibiotics promote gut health by several possible mechanisms including altering gut pH, maintaining protective gut mucins, selection for beneficial intestinal organisms or against pathogens, enhancing fermentation acids, enhancing nutrient uptake, and increasing the humoral immune response. Strategic use of these alternative compounds will help optimize growth provided they are used in a manner that complements their modes of action. Considering the proposed mechanism of action of AGPs (microbiome and immune-modulating activities), a practical alternative should possess both of these properties in addition to

having a positive impact on feed conversion and/or animal growth (Huyghebaert *et al*., 2011; Seal *et al*., 2013). Several classes of alternatives have been proposed and tested in poultry production (Table 2) such as probiotics, prebiotics, phytobiotics, enzymes, organic acids, surfactants, hyperimmune egg yolk IgY, antimicrobial peptides, bacteriophages and clay (Gadde *et al*., 2017; Jha *et al*., 2020; Krysiak *et al*., 2021).

Table 2. Advantages and disadvantages of eight classes of feed additives used as an alternative to antibiotic growth promoters (AGP) in poultry production (Jha *et al*., 2020).

Alternative to AGP	**Description**	**Advantages**	**Disadvantages**
Probiotics	Live bacteria and yeasts that provide health benefits	• Improves digestion • Strengthens immune function	• Strain and dose-dependent • Possible adverse side effects
Prebiotics	No-digestive fibers that stimulate growth or activity of certain healthy bacteria	• Improves mineral adsorptions • Enhance immune function	• Dose-dependent • Possible adverse side effects
Hyperimmune IgY	An antibody that helps transfer passive immunity	• Environmentally friendly • Reduces the number of animals required for antibody production	• Susceptibility to proteolytic degradation in the gut • High manufacturing costs
Antimicrobial Peptides	Proteins with broad-spectrum antimicrobial activities against bacteria	• Broad-spectrum beneficial activity	• High manufacturing costs • Systemic and local toxicity • Susceptibility to proteolytic • Natural resistance
Organic Acids	Different acids that have antimicrobial activity	• Improves growth performance • Strengthens immunity	• Dose-dependent • Possible adverse side effects
Pthytogenics (Oleoresin, Essential oils)	Natural growth promoters or non AGP-s used as feed additives derived from herbs, spices, or other plants	• Improves growth performance	• Potential interaction with bacteria
Enzymes	Exogenous feed enzymes that break down fiber and other (anti-nutritional) cmponents of the diet-o.g, phytase	• Improves growth performance • Strengthens immunity	• Highly sensitive to the environment
Clay	Supplement used as a binding and lubricating agent in the production of pelleted feeds	• Enhance growth performance • Combats bacterial infections in poultry	• Potential interaction with bacteria • Possible adverse side effects

Although the beneficial effects of many of the alternatives tested have been well demonstrated, there is a lack of consistency, since results vary greatly from farm to farm. Further research is needed to identify means to standardize the effects. In addition, using optimal combinations of various alternatives coupled with good management and husbandry practices could be the key to maximizing performance and maintaining animal productivity, reducing antibiotic use in the animal industry (Gadde *et al*., 2017).

2.2.1 Intestinal microbiota modulators: probiotics, prebiotics, synbiotics

Specific probiotics, prebiotics and their synergistic combination (synbiotics) could be crucial to modulate effectively the gastrointestinal system of farm animals, to limit the incidence of infections and reduce the use of antimicrobials in the future. The statement "immunity comes from the intestine" has become more significant in the poultry industry with the emerging use of probiotcs (Krysiak *et al*., 2021). Microbiota of the avian gastrointestinal tract (GIT) constitute a key factor in the development and regulation of immunity, digestion, and absorption of nutrients and their metabolism (Patterson and Burkholder, 2003; Hajati and Rezaei, 2010). GIT microbiota can be modulated by bioactive substances, such as prebiotics, probiotics, or synbiotics (Yang and Choct, 2009). These bioactive compounds can directly modulate the host microbiome and, consequently, indirectly affect host organisms (Dunislawska *et al*., 2017).

Probiotics

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 (2009) have

defined probiotics as “live microorganisms, which when administered in adequate amounts, confer a health benefit on the host”. Since the term “probiotic” is reserved for products that meet some strictly criteria of selection, in 2013, the International Scientific Association for Probiotics and Prebiotics (ISAPP) defined probiotics as “live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Hill *et al*., 2014). In recent years, some of these live probiotic cultures have begun to be described by the general term “Eubiotics”, which is related to the Greek word “Eubiosis”, referring to an optimal microbiota balance in the GIT (Miniello *et al*., 2017). Thirty probiotic preparations are currently registered in EU (Krysiak *et al*., 2021). A variety of bacteria (*Bacillus, Bifidobacterium, Enterococcus, Lactobacillus, Streptococcus*, and *Lactococcus* spp.) and in some cases yeasts (*Saccharomyces* spp.) have been tested as probiotics in poultry (Simon *et al*., 2001; Patterson and Burkholder, 2003; Griggs and Jacob, 2005; Kabir, 2009). To date, the main production practice in poultry is to provide infeed or in-water doses of bioactives as soon as possible after hatch, to help newly hatched chicks to rapidly establish a healthy gut microbiome. However, implementation of *in ovo* technology for bioactives delivery allows to provide the growing embryo with the potent microbiome stimulant as early as on 12th day of embryonic incubation. Such procedure results in improvement of beneficial bacteria count in gut with a life lasting effect, and positively influences growth and development of the adult chickens (Tavaniello *et al*., 2019). There is now a large body of literature reporting the effect of probiotics on poultry production. Faria Filho *et al*. (2006) carried out a meta-analysis of 27 studies involving 30,146 broiler chickens that were conducted in Brazil during 1995–2005 to investigate the performance effects of 12 different probiotics. The results showed that the probiotics promoted better weight gain

and feed conversion in the initial phase (1 to 20-28 days) compared with non-supplemented controls; while similar results were found in the total period (1 to 35-48 days). Weight gain and feed conversion were similar between probiotics and the positive control (with antimicrobial) both in the initial and in the total periods. A similar meta-analysis of several randomized controlled research trials that were carried out from 1980 to 2012 was conducted by Blajman *et al*. (2014) to investigate the effects of probiotics on body weight gain and feed efficiency in broilers. They concluded that probiotics inclusion increased body weight gain and improved feed efficiency, and also showed that probiotics application via water was more efficacious than through feed. The analysis also showed that there were no differences between the use of mono- or multi-strain probiotics and the effects observed may vary with the type of strain used. In a recent review, Jha *et al*. (2020) provided a summary of the use of probiotics in poultry production and the potential role of probiotics in the nutrient utilization, growth and laying performance, and gut health of poultry, summarized in Table 3. The authors reported a range of variation in the observed benefits due to different factors such as the intestinal health condition of birds, the probiotic inclusion level, the incubation conditions, feedstuff and water quality offered to birds.

Table 3. Summary of the beneficial probiotic species used in poultry production (Jha *et al*., 2020).

Strain	**Characteristics**	**Benefits**	**References**
Bacillus amyloliquefaciens	Root-colonizing biocontrol bacteria used to fight plant root pathogens in agriculture, aquaculture, and hydroponics.	Enhances gut health and growth performance.	References are reported in Jha *et al*., 2020).
Bacillus coagulans	Bacteria exhibits the characteristics of both genera Lactobacillus and Bacillus.	Improve growth performance and gut histomorphology.	
Bacillus licheniformis	Bacteria commonly found in soil.	Prevents necrotic enteritis and enhances growth performance.	
Bacillus subtilis	Bacteria found in soil and the gastrointestinal tract of ruminants and humans.	Enhances laying performance and helps the immune system and gut health.	
Bifidobacterium animalis	Bacteria found in the large intestines of most mammals.	Helps the immune system, gut physiology, and health	
Bifidobacterium bifidum	Bacteria that is one of the most common probiotic bacteria that can be found in the body of mammals.	Helps the immune system and gut health	
Lactobacillus acidophilus	Bacteria found in the human and animal gastrointestinal tract and mouth.	Enhances gut health and growth performance.	
Lactobacillus bulgaricus	Bacteria found in the gastrointestinal tract of mammals and naturally fermented products.	Enhances growth performance and improves immune functions.	
Lactobacillus bifermentans	Bacteria found in the human and animal gastrointestinal tract.	Enhances growth performance and digestive health.	

Lactobacillus fermentum	Bacteria found in fermenting animal and plant material.	Enhances growth performance, gut histomorphology, and immune functions.
Lactobacillus salivarius	Bacteria found in the human and animal gastrointestinal tract.	Improves laying performance and enhances gut histomorphology.
Lactobacillus sanfranciscensis	Heterofermentative bacteria closely related or normally present in sourdough.	Enhances growth performance.
Lactobacillus reuteri	Bacteria that naturally inhabits the gut of mammals and birds.	Enhances growth performance, gut histomorphology, immune system, and gut health.
Pediococcus acidilactici	Bacteria found in fermented vegetables, fermented dairy products, and meat.	Improves laying performance and modulates the gut microbiota.
Propionibacterium acidipropionici	Found in dairy products and the environment.	Contributes to the better development of gut mucosa.
Saccharomyces cerevisiae	A species of yeast found primarily on ripe fruits such as grapes.	Enhances growth performance and improves laying performance.
Streptococcus faecium	Bacteria inhabiting the gastrointestinal tracts of humans and other mammals.	Improves immune functions.

Although the benefits are evident in different studies, details about probiotics' mechanisms of action are yet to be unrevealed. The two most important mechanisms through which probiotics exert beneficial effects include the promoting effect on GIT health (eliciting a positive impact on GIT morphology, microbial populations, nutrient

absorption, intestinal barrier function, antioxidant capacity), and immune regulation (Gadde *et al*., 2017; El jeni *et al*., 2021). Probiotics help establish a microenvironment in the gut that favours beneficial microorganisms and reduces the colonization of pathogenic bacteria (competitive exclusion) by: (1) creating a hostile environment for harmful bacterial species (through production of lactic acid, SCFA, and reduction in pH); (2) competing for nutrients with undesired bacteria; (3) production and secretion of antibacterial substances (e.g. bacteriocins by *Lactobacillus*, *Bacillus* spp.); and (4) inhibition of bacterial adherence and translocation (reviewed in Gadde *et al*., 2017). Probiotics can enhance the function of intestinal barrier, which is the major defence mechanism used to maintain epithelial integrity and to protect the organism from the environment. Defences of the intestinal barrier consist of the mucous layer, antimicrobial peptides, secretory IgA and the epithelial junction adhesion complex (Ohland and Macnaughton, 2010). Several studies have indicated that enhancing the expression of genes involved in tight junction signalling is a possible mechanism to reinforce intestinal barrier integrity (Anderson *et al*., 2010). Probiotics improve the intestinal function also by maintaining epithelial cell homoeostasis, promoting cytoprotective responses and cell survival (through production of cytokines that enhance epithelial cell regeneration and inhibit apoptosis) and increasing mucin synthesis (reviewed in Gadde *et al*., 2017). It has been also reported that probiotics play an important role in digestion and nutrient retention by increasing digestive enzyme activity and improving the breakdown of indigestible nutrients (reviewed in Gadde *et al*., 2017). It is well known that probiotics can exert an immunomodulatory effect. These bacteria have the ability to interact with epithelial and dendritic cells and with monocytes/macrophages and lymphocytes. Probiotics act by reducing pro-inflammatory cytokines, increasing secretory IgA

production, and promoting specific and non-specific immune responses against pathogens (activation of macrophages, increase cytokine production by intraepithelial lymphocytes) (reviewed in Gadde *et al*., 2017). In light of the above-mentioned properties, probiotics are viewed as a valuable alternative to antibiotics that promote a "healthy" intestinal microbiota, improve growth performance, limit animal disease and inhibit foodborne pathogen growth (El Jeni *et al*., 2021). However, probiotics affect not only the growth performance of animals, but also the physico-chemical properties of meat, depending on composition and concentration of given probiotic. Some trials showed that the enrichment of diets with yeast could favourably improve the quality of meat from broilers. Studies on the probiotic administration in poultry showed that pH might be influenced, but the results depend on the type of microorganisms and also on the specifics of the experimental design (Popova, 2008). As an example, meats from broiler chickens fed a diet containing chromium-enriched *S. cerevisiae* or *S. cerevisiae* cell wall exhibited increased tenderness (Zhang *et al*. 2005) and increased water holding capacity (Lee *et al*. 2002). A study conducted by Zheng *et al*. (2015) has shown a significantly lower 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. It has been reported that probiotics containing *Bacillus licheniformis* in the poultry diet enhanced the meat colour, flavour and juiciness of meat (Liu *et al*., 2012).

Regarding the chemical composition of meat, it has been shown that the administration of probiotics also has a positive effect on increasing the levels of chemical elements in the liver (Ca, K, Mg, Mn, Si, and Zn) and chicken breast muscles (Ca, Na, Co, Cu, Fe, Mn, Ni, and Zn) (Duskaev *et al*., 2020). The research on the chemical composition of meat

showed that *Bacillus licheniformis* administration increased significantly the protein content and respective content of essential and flavour amino acids, while on the other hand, the fat content was decreased (Liu *et al*., 2012). The administration of probiotic microorganisms improves the profile of fatty acids by reducing their saturation. It is quite rare, but applying *Aspergillus awamori*, *Saccharomyces cerevisiae* or a combination of these promotes this effect. The TBARS tests have proven that probiotics increase the oxidative stability of meat (reviewed in Krysiak *et al*., 2021). Following probiotic administration to broilers, the reduction of cholesterol and fat content in the breast and thigh meat has been observed (Hossain *et al*., 2012).

The reduction of cholesterol could be achieved through its assimilation by bacterial growing cells or incorporation in the cellular surface of the probiotic microorganism, thus inhibiting the absorption of the cholesterol back in the body. In addition, increased deconjugation of bile acids by bacterial hydrolases has been reported. Furthermore, the short chain fatty acids produced by probiotics, could exert controlling effect on lowering hepatic lipogenesis and the inhibition of lipogenesis process related to the decrease in meat fat content (reviewed in Popova, 2017).

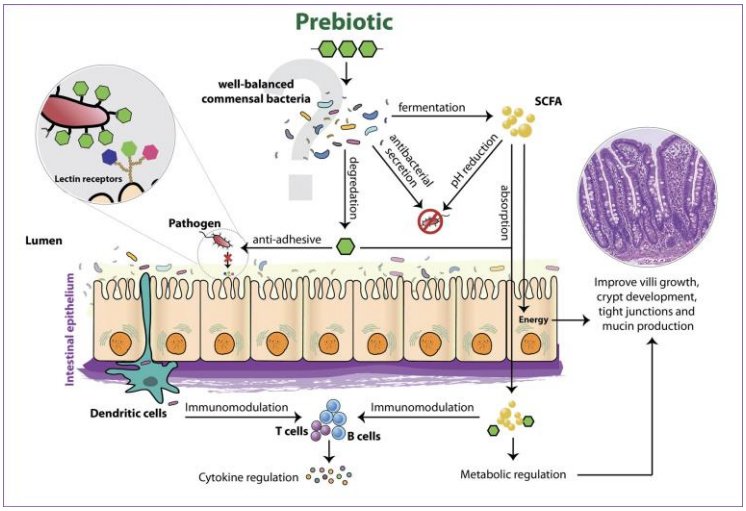
Prebiotics

The prebiotic concept is much younger than that of probiotic and was first introduced by Gibson and Roberfroid (1995) as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health." As for probiotics, the original definition has been modified frequently, but so far, no consensus has been reached. In 2007, the FAO Technical Meeting on Prebiotics

defined the term “prebiotic” as “a non-viable food component that confers a health benefit on the host associated with the modulation of microflora” (Pineiro *et al*., 2008). In 2017, the International Scientific Association of Probiotics and Prebiotics (ISAPP) defined prebiotics as “a substrate that is selectively utilized by host microorganisms, conferring a health benefit” (Gibson *et al*., 2017). For a dietary substrate to be classified as a prebiotic, at least three criteria are required: (1) the substrate must not be hydrolysed or absorbed in the stomach or small intestine, (2) it must be selective for beneficial commensal bacteria in the large intestine, and (3) fermentation of the substrate should induce beneficial luminal/systemic effects within the host (Gibson *et al*. 2004). Compounds that meet these criteria are certain non-digestible carbohydrates such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), mannan-oligosaccharides (MOS), and related carbohydrate polymers (Patterson and Burkholder, 2003; Ricke, 2015, 2018a). Considering that the definition of what constitutes a prebiotic continues to be refined other compounds such as resistant starch, cereal grain components, lactulose, and other sources have also been considered as potential prebiotics or at least compounds that exhibit some characteristics that could be considered prebiotic-like (Bird *et al*., 2010; Ricke, 2015, 2018b, 2021; Roto *et al*., 2016; Hutkins *et al*., 2016; Gibson *et al*., 2017). Traditionally, prebiotics were believed to favour certain beneficial GIT bacteria such as *Lactobacillus* and *Bifidobacterium*. However, the introduction of 16S rDNA based Next-Generation sequencing has revealed that the poultry GIT microbiome response to dietary prebiotics may involve more members of the GIT microbial community than just a select few (reviewed in Ricke *et al*., 2021).

Several studies have shown that dietary supplementation of prebiotic had beneficial effects on gut health and productive traits. Prebiotics stimulate the proliferation of beneficial bacteria, inhibit the colonization of pathogenic bacteria, improve nutrient absorption, promote growth rate and feed utilization efficiency. However, at present, the exact mechanisms for pathogen inhibition have not been elucidated. Prebiotics are metabolized through commensal microorganisms, leading to host health benefits (Gibson *et al*., 2017). Most of the prebiotic impact occurs in the lower parts of the GIT, particularly the birds' ceca, with some microbial hydrolysis that could occur in the upper sections, such as the crop (Ricke, 2018a). Various potential mechanisms have been proposed for health benefits of prebiotic-mediated changes in the gut microbiota (summarized in Figure 3; Pourabedin and Zhao, 2015) such as: competitive exclusion of pathogens (Callaway *et al.* 2008); production of antimicrobial factors (Chen *et al.*, 2007; Munoz *et al.*, 2012); stimulation of host adaptive immune system (Babu *et al.* 2012; Yitbarek *et al.* 2012); improving gut morphological structure (Chee *et al.* 2010; Pourabedin *et al.* 2014).

Figure 3. Potential mechanisms of action of prebiotics (Pourabedin and Zhao, 2015).



Probably among the best characterized properties identified with GIT microbial antagonism of foodborne pathogens is the production of short chain fatty acids (SCFA) and lactate during fermentation. Since 1992, Russell hypothesized that some bacteria such as the lactic acid bacteria could tolerate lower intracellular pH levels than their pathogen co-inhabitants such as *E. coli* which strive to maintain a more neutral intracellular pH. Van Immerseel *et al*. (2006) suggested that the presence of certain SCFA such as butyrate may down regulate *Salmonella* invasion genes while propionate, but not acetate, can inhibit epithelial cell invasion. However, acetate may elicit other impacts on foodborne pathogens. It has been reported that prebiotics can limit *Salmonella* establishment either by altering the microbial taxonomic composition and/or fermentation activities in GIT that lead to a hostile GIT environment against *Salmonella* establishment or, in the case of MOS, directly interfere with mannose-specific type 1 fimbriae attachment by *Salmonella* (Micciche *et al*., 2018). MOS have also been reported to improve overall gut health through increasing villi length and providing an adjuvant-like effect by acting as a microbial antigen (reviewed in Micciche *et al*., 2018). In light of this, prebiotics can improve the safety of poultry products by promoting the overall health and well-being of the bird as well as provide for an intestinal environment that is unfavourable for foodborne pathogens such as *Salmonella* (Micciche *et al*., 2018).

As mentioned above, there is no exact mechanism of action for beneficial effects of prebiotics, so that stimulation of poultry performance results from the very complex interactions of all mechanisms previously described, for instance, by decreasing pathogen colonization. It has been reported that pathogens depress performance by interfering with nutrient digestion, absorption, and utilization. At least some prebiotics can also directly serve as immuno-modulatory agents (Teng and Kim, 2018). The combined influence of

feeding prebiotics on the GIT microbial population and corresponding host responses would presumably be reflective in detectable differences in poultry performance (Ricke, 2021). Hajati and Rezaei (2010) suggested that along with limiting pathogens, prebiotics could result in better overall bird performance, improved GIT health and enhanced nutrient utilization accompanied by decreases in environmental pollution and production costs. However, poultry production responses across experimental studies can still be inconsistent and depend upon numerous factors, making predictions and recommendations difficult (Ricke, 2021). Fructooligosaccharides (FOS) are naturally occurring, typically of plant origin (onion, chicory, garlic, asparagus, banana, artichoke as well as other sources), contain β-(2,1) linkages, and can be food ingredients, functional foods, and prebiotics (Gibson and Roberfroid, 1995; Ricke, 2015). Due to the β-(2,1)-linkages, enzymatic degradation is difficult in the upper GIT, leading to primary breakdown occurring in the ceca (Micciche *et al*., 2018). FOS is fermented by *Bifidobacteria* and *Lactobacillus* species, which could boost the host's gut health (Ricke, 2015, 2018). FOS may increase lactic acid and SCFA concentration and inhibits the growth of pathogens such as Clostridium perfringens, one of the major causes of high mortality in poultry production (Ricke, 2015; Kumar *et al*., 2019). Several studies reported that FOS administration in diet significantly improved bird's performance (Xu *et al*., 2003; Kim *et al*., 2011; Shang and Kim, 2017) demonstrated a positive effect of FOS on the broilers' body weight gain and feed efficiency. The improvement in feed conversion ratio (FCR) was positively associated with the increased enzymatic activity of protease, amylase, and leucine aminopeptidase (Xu *et al*., 2003; Ricke, 2015, 2018a; Micciche *et al*., 2018; Kim *et al*., 2019). However, the adverse influence of FOS was revealed by Ten Bruggencate *et al*. (2003), claiming that these components may

encourage some harmful bacterial growth. Other classes of prebiotics showing promise as an antibiotic alternative owing to their efficacy in improving bird's performance, such as isomaltooligosaccharide (Mookiah *et al*., 2014), lactulose (Cho and Kim, 2014), lignin (Baurhoo *et al*., 2007), inulin (Rebol *et al*., 2010), and palm kernel extract (Rezaei *et al*., 2015). In contrast to the previous results, several authors reported that prebiotic supplementation had no effect on performance (reviewed in Gadde *et al*., 2017). Mannanooligosaccharides (MOS) are found in the cell wall of numerous fungal species including brewer's yeast (*Saccharomyces cerevisiae*) and *Saccharomyces boulardii*, as well as certain plants, which are associated with improved broilers' growth performance (Hooge, 2004; Rosen, 2007). Hooge (2004) used meta-analysis to summarize results from global pen broiler trials of birds fed MOS over a range of diets and different environmental conditions that had been conducted for over 10 y. Several criteria were used to select the studies to include appropriate controls (negative control versus positive control consisting of antibiotic supplementation), feeding MOS for the duration of the trial, and proper replication, among others. Based on these analyses, Hooge (2004) detected improvements in feed conversion and body weight compared to the corresponding control. Still, mortalities were significantly lowered in MOS fed birds and were considered the predominant beneficial outcome of MOS supplementation to broilers compared to antibiotic fed birds (Ricke, 2021).

The galactooligosaccharides (GOS) are synthesized from lactose by glycosyl transfer of the D-galactosyl unit to the D-galactose moiety of lactose, and the catalysis of the hydrolysis of β-galactosides via β-galactosidase (Ricke, 2015,2018; Micciche *et al*., 2018; Kim *et al*., 2019). Several studies suggest the gut health-promoting effect of dietary GOS. Varasteh *et al*. (2015) found that GOS could not mitigate the alterations in the

ileum, but successfully prevented all heat-stress induced changes in the jejunum. Recently, Slawinska *et al*. (2019a) found that GOS delivered *in ovo* had a bifidogenic effect in adult chickens; it also increased the expression of the cytokine genes, barrier function genes and free fatty acid receptors, and varied the expression of the glucose transporter genes in the intestinal mucosa. Moreover, in a subsequent study, Slawinska *et al*. (2019b) found that stimulation *in ovo* with GOS prebiotic dampened heat-induced immune- and stress-related gene expression signatures in spleen of chickens exposed to acute heat stress. Promotion of the intestinal health through embryonic stimulation of the intestinal microflora with GOS has not only a beneficial effect on host systems but also on growth performance. In fact, GOS delivered *in ovo* significantly improved the resilience of birds exposed to heat stress and improved feed and growth efficiency (Slawinska *et al*., 2019c). As for welfare traits, Slawinska *et al*. (2019 c) also found that *in ovo* stimulation with GOS dampened body temperature in both thermoneutral and heat stress conditions and numerically improved survivability during heat stress; in addition, it was found that GOS delivered *in ovo* decreased the prevalence of food-pad dermatitis in thermoneutral conditions by 20% (no lesions in 81% in GOS vs. 60% in C). Considering all this evidence, the aim of this study was to evaluate the effect of *in ovo* GOS prebiotic injection and chronic heat stress on meat quality traits of fast-growing broiler chickens.

Given the economics and large quantities potentially required for poultry nutrition, less purified and therefore cruder fractions of non-digestible oligosaccharides (NDO) sources could be potentially attractive. Certain cereal grains represent a source that is already a major part of poultry diets, generally contain non-starch polysaccharides. Cereal grains and specific components such as the bran fraction provide potential sources of prebiotic ingredients. Brans derived from rice and wheat have been shown to exhibit modulation of

cecal microbiota composition and metabolic activities in chicken cecal contents (Ricke, 2018b, 2021). As stated by Ricke *et al*. (2021), more research are needed to better understand the mechanisms of various prebiotics by evaluating the effects of prebiotics at the molecular level, while focusing on assessment of GIT microbiome analyses, host GIT responses from metabolism, and immune response stand-point.

Synbiotics

The synbiotics, developed to overcome possible survival difficulties for probiotics, are the synergistic combination of probiotics and prebiotics (de Vrese and Schrezenmeir, 2008). In 1995, Gibson and Roberfroid introduced the term "synbiotic" referring to "a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the GIT, by selectively stimulating the growth and/ or activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare". Two types of synergism between prebiotic and probiotic have been defined. Both compounds can be: i) synergistic with each other as the prebiotic stimulates growth of probiotic bacteria; ii) synergistic with the host, assuming that the prebiotic and probiotic act independently in the GIT where they stimulate development of the host microbiota. Indigestible oligosaccharides (prebiotics) are fermented in the GIT, while beneficial live microorganisms (probiotics) colonize the GIT (reviewed in Dunislawska *et al*., 2017). Regarding the effect of synbiotics (delivered *in ovo* or in feed) on growth performance and meat quality different are the studies yielding sometimes contradictory results. Some studies (Awad *et al*., 2009; Mookiah *et al*., 2014; Ghasemi *et al*., 2016; Cheng *et al*., 2017), using different kind of synbiotics supplemented in feed, reported beneficial effects

on growth performance, feed efficiency, carcass, and some meat quality traits. Differently, other authors found minimal (Maiorano *et al*., 2012; Tavaniello *et al*., 2019) or none (Jung *et al*., 2008; Midilli *et al*., 2008) effect of synbiotics, delivered *in ovo* or in feed, on growth performance and on meat quality traits. These results reveal the complexity of the interaction taking place in the gastrointestinal tract, also related to the kind of bioactive administered. There is a great potential for synbiotics to be used as antibiotic alternatives for improving performance and reducing pathogenic load in the intestines of poultry. Careful consideration must be given when selecting the combinations of various prebiotics and probiotics to be used as synbiotics, and research trials should be conducted to demonstrate their synergistic effect compared with the use of either product alone (Gadde *et al*., 2017).

3. *IN OVO* STIMULATION OF EMBRIONIC CHICKEN MICROBIOME

An ideal route for optimizing bird performance and health would be to establish a healthy and balanced GIT microbiome at the beginning of its formation rather than trying to alter an already established GIT microbiome (Roto *et al*., 2016). For an effective microbiome stimulation, bioactive substances such as pre-, pro-, and synbiotics need to be delivered as early as possible, since the microflora of the hatched chicks is very poorly developed. In fact, during the period immediately following hatch, the immune system of the chick is immature and inefficient, rendering birds vulnerable to environmental threats. Considering this, immunomodulators are being sought after and studied in order to protect these birds during this immunologically sensitive time (Pender *et al*., 2017). Stimulation of commensal microbiota is critical, as it affects, to a great extent, the entire life-span of an individual, and also because the nutritional manipulations of the GIT microbiome to enhance productivity and health are rather limited by the resilience of the ecosystem once established in the chicken's gut (Siwek *et al*., 2018; Rubio, 2019). The main functions ascribed to the intestinal microbiota in broilers are: (i) nutrient exchange, (ii) modulation of the immune system, (iii) physiology of the digestive system, and (iv) pathogens exclusion (Oakley *et al*., 2014; Stanley *et al*., 2014). It is generally accepted that the establishment of an adequate microbiota is an effective barrier to colonization by opportunistic pathogens, provides metabolic substrates required by the animal (vitamins, SCFA, etc.), and is a stimulus for proper development of the immune system (Lan *et al*.,

2005). To avoid random composition of chicken microbiota, a planned microbiome stimulation might be introduced through direct supplementation of chicken embryo with proper bioactive substances (Siwek *et al*., 2018). To date, the main production practice in poultry is to provide in-feed or in-water doses of bioactives as soon as possible after hatch, to help newly hatched chicks to rapidly establish a healthy gut microbiome. In this case, the whole embryonic and perinatal period is completely ignored. The perinatal period, which lasts from the last few days prior to hatch to the first few post-hatching days, is currently recognized as the most crucial time in the development of the chicks; this is a transitional time in which the chicks undergo metabolic and physiological shifts from the utilization of egg nutrients to exogenous feed (Roto *et al*., 2016). In addition, in modern commercial settings, hatchlings may be fasted for 48-72 hours until being delivered to rearing farms. For a commercial broiler, the incubation and neonatal periods represent approximately 50% of the productive lifespan of the bird. The time from embryonic day 18 to 4 days post hatch is critical for the survival and development of the chick (Ferket, 2006). Uni and Ferket (2003) proposed providing nutrients during embryonic development to minimize the negative effects of post-hatch fasting and transition phase, thereby ensuring maximum expression of the genetic potential throughout the production cycle. *In ovo* technology represents a viable way for modulating the condition inside the egg through vaccines, nutrients and bioactive substances. This technique may represent a means to both compensate for the starvation period of hatchlings and facilitate early establishment of a healthy GIT microbiome before it is exposed to any pathogens (Roto *et al*., 2016). Early access to substances (pre- and post-hatch) is particularly relevant here because in broilers, which are selected for rapid early growth, much of the immune system development occurs early in life.

In ovo technology was first applied to the delivery of immunostimulants (vaccines) to confer poultry immunity against economically important diseases such as Marek's disease. The Inovoject® machine, manufactured by Embrex, Inc., was the first automated system to be introduced in the United States (Ricks *et al*., 1999). *In ovo* injection machines now manufactured by 4 different companies are currently in use in more than 90% of U.S. hatcheries. The use of *in ovo* injection for the vaccination of late-stage embryos (17.5 -19.2 days of incubation) has proven to be safe with minimal effects on hatchability (Peebles, 2018). *In ovo* vaccination has a number of biological and commercial advantages over subcutaneous vaccination on day of hatch:

- immunity against disease is generated as early as possible (Sharma and Burmester (1982), recognizing the ability of late-stage embryos and foetuses to support immune responses to viral and bacterial antigens, used the *in ovo* injection for the Marek's disease vaccine in embryonic chickens);
- vaccine is delivered reliably and accurately in carefully controlled, hygienic conditions;
- the process is less labour-intensive and less prone to human error;
- chick handling is minimized, reducing bird stress and improving bird health;
- birds can be transferred out of the hatchery more quickly and grow-out conditions established sooner.

Because of the success with *in ovo* vaccination, extensive experimentation has been conducted with the injections of various biologics, such as nutrient supplementation, hormones, and immunostimulants. *In ovo* injection has been reported to be an effective way to deliver nutrients into the amniotic cavity to compensate for the energy

deficiencies that occur during the hatching process. However, scepticism of the technique was also raised based on lack of:

- optimization in deliverance such as: age, site of injection (amnion, allantoic cavity, yolk sac, and air sac), chemical and physical characteristics of the injectable solution, volume;
- stress caused to the embryo by disruption of the internal environment or osmotic balance;
- insufficient evaluation for the optimal individual or mixed substances for injection or their appropriate concentrations for delivery (Roto *et al*., 2016).

In ovo feeding firstly patented by Uni and Ferket (2003) (USA Patent # 6.592.878 B2) provides nutrient solutions in the amniotic fluid of broiler and turkey embryos. This technique was optimized in a series of studies and several patents for automated deliverance with variations in site of injection, solution injected, age of injection, and method of automation were approved. The *in ovo* technology was applied to the delivery of nutrients (carbohydrates, vitamins, amino acids, trace elements, growth hormones, etc.) as an early feeding strategy that guarantees early growth start-off and improved bird performance. Uni and Ferket (2004) have stated that the mortality experienced by hatchlings during the critical post-hatch period, when they are having to adjust to new environments and nutrient sources, may be alleviated by the *in ovo* administration of nutrients when they are in the late-term embryonic stage. A recently emerging field, *in ovo* technology involving the delivery of bioactives (probiotics, prebiotics and synbiotics) directly to the developing embryo, presents an opportunity to develop effective alternatives to AGP for the poultry industry. *In ovo* technology can be defined as the direct inoculation of bioactive substances to the developing embryo to obtain superior

lifelong effects while considering the dynamic physiology of the chicken embryo. The method allows the accurate and precise delivery of the bioactives at very low doses to embryo 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). Such *in ovo* injection can be automated without losses in eggs hatchability (Bednarczyk *et al*., 2011). It is based on the concept of supplementing the chick embryo with bioactive substances to establish lifelong phenotypes, including superior performance, immunity, and healthy gut microbiome in the bird (Siwek *et al*., 2018). The delivery of bioactives is better defined as "*in ovo* stimulation" rather than "*in ovo* feeding". This method has been developed and patented by Gulewicz and Berdarczyk in 2008. The main differences between *in ovo* stimulation and *in ovo* feeding refer to strategy, biological mechanisms and technical tools which are briefly described in the Table 4.

Table 4. *In ovo* feeding vs *in ovo* stimulation

	In ovo feeding	*In ovo* stimulation
Aim	To compensate for the energy deficiencies that occur during the hatching process.	To program lifelong phenotypes (e.g. immunity, gut microbiome, performance) already during the embryonic phase.
Days of injection	Day 17/18 of egg incubation (late-term chicken embryo)	Day 12 of egg incubation (early-stage chicken embryo)
Site of injection	Amnion	Air cell
Volume injected	1-1.7 ml solution of nutrients (carbohydrates, proteins….)	0.2 ml solution of bioactives (pro-, pre-, synbiotics)

The assumption of chickens being hatched germ-free is no longer valid, since it was recently shown that the microbial colonization of the digestive system, may start during

the last stage of embryonic development. 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 (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). It has been shown that day 12 of incubation is the optimal time for prebiotic injection into the air cell of the incubating egg (Villaluenga *et al*., 2004). At this time, embryo is totally immersed in amniotic fluid. Allantochorion is completely developed and highly vascularized, allowing for transfer of the bioactive solution from air cell to embryonic gastrointestinal tract. This method has been successfully used for prebiotic (Pilarski *et al*., 2005; Bednarczyk *et al*., 2011; Tavaniello *et al*., 2018, 2020; Slawinska *et al*., 2019, 2020) or synbiotic (Maiorano *et al*., 2012; Slawinska *et al*., 2014a and 2014b; Madej and Bednarczyk, 2016; Madej *et al*., 2015; Pruszynska-Oszmalek *et al*., 2015; Tavaniello *et al*., 2019) *in ovo* delivery. It was demonstrated that prebiotic migrates through the shell membrane and enters the blood circulation on day 3 after injection (i.e., day 15 of egg incubation onwards). Unlike the prebiotic, the probiotic bacteria stay in the air cell until the beginning of hatching (i.e., day 19 of egg incubation) (Siwek *et al*., 2018). Considering this and the distinction between *in ovo* stimulation and *in ovo* feeding, only prebiotic compound is used for

actual stimulation of the indigenous flora in the embryo. Probiotic, even though it might be injected on day 12 of egg incubation (in the form of synbiotic), should be considered *in ovo* feeding due to its availability to the late-stage embryo (after pipping) (Siwek *et al*., 2018).

Life-long phenotypic effects that followed *in ovo* stimulation were determined on multiple levels. It has been shown that prebiotics executes several beneficial effects through both direct and indirect mechanisms. *In ovo* injection of prebiotics at an early stage of development increases the population of beneficial microflora on the day of hatch compared to dietary inclusion (Bednarczyk *et al*., 2016). This leads to a high and stable level of Bifidobacteria throughout the growing period of broiler chickens (Villaluenga *et al*., 2004). *In ovo* technology enables delivery of sustainable bioactives such as pre/synbiotics as early as possible, namely at d 12 of embryonic incubation, and influences the microbiome structure in newly hatched chicks (Pilarski *et al*., 2005; Bednarczyk *et al*., 2011; Slawinska *et al*., 2014; Madej *et al*., 2015; Plowiec *et al*., 2015; Madej and Bednarczyk, 2016). On a large scale (using 25,000 chickens), Sobolewska (2017) substantiated the positive effect of prebiotic DiNovo (BioAtlantis Ltd., Bioatlantis Ltd., Tralee, Co., Kerry, Ireland; an extract of Laminaria spp. containing laminarin and fucoidan) on intestinal morphological parameters (duodenal villi width and crypt depth), which were positively improved, suggesting improved intestinal secretion and absorption rates. The immunomodulatory effect of prebiotics has also been confirmed by Angwech *et al*. (2019) who observed that Bi^2tos (3.5 mg/embryo) reduced the incidence of intestinal lesions and oocyst excretion in tropical Kuroiler chickens exposed to natural Coccidiosis challenge. Recently it was demonstrated that GOS used for *in ovo* stimulation improves gene expression signatures of innate immunity (cytokine gene expression),

barrier function (mucin and host defence peptides gene expression), and intestinal integrity (tight junctions gene expression) in jejunum and cecum of the broiler chickens (Slawinska *et al*., 2019a). GOS also increased expression of Bifidobacteria spp. in jejunum and cecum, which confirmed its bifidogenic effects upon delivery *in ovo* (Slawinska *et al*., 2019a). When combined with the HS, stimulation with GOS *in ovo* proved to mitigate heat-induced immune responses and oxidative stress in the spleen of broiler chickens (Slawinska *et al*., 2019b). Promotion of the intestinal health through embryonic stimulation of the intestinal microflora with GOS has not only a beneficial effect on host systems but also on growth performance. In fact, GOS delivered *in ovo* significantly improved the resilience of birds exposed to heat stress and improved feed and growth efficiency (Slawinska *et al*., 2019c). As for welfare traits, Slawinska *et al*. (2019c) also found that *in ovo* stimulation with GOS dampened body temperature in both thermoneutral and heat stress conditions and numerically improved survivability during heat stress; in addition, it was found that GOS delivered *in ovo* decreased the prevalence of footpad dermatitis in thermoneutral conditions by 20% (no lesions in 81% in GOS vs. 60% in C). Finally, GOS delivered *in ovo* modulated positive profile of the fatty acids in pectoral muscle of the heat stressed chickens (Tavaniello *et al*., 2020).

As for *in ovo* synbiotics administration, it is a promising approach in chicken immune system enhancement, as it combines advantages of the synergism between prebiotics and probiotics and by early administration into the embryo; it supports development of immune organs (Slawinska *et al*., 2014; Madej *et al*., 2015; Madej and Bednarczyk, 2016), influencing also the immunomodulatory gene expression in gut-associated lymphatic tissue (Dunislawska *et al*., 2017). It has also been shown that the *in ovo* delivery of pre- or synbiotics significantly increases the total activity of pancreatic

enzymes (amylase, lipase, and trypsin) (Pruszynska- Oszmalek *et al*., 2015) and influences the histological structure of chicken intestinal tissue (Bogucka *et al*., 2016; Sobolewska *et al*., 2017). It is clear that the intrinsic crosstalk between the microbiome and its host is not limited only to the gastrointestinal tract but it involves all the organisms, affecting also growth performance and meat quality traits (Tavaniello *et al*., 2019).

Despite *in ovo* stimulation provides beneficial and long-lasting effects to the embryo, it is still a new technology on the market. A prototype of such an automated injection system for early-stage embryos exists and has been tested in commercial settings (Bednarczyk *et al*., 2011). The major obstacle to using *in ovo* stimulation is the need for technological adjustment of production lines in hatcheries to add another time point for egg manipulation (day 12 of egg incubation). The injection machine for *in ovo* injection of early-stage embryos needs to be adjusted so that the injection is done into the air cell and the puncture hole is sealed to avoid embryo evaporation.

4. POULTRY MEAT QUALITY AND ITS DETERMINANTS

Poultry products are in demand in all parts of the world: poultry meat continue to be the most efficient and economical way to convert feed grains to animal protein. When there are no religious or cultural barriers, poultry meat usually leads in consumer preference. Poultry meat also enjoys popularity in developed markets, due to its price and perceived safety health advantages compared to other meat sources. The following five factors are believed to have contributed to the increasing popularity of chicken meat:

- value/price compared with other foods;
- good nutritional profile/low in fat;
- convenience/ease of preparation;
- versatility and well suited for quick-service.

Overall, poultry meat is distinguished for its low energy concentration and its high nutrient density, although many different intrinsic and extrinsic factors including genotype, diet, rearing system (organic, free range, intensive), pre- and post-slaughter handling, as well as the cut, the presence of skin, the cooking procedure have been shown to influence meat quality traits of the meat. A recent review article by Mir *et al*. (2017) reports the main determinants of broiler chicken meat quality, here briefly summarized. Genotype is one of the main determinant factors affecting growth performance and meat quality traits. The heritability estimates of various parameters like meat quality traits (0.35–0.81), *post-mortem* pH decline (0.35–0.49), lightness (0.50–0.75), redness (0.57–

0.81), yellowness (0.55–0.64), drip loss (0.39), etc. suggest that genetic selection is a best tool for improvement of broiler meat quality (Mir *et al*., 2017). Management of poultry meat production is reflected mostly on sensory attributes of meat (juiciness, tenderness, flavour). Nutrition of birds has a significant impact on poultry meat quality and safety, in particular, it is well known that dietary fatty acid profiles are reflected in tissue fatty acid. Poultry meat can be easily enriched in n-3 PUFA using appropriate feeding. Recently, consumers have become cautious of using GMO (genetically modified organisms) feed components in animal nutrition, although no dangerous transfer of plant DNA or recombinant DNA to tissues has been proven yet. Though, no significant differences were observed in broilers fed transgenic corn compared to non-modified corn variety in terms of fattening performance, slaughter performance and nutrient contents in broiler tissues (Taylor *et al*., 2003), the potentially dangerous effects of GMO in animal diets on human health by consumption of animal products cannot be fully ignored. After slaughter, biochemical changes, causing the conversion of muscle to meat, determine final meat quality. In addition, further processed product which can be either "ready to eat" or "ready to cook" product have become a matter of concern with respect to nutritional quality of broiler meat, since critics have implied that further processing reduce the nutritional value of poultry meat (Mir *et al*., 2017).

4.1 Chemical and nutritional composition of poultry meat

Poultry meat meets multiple nutritional requirements. Besides containing good amounts of proteins and many micronutrients, poultry meat is relatively low in fats and

cholesterol, particularly when it is consumed without skin. An interesting nutritional characteristic of the poultry meat is to be rich in n-3 polyunsaturated fatty acids. Poultry meat also contains several bioactive components such as glutathione, taurine, anserine, and others, and the content of most of them can also be increased by appropriate feeding. Future perspective indicates poultry meat as a promising functional food (Bordoni and Danesi, 2017).

Poultry meat, as well as other meats, is a good source of high-biological value proteins (20% 22%). In addition to the high protein content, meat can be especially distinguished by its higher levels of essential amino acids and especially in branched chain amino acids (valine, isoleucine, and leucine) (Pereira and Vicente, 2013). The low content of collagen (a structural protein) is another favourable characteristic of poultry meat. In fact, collagen reduces the digestibility of meat, and high levels of this protein in muscular meat are associated with a lower percentage of digested product per unit of time (Marangoni *et al*., 2015).

Compared to other types of meat, poultry appears to be relatively low in fats, which can increase during preparation due to the addition of butter or oil, and to the water loss during cooking. Lipid and cholesterol content depends on the cut, and mainly on the presence of the skin (which can be easily removed). Regarding the fatty acid composition of poultry meat, the ratio of saturated fatty acids to unsaturated fatty acids (SFA/UFA) is about 1:3, being lower in breast than in other cuts, and when the skin is removed. An interesting nutritional characteristic of poultry meat is the high content of long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA) (Bordoni and Danesi, 2017). 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. It has been shown, that the content of poultry meat in n-3 fatty acids (important for their therapeutic and preventive benefits for human health), particularly in α-linolenic acid, can be readily improved by increasing the levels of n-3 PUFA in poultry diets through the incorporation of oily fish by-products (Lopez-Ferrer *et al*., 2001). However, when fish oils are used in the feed formulations of birds to improve the nutritional value of the products there is some decrease in sensory quality reported as fishy off-flavours. In order to avoid organoleptic problems, a reduction of fish products in the diet during the last days before slaughtering has been tested. When linseed oil replaced fish oil for 1 or 2 weeks before slaughter, the sensory quality of the meat improved and maintained an important quantity of n-3 LC-PUFA (Lopez-Ferrer *et al*.,1999). Boschetti *et al*. (2016) have highlighted the possibility of a genotype-based selection of strains to produce meat with increased content of n-3 LC-PUFA, due to a higher ability to desaturate/elongate the dietary precursor α-linolenic acid (ALA). 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, improves the immune system, and reduce plasma cholesterol and fatness (reviewed in Grashorn, 2007). Besides fatty acids, cholesterol is another nutritionally important component of meats. Cholesterol exists in meat as free cholesterol and esterified cholesterol. Free cholesterol is associated chiefly with cellular and subcellular membranes of muscle and intramuscular adipocytes. Because intramuscular adipocytes are essentially lipid-filled spheres with very little

membrane content, the amount of cholesterol associated with membranes is small (usually about 25 %). Esterified cholesterol, located within the triacylglycerol-rich central lipid vacuole, comprises about 75 % of the total cholesterol in adipose tissue. According to Smith *et al*. (2004), muscle fibers have nearly 75 % of their total cholesterol associated with membranes and the rest are in the form of neutral lipids. Poultry meat is characterized by a low cholesterol content (broiler Pectoralis muscle, 47.41 mg/100 g muscle; Chizzolini *et al*., 1999) making it healthier than other meat products i.e., beef 66 mg/100 g, pork 65 mg/100 and lamb 50 mg/100 (Chizzolini *et al*., 1999). However, other studies on broiler chicken reported higher cholesterol values; for example, Salma *et al*. (2007) reported an average cholesterol content of 93.6 mg/100g of meat in Pectoralis major of 56-day-old male Chunky broilers; while, Maiorano *et al*. (2012) reported cholesterol values ranging from 70.45 to 78.12 mg/100g in 42 days old broiler chickens and de Oliveira *et al*. (2016) reported values ranging from 47.88 to 68.15 mg/100 g in breast and thigh respectively. The reported discrepancies in cholesterol content could be explained by the use of different analytical methodologies for cholesterol quantification and sampling (Bragagnolo and Rodriguez-Amaya, 2002), diet, breed (de Oliveira *et al*., 2016), diet, age, and sex (Wang *et al*., 2005).

The energetic value of poultry meats is within the range of other meats, although it varies according to the presence/absence of the skin. In raw meat, the highest value is in chicken thighs (196 kcal/100 g), and the lowest in chicken breast without skin (100 kcal/100 g). In general, the presence of skin increases the caloric value by around 25% 30% (Bordoni and Danesi, 2017). Poultry meat represents an excellent source of the majority of hydrophilic vitamins, and it is the ideal dietary source of vitamin B12. The amounts of B-group vitamins (e.g., niacin, vitamin B6, and pantothenic acid) in poultry are very similar

to those of other meats (Marangoni *et al*., 2015). The concentration of lipophilic vitamins is lower in meat than in plant-based foods. Poultry meat can be enriched with vitamin E by providing it with feed (Bordoni and Danesi, 2017). Poultry meat also provides several minerals. Chicken meat is an excellent source of selenium. Sodium is only minimally present in fresh meat and in poultry too, and does not significantly contribute to total dietary intake. Processed meat products, on the other hand, can contain high or very high quantities of sodium, added as a preservative or flavour enhancer. Among meats, horse meat has the highest iron content (3.9 mg/100 g), and raw chicken breast has the lowest (0.4 mg/100 g). Percentage of heme iron in total iron in raw chicken breast is 30%. Both total and heme iron markedly differed among the meat cuts (Bordoni and Danesi, 2017).
appearance is the most important quality attribute of cooked or raw poultry meat because consumers associate it with the product's freshness, and they decide whether or not to buy the product based on their opinion of its attractiveness. Poultry meat is unique because it is sold with intact skin or without skin.

4.2 Chicken meat technological and sensory quality traits

It is well known that the ultimate pH of the muscle is an important contributing factor to meat quality expressed as tenderness, color, and storage life (Van Laack *et al*., 2000). *Post-mortem* metabolism of the muscle tissue influences the characteristics of the meat. In particular, the rate and the amplitude of acidification have a strong effect on both sensory and technological parameters of meat (Duclos *et al*., 2007). Meat pH changes dramatically during the first hours after slaughter. This is the period of most enhanced

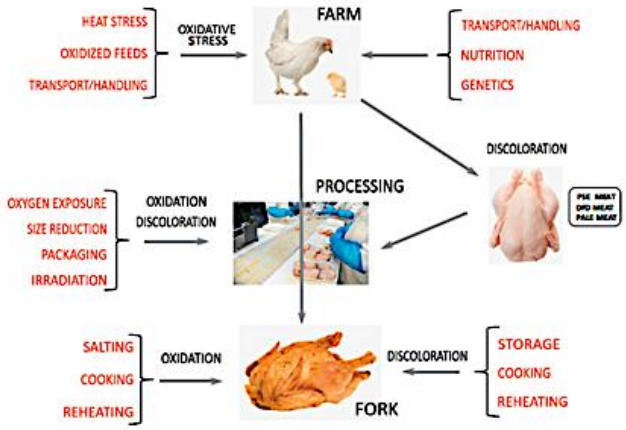
glycolysis and lactate accumulation into muscle tissue. Glycolysis occurs very fast in poultry muscles. In the chicken, normal pH values at 15 min *post-mortem* are around 6.2 to 6.5, whereas normal ultimate pH values are around 5.8 (reviewed in Duclos et a., 2007). As a consequence of being predominantly constituted of type IIB-white glycolytic fibers, the ultimate pH values of broilers Pectoralis major muscles (5.8 achieved 2-3 h *post-mortem*) significantly differ from the ones measured in red leg muscles (5.6 at 8 h *post-mortem*) (Stewart *et al*., 1984). Several *ante-mortem* factors could affect the rate of *post-mortem* glycolysis and the final pH, with direct consequence on color, water-holding capacity, juiciness and shelf life of meat. Differences in pH could be due to the variation in muscle glycogen content (Berri *et al*., 2005). However, ultimate pH value is also dependent on the *ante-mortem* stress, type of breed and the genetic variation within breeds (Terlouw, 2005). In some cases, as a consequence of glycogen stores depletion before slaughtering, lactic acid does not accumulate within the muscle tissue resulting in high ultimate pH values (around 6.0) achieved in very short time. The resulting meat, referred to as DFD (Dark, Firm and Dry)-like, exhibit dark color, firm texture and dry appearance. On the opposite, a rapid pH decline occurring at the beginning of the *post-mortem* process (within the first hour after slaughtering) when the muscle temperature is still high (> 35°C) leads to protein denaturation resulting in the development of the PSE (Pale, Soft and Exudative)-like condition. In detail, the impaired functionality of the partially denatured myosin molecules leads to an overall reduced water holding capacity of meat displaying increased fluid losses (exudative meat) (reviewed in Duclos *et al*., 2007). As regards water holding capacity (WHC) is known to be one of the major quality characteristics of fresh meat, as it affects some major characteristics of the cooked meat such as potential drip loss, technological quality, appearance and sensory properties. It

can be said that appearance and technological characteristics are connected. WHC refers to the ability of meat to hold inherent or added moisture throughout fabrication, processing, and storage. For consumers, poor WHC in fresh poultry meat products results in diminished visual appeal due to excess purge in packages and inferior palatability traits related to juiciness and tenderness. For processors, improved WHC in raw poultry meat leads to greater marinade pick-up and retention, better protein functionality, and greater processing and cooking yields (Bowker, 2017). Although WHC attributes are a manifestation of biochemical and structural alterations that occur within the muscles after the birds are slaughtered, *ante-mortem* factors such as preslaughter activity and environmental conditions can influence *post-mortem* changes in muscle tissue and WHC in the final meat product. The modern hybrids intensively selected for growth rate and carcass part yields seem to be more stress-susceptible and prone to developing myopathies that have a detrimental effect on WHC and other meat-quality traits (Petracci and Cavani, 2012).

Regarding meat sensory attributes, appearance is the most important quality attribute of cooked or raw poultry meat because consumers associate it with the product's freshness, and they decide whether or not to buy the product based on their opinion of its attractiveness. Poultry meat is unique because it is sold with intact skin or without skin. Skin colour appears to be critical for the marketing of fresh whole birds or cut portions. The colour of the meat is more relevant to deboned and skinless. Visual defects include bruises and haemorrhages of varying severity have a significant impact on consumers' choice. Consumer preference for skin colour shows some interesting variation for broilers, with preferred color ranging from white, through pale yellow to deeply pigmented, and choice being based on traditional market forms (Fletcher, 2002).

Preferences for one type of color or another have tended to show a regional pattern in Italy, e.g. in the North Italy yellow skinned birds are preferred to those with the usual whiter appearance, on the assumption that such birds have a better eating quality. A plethora of factors affect poultry meat color. These include: bird's sex, age, strain, processing procedures, chemical exposure, scalding temperature, cooking temperature, irradiation, and freezing conditions, pre-slaughter conditions, reaction of the major meat pigment, myoglobin, as well as effects of nitrates and nitrites, ovens and environmental gasses (primarily carbon monoxide and nitric oxide), haemachromes, and cytochrome C (Maga, 1994; Froning, 1995). Estevez (2015) reported the relevance and consequences of the oxidative reactions throughout the production of poultry meat (Figure 4) which can affect also poultry meat color causing the discoloration. Discoloration is associated with oxidative damage to poultry and can cause meat defects during poultry production and handling on the farm as well as during meat processing: chilling, storage, cooking, and others. The impairment of the homeostasis of lipids and proteins leads to oxidation in muscle promoting changes in meat color due to modification of pigments state (Carvalho *et al*., 2017).

Figure 4. Source of oxidative stress and discoloration to poultry and poultry meat from farm to fork (Carvalho *et al*., 2017).



Poultry-meat colour is dependent on the concentration and chemical state of meat pigments, especially myoglobin, haemoglobin, cytochrome C and their derivatives, presence of ligands complexing with heme pigments (Fletcher, 2002). Briefly, colour of meat depends on:

- the presence and the quantity of pigments in muscle and their chemical status;
- the type of muscle fibers and their spatial relationships, which determine the scattering grade of light and thus its deepness of penetration;
- the intramuscular fat and surface dehydration which confer different degrees of glossiness and thus affect light scattering and reflection.

Myoglobin content is strictly depended by animal species, muscle type, and animal age. About poultry meat in particular, white meat from 8-week-old poultry has the lowest Mb content (0.01 mg Mb/g meat) follow by 26-week-old male poultry white meat (0.10 mg/g), young turkey white meat (0.12 mg/g), 8-week-old poultry dark meat (0.40 mg/g), 26-week-old male poultry dark meat (1.50 mg/g), and 24-week-old male turkey dark meat (1.50 mg/g). These values are considerably lower than Mb concentrations reported in other animals' species such as young lamb (2.50 mg/g), dark meat fish species (5.3 24.4 mg/g), 3-year-old beef (4.60 mg/g), and old beef (16 20 mg/g) (Carvalho *et al*., 2017). The chemical forms of myoglobin are primarily responsible for meat color: purple-red deoxymyoglobin in fresh meat in the absence of air; bright red oxymyoglobin formed in the presence of oxygen; brown metmyoglobin, the result of myoglobin oxidation. The pigments myoglobin, oxymyoglobin and metmyoglobin can be changed from one to the other, depending on the store conditions of meat. This reaction is reversible and dependent on the availability of oxygen, active enzymes and reducing

compounds in the muscle (Mancini and Hunt, 2005). 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), and meats with an L* value below 46 are called to be dark, firm, dry, which means they have a dark color, high water holding capacity, and short shelf life; while, meat with L* value higher than 53 are called to be pale, soft and exudative (Bianchi *et al*., 2005).

As mentioned before, the appearance and tenderness are two extremely important traits in poultry meat quality (Fletcher, 2002). In particular, meat tenderness is the single most important sensory property affecting final quality assessment (Fletcher, 2002). Texture is probably the single most critical quality factor associated with the consumer's ultimate satisfaction with a poultry meat product. The two major contributors to poultry meat tenderness are the maturity of the connective tissues and contractile state of the myofibrillar proteins. The myofibrillar protein impacts on ultimate meat tenderness are primarily a function of the biochemical predisposition of the muscle at the time of slaughter, the rate and severity of rigor mortis development, and the physical handling of the carcass and muscle during rigor development (Fletcher, 2002). The maturity of the connective tissue involves the chemical cross bonding of the collagen in the muscle. Since collagen cross-linking increases with age, meat is generally tougher from older animals. Collagen and its hydroxypyridinoline crosslinks (the main intramuscular collagen mature crosslink) contribution to meat toughness was reviewed by McCormick (2009), reporting that collagen (content and cross-linking) affects the background toughness of meat. As previously reported, McCormick (1999) suggested that mature crosslinks and collagen concentration have an additive effect on the toughening of meat. In other words, the role of collagen on meat tenderness depends not only on the

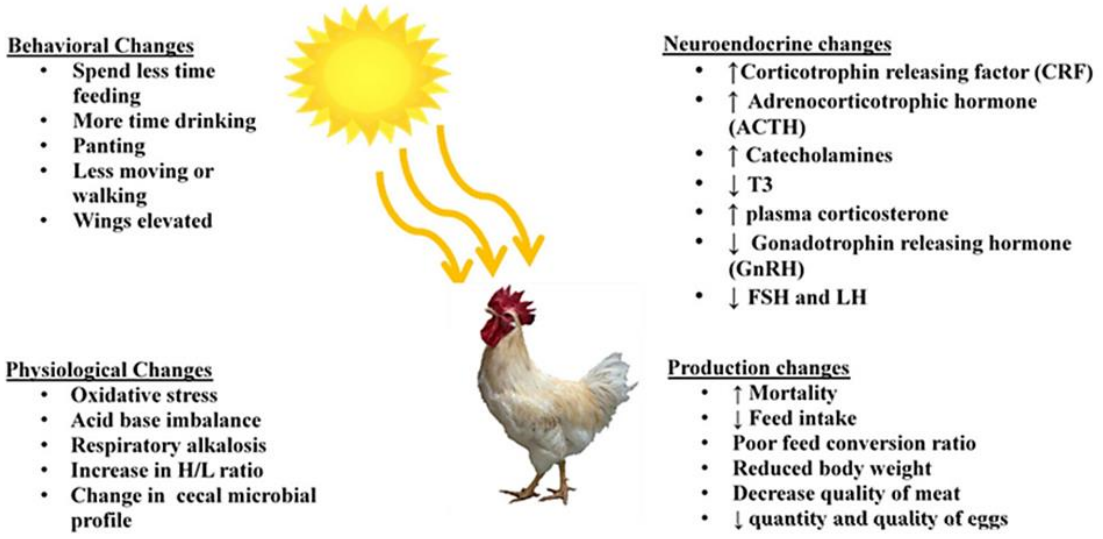
crosslinks but also on the amount of collagen. It is well known that the proportion of mature to reducible crosslinks increases with age, resulting in older animals that often have less tender meat than younger animals. However, the expression of connective tissue within muscles is greatly variable, depending on developmental stage, muscle position/function, animal breed, nutrition, exercise and injury (Purslow, 2005). Today, broiler chickens are slaughtered in 6-7 weeks to meet the needs of consumers, so the age-related toughness problem (connective tissue cross-linking) has disappeared. In addition, in recent years was reported a new emerging quality issue in poultry that is the poor cohesiveness of meat due to immaturity of intramuscular collagen tissue, in relation to the very early slaughter age of modern chicken and turkey strains (Petracci and Cavani, 2012).

5. HEAT STRESS

Heat stress is an important environmental determinant which adversely affects the animal production worldwide. Because recent climate models predict an increase in average worldwide ambient temperatures and more frequent and intense heat events, the negative impact of heat stress on animal production is expected to increase substantially. Although heat stress conditions are commonplace in tropical and subtropical regions, climate change may increase the frequency of heat waves and extend heat stress conditions to regions of the world where HS occurs only during certain times of the year (i.e., temperate regions). Heat stress is currently considered to be a major environmental factor impairing welfare and productivity of poultry, causing huge economic losses to the poultry industry. St-Pierre (2003) estimated a total annual economic loss of 128-165$ million to the US poultry industry due to heat stress. Exposure of poultry to high ambient temperature leads to physiological, behavioural, and immunological responses which directly or indirectly have adverse effects on health and performance, due to the inability of chickens to maintain a balance between body heat production and heat loss. Heat stress results from the interaction of different factors such as high environmental temperature, humidity, radiant heat, and airspeed. The normal body temperature of the chicken is around 41-42 °C, and the thermoneutral temperature to maximize growth is between 18-21 °C. Variations in tolerance of temperature beyond thermoneutral zone may be associated with age, sex, breed, body weight, physiological activity, molting period, broodiness, feeding status and external environment. Heat stress is notified as a major poultry welfare concern for both backyard poultry farmers and commercial enterprises

(Kumar *et al*., 2021). The main effect of heat stress on behavioural, physiological, neuroendocrine and production traits are summarized in the Figure 5.

Figure 5. Effects of heat stress on behavioural, physiological, neuroendocrine and production traits (Wasti *et al*., 2020).



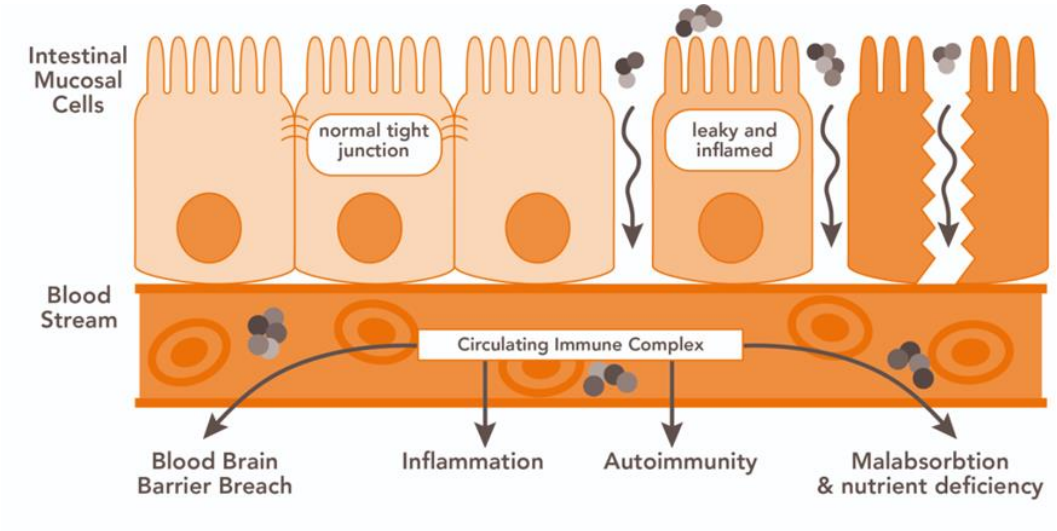
As for behavioural changes, it has been shown that birds subjected to heat stress conditions spend less time feeding, more time drinking and panting, as well as more time with their wings elevated, less time moving or walking, and more time resting (Lara and Rostagno, 2013).

Birds are characterized by the lack of sweat glands and the presence of feathers throughout the body; as a consequence, they need to disperse heat through active mechanisms such as panting. Birds activate also a series of mechanisms to maintain the homeostasis as increasing radiant, convective, and evaporative heat loss by vasodilatation and perspiration (Mustaf *et al*., 2009), but also promoting the circulation in the air sacs, a mechanism typical in the birds (Fedde, 1998). Increased panting under heat stress conditions leads to increased excretion of CO2 which alters the standard bicarbonate

buffer system in the blood. The reduction of CO_2 leads to a decrease in the concentration of carbonic acids (H_2CO_3) and hydrogen ions (H^+). On the contrary, there is an increase in the concentration of bicarbonate ions (HCO_3) which causes an increase in the pH of the blood, i.e., the blood becomes alkaline. To maintain the normal blood pH, birds will start excreting more amount of HCO_3 and retain H^+ from the kidney. The elevated H^+ alters the acid-base balance leading to respiratory alkalosis and metabolic acidosis (Wasti *et al*., 2020). Another physiological change caused by heat stress is the oxidative stress which is associated with biological damage with severe health disorders, lower growth rates and consequently economic losses (Estevez, 2015). Briefly, heat stress is associated with a higher production of reactive oxygen species (ROS) with consequent damage to DNA, proteins and lipids (Wasti *et al*., 2020).

The physiological symptoms of heat stress are manifested also by dysbiosis of the intestinal microflora, which brings immediate pressure to intestinal integrity Dysbiosis in microflora composition (Shi *et al*., 2019), reduction in mucus layer (Burkholder *et al*., 2008), and alteration of tight junctions in intestinal epithelia (Varasteh *et al*., 2015) indicate a compromised gut barrier function and integrity. Due to the compromised barrier function of the gut, intestinal bacteria and their toxins access internal milieu and trigger acute pro-inflammatory immune responses (Figure 6). This condition is referred to as endotoxemia and leads straight to the heat stroke (Leon and Helwig, 2010). In addition, birds experiencing heat stress are hypersensitive to corticosterone, which can delay the proliferation of intestinal cells, which in turn leads to a decrease in the height of intestinal villi and a decrease in the depth of intestinal crypts. Furthermore, corticosterone is an activator of pro-inflammatory reactions in the intestine (reviewed in Krysiak *et al*., 2021).

Figure 6. The damaging effect of heat stress on intestinal barrier



Heat stress is known to suppress immunity in the chicken, it has been reported that the size of immune-related organs such as the spleen, thymus, and lymphoid organs are also regressed in the heat-stressed birds (Ghazi *et al*., 2012; Quintero-Filho *et al*., 2010). A low level of antibodies was also observed in the heat-stressed birds (Bartlett *et al*., 2003). Regarding the neuroendocrine system, heat stress results in activation of the hypothalamic-pituitary-adrenal (HPA) axis. The adrenal medulla increases the secretion of catecholamines, which cause a surge of glucose release in the blood, deplete liver glycogen, reduce muscle glycogen, increase respiration rate, vasodilate the peripheral blood vessels, and increase neural sensitivity to cope with the stress. In response to the stress for a more extended period, corticotrophin-releasing hormone (CRH) is secreted from the hypothalamus, which triggers the release of an adrenocorticotrophic hormone (ACTH) from the pituitary. ACTH increases the production and release of corticosteroid by the adrenal glands which stimulates gluconeogenesis to increase plasma glucose levels (reviewed in Wasti *et al*., 2020). Body temperature and metabolic activity are regulated by the thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4), and their balance. The

reduction of T_3 concentration during heat stress is due to a decrease in peripheral deiodination of T_4 to T_3 (Lara and Rostagno, 2013). Alteration of thyroid hormones and neuroendocrine system due to heat stress have effects on the reproduction function of birds: in females dysregulating the normal status of reproductive hormones at the hypothalamus and at the ovary, reducing systemic levels and functions (Elnagar *et al*., 2010); in males decreasing semen volume, sperm concentration, number of live sperm cells and motility (McDaniel *et al*., 2004). In summary, heat stress impairs overall poultry and egg production by modifying the bird's neuroendocrine profile both by decreased feed intake and by activation of the HPA axis.

The above-mentioned changes caused by heat stress led to increased mortality, reduced feed intake, body weight and a consequently higher feed conversion ratio (FCR), as well as effect on egg and meat quality. A recent study (Sohail *et al*., 2012) reported that broilers subjected to chronic heat stress had significantly reduced feed intake (16.4%), lower body weight (32.6%), and higher feed conversion ratio (+25.6%) at 42 days of age. Many additional studies have shown impaired growth performance in broilers subjected to heat stress (reviewed in Lara and Rostagno, 2013). However, it is important to consider that stocking density has a major role as a potential compounding factor, both from the standpoint of productivity as well as welfare. Several studies (Lu *et al*., 2007; Zhang *et al*., 2012; Cramer *et al*., 2018) found a lower weight of the breast muscle in response to heat stress, due to heat-induced suppression of growth. As already mentioned, heat stress stimulates the hypothalamic-pituitary-adrenal axis in poultry and increases the concentration of circulating corticosterone hormone, which increments protein degradation and breakdown of skeletal muscle (Yunianto *et al*., 1997; Scanes, 2016). Heavy-weight broiler chickens become very sensitive to heat compared to other breeds.

Modern poultry genotypes are more susceptible to heat stress than even before. Because fast-growing broilers produce more heat and have a higher heat load, the effect of heat stress is more pronounced in commercial broiler stocks and in broilers with high growth potential compared to the slower-growing chickens (Lin *et al*., 2006). The development of the thermoregulatory systems could not match to the rapid growth rate of muscle, resulting in inability of the modern birds to control their body heat with the fluctuating environmental temperature and high metabolic rates. Furthermore, broiler chickens are particularly sensitive to heat in the last period of rearing when their circulatory system is inefficient in relation to body weight (Drain *et al*., 2007). Heat-resistance depends on the genetic adaptation of the chickens. Native chickens from tropical and sub-tropical regions are more tolerant to high ambient temperatures than fast-growing lines. Since they are smaller and lighter and have not been subjected to selective pressure for meat-related traits, they have retained their genetic adaptation to handle high temperatures. Studies on Brazilian breeds (Pelaco and Caneluda) and Egyptian breeds (Fayoumi, Dandarawi, and Sinai) have shown a good tolerance to elevated ambient temperature, manifested by the increased expression of heat shock proteins (Pietrzak *et al*., 2020). In some countries, native breeds are crossbred with commercial broiler lines to obtain heat-resistant hybrids with good meat production such as the hybrid used in the trial 2 reported in this thesis which is obtained by crossing a Hubbard RedBro male with a Hubbard JA57 female. These free-range poultry hybrids are distinguished by a good adaptation to a warm climate and a high disease resistance (Federico Sirri, unpublished data).

Either acute or chronic heat stress could lead to meat quality issues due to an increased *ante/post mortem* glycolytic metabolism coupled with a reduced protein synthesis and turnover, enhanced fat deposition and overproduction of reactive oxygen species (Temim

et al., 2000; Lu *et al*., 2007; Zhang *et al*., 2012; Zaboli *et al*., 2019). Exposure of broilers to high temperatures can induce a lower ultimate pH with variation in meat color, water holding capacity and tenderness of meat (Berri *et al*., 2005; Aksit *et al*., 2006; Zhang *et al*., 2012; Wang *et al*., 2017), resulting in a lower consumer's acceptability. Several studies suggest that heat stress could increase the rate of glycolysis in skeletal muscles causing a built-up of lactic acid within the muscle tissue (Zhang *et al*., 2012), which induces a faster pH decline with a lower ultimate pH (Zhang *et al*., 2012; Cramer *et al*., 2018; Zaboli *et al*., 2019). It has been reported that the acute heat stress can increase lightness (L*) and reduce redness (a*) and yellowness (b*) of breast meat. This could be due to the denaturation of sarcoplasmatic proteins which results in scattering of light (reviewed in Zhang *et al*., 2012). Heat stress also affects the chemical composition of meat. It has been reported that high ambient temperature significantly decreased body protein content, protein gain, protein retain and intake, due to a decreased muscle protein synthesis and increased protein catabolism (reviewed by Zhang *et al*., 2012). Temim *et al*. (2000) found that protein synthesis is more susceptible than proteolysis to high environmental temperature (32 °C); furthermore, it was demonstrated that heat stress determines changes in ribosomal gene transcription lowering the protein synthesis (Jacob, 1995; Temim *et al*., 1998). Exposure to high ambient temperature has been recognized as responsible of increased abdominal, subcutaneous, and intermuscular fat deposits (Ain Baziz *et al*., 1996; Geraert *et al*., 1996). The increased fat deposition could be related to reduction in basal metabolism and physical activity to reduce metabolic heat production and maintain homeothermy (Geraert *et al*., 1996). Regarding fatty acid profile limited is the information regarding the effect of heat stress on FA composition of chicken meat. Tavaniello *et al*. (2020) found a marginal effect of heat stress on FA composition of meat

from Ross broiler chickens subjected to heat stress. In a study conducted on French local broiler chicken, Ain Baziz *et al*. (1996) found that meat from heat-exposed birds (32 °C from 4 to 7 weeks old) had the same FA profile than that of control chickens with ad libitum feeding; while, in pair-feeding conditions, heat-exposed birds showed a higher SFA and lower PUFA contents compared to control chickens.

In recent years, poultry industry has made effort to mitigate the negative effects of heat stress on poultry production to reduce economic losses. There are different approaches to mitigate the problem of heat in poultry, including adjustment in ventilation infrastructure, genetic breeding (Lin *et al*., 2006) and dietary interventions (Renaudeau *et al*., 2012). Nutritional solutions can help poultry to cope with heat stress, with two objectives: i) to reduce diet induced thermogenesis by selecting nutrients having a low heat increment; ii) to provide birds with specific bioactives that correct the physiological dysfunctions associated with heat stress. In particular, feed additives, such as probiotics, prebiotics, and synbiotics, have been proposed as a nutritional strategy to improve the resilience of animals against heat stress. The rationale for the administration of such bioactives is to improve intestinal health, which is one of the main factors influencing the vulnerability of the chickens to heat (Ashraf *et al*., 2013; Varasteh *et al*., 2015; Sugiharto *et al*., 2017; Cramer *et al*., 2018). Currently, there is growing evidence that the supplementation with prebiotics can be effective in alleviating the detrimental effects of heat stress in chickens. Prebiotics such as fructooligosaccharides, galactooligosaccharides (GOS), and mannanoligosaccharides are considered preventative agents since they can select for a gastrointestinal microbiota which not only benefits the host but can serve as a barrier to pathogen colonization (Ricke, 2018a). The approach to prevent the detrimental symptoms of heat stress is to promote eubiotic microflora and improve intestinal health of the host.

Further, while studying to modulate chicken intestinal microflora by *in ovo* stimulation with galactooligosaccharides (GOS) prebiotic, Slawinska *et al*. (2019a) reported a bifidogenic effect in adult chickens and an increased expression of the cytokine genes, barrier function genes, and free fatty acid receptors, and a variation in the expression of the glucose transporter genes in the intestinal mucosa. Furthermore, in a subsequent study, Slawinska *et al*. (2019b) found that stimulation *in ovo* with GOS prebiotic dampened heat-induced immune- and stress-related gene expression signatures in spleen of chickens exposed to acute heat stress.

Several strategies have been tested to mitigate heat stress in poultry. However, considering that heat stress results from the interplay of several factors (high environmental temperature, humidity, radiant heat, and airspeed) and causes several physiological, neuroendocrine, and behavioural changes, no single approach alone is enough to mitigate the detrimental impacts of heat-stress on poultry. Therefore, there is a need for a holistic approach to attenuate the negative effect of heat stress in poultry.

6. AIM OF THE THESIS

In last decades, the increased worldwide demand for poultry has led to the replacement of small and medium-sized company with large poultry industries that dominate the market. Indeed, the expansion of the poultry sector was such that it did not slow down, as happened for some other industrial sectors, even following the economic and health crisis caused by the pandemic SARS-CoV-2 virus.

Although the large poultry industry has moved towards the marketing of fast-growing hybrids to sustain the cadence of market, the consumer seems to have had an opposite trend in recent decades. In fact, the consumers have become more health attentive to nutrition, choosing niche products such as organic or products that do not include the use of GMOs in animal's diet (Fanatico *et al*., 2007). Consequently, the poultry sector had to reshape its productions, moving from intensive ones with fast-growing commercial hybrids to semi-extensive or extensive productions with medium-slow-growing commercial hybrids, which better meet the new consumer demand: in fact, medium-slow-growing chickens have a higher protein content and lower fat content compared to fast-growing broilers (Poltowicz and Doktor, 2012; Sirri *et al*., 2011). However, this change of direction and the return to old farming techniques, involves a greater effort in economic terms for the companies, since the growth performance is less efficient (Fanatico *et al*., 2005; Sirri *et al*., 2011), the demand for wide space is greater, the regulations in terms of pollution are more restrictive. Considering the changing demands of the consumer and the rediscovered breeding techniques, which require particular kind

of genotypes more adaptable to outdoor life, but not for this reason not subjected to environmental stress (such as heat stress), it would be desirable introduce innovative technologies that increase the resilience of the animals.

Among these technologies, the *in ovo* injection of prebiotics, object of the present study, have benefits already explained in the introduction. As a matter of fact, as reported in literature (Bednarczyk *et al*., 2016; Roto *et al*., 2016; Maiorano *et al*., 2017), this technology has a positive impact on the resilience of animals and production performance.

The aim of the present thesis has been two-fold:

a) to study the effects of the *in ovo* injection of prebiotics (Galactooligosaccharides, GOS) on growth performance and meat quality in slow-growing chickens exposed to heat stress;

b) assess the quality characteristics of poultry meat products placed on the market. In particular, the qualitative assessment of the commercial products was carried out on two different production lines of the Amadori company: the Vegetale® line and the Campese® line, respectively consisting of fast-growing commercial hybrids reared with intensive techniques, and slow-growing hybrids reared semi-extensively, both fed with a plant-based diet without GMOs. The evaluation was carried out through considering different factors, such as genotype, sex and slaughter age.

7. RESEARCH N. 1

Galactooligosaccharides delivered *in ovo*: effect on performance and meat quality traits of slow-growing broiler chickens exposed to heat stress.

Background and aims

The key role of the intestinal microbiota has become increasingly relevant in various aspects of animal physiology and well-being. In recent years, *in ovo* injection technology allowed the precise delivery of probiotics, prebiotics and synbiotics, at very low doses into the egg air chamber, to the embryo at an early stage of development, influencing the structure of microbiota in newly hatched chicks, allowing a greater protection against the risk of gastrointestinal infections and improving productive performance and meat quality traits of treated chickens. The balance of the intestinal microbiota can be seriously compromised by several environmental factors and incorrect management procedures. This aspect assumes particular importance in case of fast-growing genotypes, especially considering the frequency of metabolic disorders, immunological problems and above all, the negative effects regarding the qualitative and sensory characteristics of meat (Castellini *et al*., 2002; Blagojević *et al*., 2009; Petracci *et al*., 2015). Most evidence of the positive influence of prebiotics is based on results from meat-type chickens (broilers). Beside from commercial broilers, chickens are the extremely rich source of genetic diversity and could have different reaction to microbiome stimulation by *in ovo* delivery

of prebiotics. The broilers, egg layers, fancy breeds and indigenous breeds are not only genetically distant, but also enormously different in their physiology. Considering this, the administration of bioactives through the *in ovo* injection technology could be of particular interest in the case of slow-growing genotypes because never been studied especially in conditions of thermal stress.

The aim of this research was to evaluate the production traits in slow-growing chickens stimulated *in ovo* with GOS prebiotic and exposed to chronic heat stress (in the last phase of rearing period) in order to define the alleviating effects of *in ovo* prebiotics delivery with respect to detrimental heat stress effects in chickens.

7.1 Materials and Methods

Ethical Statement

The animal procedures were conducted in compliance with decision of the Ethical Committee in Rome (Italy), decision number 503/2016.

Birds and experimental design

The experimental material was slow-growing free-range chickens that are obtained by crossing a Hubbard RedBro male with a Hubbard JA57 female. On d 12 of incubation, after candling, 3,000 eggs with viable embryos were randomly divided into 3 experimental groups: prebiotic group (GOS) injected with a single dose of 3.5 mg GOS/egg suspended in 0.2 mL of physiological saline; saline group (S) injected with 0.2 mL of physiological saline (0.9 % NaCl); control group (C) uninjected. Saline and GOS

solution were injected into the air chamber and the hole was sealed with organic glue. GOS prebiotic used in this study (trade name: Bi2tos, Clasado Biosciences Ltd., Jersey, UK) is manufactured by enzymatic transgalactosylation of the milk lactose by the whole cells of *Bifidobacterium bifidum* 41171 (Tzortzis *et al*., 2005). At hatching, chicks were sexed and vaccinated against coccidiosis, infectious bronchitis virus, Marek's disease virus, Newcastle disease, and Gumboro disease, and they received food and water ad libitum. The composition of the diets is presented in Table 5. Hatchability was calculated as the number of chicks being hatched and expressed as percentage of fertile eggs with apparently live embryos selected at 12 days of incubation by candling. Male chicks (n=900, 300 per treatment) were then transferred to an environmental controlled poultry house and divided in 6 groups of 150 chicks/treatment/environmental condition. Each group was composed of 6 replicates of 25 birds each. All the birds received the same commercial diet composed by 3 feeding phases: Starter (0-14 d), Grower (15-36 d) and Finisher (37-50 d). Chronic heat stress (30°C) was applied from 36 to 50d. Body weight (BW) was determined on a pen basis at 0, 14, 36, and 50 d, while feed intake (FI) was recorded on a pen basis at the end of each feeding phase (14, 36, 50 d). Daily weight gain (DWG), daily feed intake (DFI) and feed conversion rate (FCR) were calculated accordingly. Birds died during the trial were recorded and weighed daily to calculate mortality rate and to correct productive data.

Table 5. Composition of the diet supplied to the birds of all the experimental groups.

	Period		
	Starter (0-14) d)	Grower (15-36)	Finisher (37-50 d)
Ingredients (%)			
Sodium bicarbonate	0.15	0.10	0.15
Salt	0.27	0.27	0.25
Coline chloride	0.10	0.10	0.10
Lysine solfate	0.59	0.55	0.46
Dl-methionine	0.27	0.29	0.30
Threonine	0.15	0.14	0.14
Enzyme-roxazyme g2g	0.08	0.08	0.08
Phytase 0.1%	0.10	0.10	0.10
Coccidiostat			
Vit-min premix[1]	0.50	0.50	0.50
Dry matter			
Calculated nutritional value of the diet (%)			
Dry matter	88.57	88.65	88.64
Protein	22.70	21.49	19.74
Lipid	7.06	8.24	9.74
Fiber	3.08	3.04	3.07
Ash	5.85	5.17	4.49
Lys	1.38	1.29	1.21
Met	0.67	0.62	0.59
Met + Cys	1.03	0.97	0.91
Calcium	0.91	0.80	0.59
Phosphate	0.63	0.57	0.46
Metabolize energy (kcal/kg)	3.076	3.168	3.264

[1]Provided the following per kg of diet: vitamin A (retinyl acetate), 13000 IU; vitamin D3 (cholecalciferol), 4000 IU; vitamin E (DL-α_tocopheryl acetate), 80 IU; vitamin K (menadione sodium bisulfite), 3mg; riboflavin, 6 mg; panthothenic acid, 6 mg; niacin, 20 mg; pyridoxine, 2 mg; folic acid, 0.5 mg; biotin, 0.10 mg; thiamine, 2.5 mg; vitain B_{12} 20 µg; Mn, 100 mg; Zn, 85 mg; Fe, 30 mg; Cu, 10 mg; I, 1.5 mg; Se, 0.2 mg; and ethoxyquin, 100 mg.

Slaughter Surveys

At slaughter (50 d), all the birds were slaughtered in a commercial processing plant and carcass yield were assessed on all the processed birds and the results reported on a group basis. Right pectoral muscle (PM) was removed from 15 carcasses, randomly chosen

from each group, for the evaluation of physico-chemical properties of meat. Pectoral muscle (PM), including *Pectoralis major* and *Pectoralis minor*, was removed from the carcass and weighed. The pH was measured 24 h *post-mortem* on the upper part of the left-side breast fillet using a a portable pH meter (FiveGo, Mettler-Toledo, Switzerland) equipped with a penetrating glass electrode. Tri-stimulus color coordinates (lightness, L*; redness, a*; yellowness, b*) were measured 24 h *post-mortem* on the bone-side surface of left-side breast fillet using a Chroma Meter CR-300 (Minolta Corporation, Italia s.r.l., Milano).

Water holding capacity, cooking loss and Warner-Bratzler shear force analyses

Water holding capacity (WHC), expressed as expressible juice, was measured on PM 24 h after chilling using the press method (Grau and Hamm, 1953). As for cooking loss determination, PM samples were individually weighed, placed in metallic trays and introduced in the oven. All cooked samples (internal temperature 75 °C) were drained from the excess liquid in a plastic net, then again individually weighed. Cooking loss was expressed as g/100 g by weight difference between uncooked and cooked samples. For the determination of meat tenderness, meat samples were cut into 6 cores with similar sizes; each core was sheared perpendicular to the longitudinal orientation of the muscle fiber using a Warner-Bratzler shear blade with the triangular slot cutting edge mounted on Salter model 235 (Warner-Bratzler meat shear, G-R manufacturing Co. 1317 Collins LN, Manhattan, Kansas, USA) to determine the peak force (kg) when the samples were sheared. Shear force was determined as the average of the maximum force of the 6 replicates from each sample.

Nutrient content, fatty acid profile and cholesterol content

Proximate composition (moisture, crude protein, total fat and crude ash) of PM was determined following standard methods. In particular, moisture content was calculated as the percentage of weight lost after drying 5 g of sample in oven (103±2°C for 16 h) (AOAC, 1990). Crude protein content was assessed according to the Kjeldahl method by using copper sulphate as catalyst (AOAC, 1990), whereas lipids were extracted following the chloroform:methanol extraction procedure (Folch *et al*., 1957). Crude ash content was assessed by weighing samples after incineration at 525°C (AOAC, 1990). Following lipid extraction, fatty acids (FA) were quantified as methyl esters (FAME) using a gas chromatograph GC Trace 2000 (ThermoQuest EC Instruments) equipped with a flame ionization detector (260 °C) and a fused silica capillary Column (SGE Forte BP×90, Phenomenex, Torrance, CA, USA) 100 m × 0.25 mm × 0.25 μm film thickness. Helium was used as carrier gas. The oven temperature program was 100 °C for 5 min then increasing at 4°C/min up to 240 °C where it was maintained for 20 min. The individual FA peaks were identified by comparison of retention times with those of FAME authentic standards run under the same operating conditions. Results were expressed as percentage of the total FA identified. To assess the nutritional implications, the ratio of n-6 to n-3 FA (n-6/n-3) and the ratio of polyunsaturated fatty acids to saturated fatty acids (P/S) were calculated. Moreover, to evaluate the risk of atherosclerosis and the potential aggregation of blood platelets, the atherogenic index (AI) and the thrombogenic index (TI) were calculated according to the formulas suggested by Ulbricht and Southgate (1991).

Cholesterol was extracted using the method of Maraschiello *et al*. (1996) and then quantified by HPLC. A Kontron HPLC (Kontron Instruments, Milan, Italy) model 535, equipped with a Kinetex 5μ C18 reverse-phase column (150 x 4.6mm x 5 μm;

Phenomenex, Torrance, CA), was used. The HPLC mobile phase consisted of acetonitrile: 2-propanol (55:45, vol/vol) at a flow rate of 1.0 mL/min. The detection wavelength was 210 nm. The quantitation of muscle cholesterol content was based on the external standard method using a pure cholesterol standard (Sigma, St. Louis, MO).

Intramuscular collagen properties

At analysis, muscle samples were thawed, at room temperature, trimmed of fat and epimysium, lyophilized for 48 h, and hydrolysed 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 analyses were carried out in duplicate. Intramuscular collagen concentration was calculated assuming that collagen weighed 7.25 times the measured hydroxyproline weight (Eastoe and Leach, 1958) and expressed as micrograms of hydroxyproline per milligram of lyophilized tissue. Hydroxylysylpyridinoline (HLP) concentration, the principal nonreducible crosslink of muscle collagen and highly correlated with the thermal stability of collagen (McCormick, 1999), was determined 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 × 4.6 mm × 5 μm; Phenomenex, Torrance, CA), was used. The concentration of HLP residues in the samples was calculated based on the concentration of collagen in each hydrolysate, assuming that the molecular weight of collagen was 300,000 and the molar fluorescence yield of pyridoxamine (internal standard) was 3.1 times that of HLP (Eyre *et al*., 1984). Crosslink concentration was expressed as moles of HLP per mole of collagen.

Statistical analyses

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

7.2 Results and discussion

Hatchability and growth performance

Hatchability is one of the most relevant parameters to estimate successful *in ovo* intervention. In the present study, hatchability of the *in ovo*-injected eggs resulted similar in the experimental groups, ranging from 92.6 and 91.8%, respectively for CON and GOS, with intermediate values for SAL (92.1%). In our early studies, was demonstrate that day 12 of incubation is the optimal time for prebiotic injection into the air cell of the incubating egg (Ross 308) that allows for high hatchability scores (Bednarczyk *et al*., 2016; Slawinska *et al*., 2020). This due to the bioactive solution that is deposited in the air cell, from which the prebiotic diffuses into the bloodstream, without penetrate the inner parts of the egg and potentially decrease the embryo viability and consequently the eggs hatchability (Siwek *et al*., 2018). At placement, C chicks were heavier than those of GOS and S groups (40.7 vs. 39.8 and 39.8 g, respectively; $P<0.001$), due to the egg's manipulation at d 12 of incubation; the same results were obtained in our previous study on Ross 308 (Slawinska *et al*., 2020), but such negative effects of *in ovo* treatment were transient and did not last during the other rearing phases.

Considering, the whole rearing period (0 – 50 d), S birds exhibited higher BW than GOS

(2,190 vs. 1,996 g, respectively; P<0.05) (Table 6). Otherwise, Slawinska *et al*. (2020) found a significant increase in BW on day 42 in GOS compared to C group (2,892 g in GOS vs 2,758 g in C). In literature, results on the effect of prebiotic administration in feed showed improved growth performance of birds but positive effects are not always observed as reported in the review by Yang *et al*. (2009). Sohail *et al*. (2012) and Sherif *et al*. (2012) noted that the usage of prebiotic in broiler diet had no significant effect on feed intake and feed conversion ratio. Also, Midilli *et al*. (2008) observed no significant improvement in productive traits. It has been reported that the type of diet (i.e., the content of non-digestible oligosaccharides), the type and inclusion level of the supplements, the animal characteristics (species, age, stage of production), and the hygiene status of the farm could influence the efficacy of prebiotic administration (Verdonk *et al*., 2005). Most probably, the lack of growth promoting effect of GOS observed in our previous studies (Slawinska *et al*., 2020; Tavaniello *et al*., 2018; Maiorano *et al*., 2017; Bednarczyck *et al*., 2016) is related to the different chicken genotype used in the present study. DFI was greater in S groups than in GOS ($P < 0.01$) and C ($P < 0.05$) groups, while FCR was similar among groups (ranging from 2.24 to 2.30; $P > 0.05$). The total mortality ranging from 1.44 % (GOS) to 2.01 (SAL) % was not affected by treatment ($P > 0.05$).

As expected, thermal challenge applied for the last 14 days of finisher feeding phase, had a dampening effect on growth performance. Heat-stressed group reported lower BW (1,943 vs. 2,208 g, respectively for HS and TN; P<0.001), lower DFI (79.9 vs 88.0 g/bird/d, respectively; P<0.001), while FCR was similar among groups ($P > 0.05$). Lower growth performance of chickens in hot conditions are well documented in literature (Lara and Rostagno, 2013). The growth rate in broilers mainly depends on the amount of feed

intake (Awad *et al*., 2018). In the present work, feed consumption and efficiency decreased in response to HS. It can be explained by the fact that digestion, absorption, and metabolism of the nutrients increase metabolic heat production. Animals in high ambient temperature condition need to minimize the production rate of metabolic heat and therefore they reduce feed ingestion.

Mortality is one of the basic welfare parameters that indicate the health status of the flock. Increased mortality of broiler chickens during heat is a major economic and welfare concern. The heat can be detrimental in form of heat waves in moderate climates, more frequent due to climatic changes (acute HS). In the present study, mortality was similar among groups (1.72 %), exhibiting no significant difference attributable to the higher environmental temperature. This observation seems to suggest that slow-growing broilers are more resilient and resistant to high environmental temperature than the fast-growing ones from our previous study (Slawinska *et al*., 2020), even though productive aspects were dampened in both the genetic lines. In this trial was also conducted a study on splenic gene expression signatures (Pietrzak *et al*., 2020) where it was demonstrated that the genetic adaptation of slow-growing chickens to HS combined with *in ovo* stimulation with GOS has mitigating effects on the molecular pathways that are associated with immune and stress responses. In fact, slow-growing chickens proved to be well adapted to acute HS, which did not trigger immune related or stress-related gene expression in the spleen. On the other hand, chronic HS activated genes that are associated with inflammation and oxidative stress (i.e., OxInflammation). GOS that were delivered *in ovo* mitigated heat-induced OxInflammation and decreased Th2 responses (down-regulation of IL-4). The hybrids that were used in our experiment are slow-growing free-range chickens that are obtained by crossing a Hubbard RedBro male with a

Hubbard JA57 female. These free-range poultry hybrids are distinguished by a good adaptation to a warm climate and a high disease resistance (Federico Sirri, unpublished data). No significant interaction between the *in ovo* treatments and the environmental condition was observed for productive parameters.

Table 6. Productive performance of slow-growing broiler chickens injected *in ovo* with GOS in response to heat stress.

	Treatment (Tr)			Temperature (T)			Significance		
	CON[1]	SAL[2]	GOS[3]	TN[4]	HS[5]	SEM	Tr	T	TrxT
Body weight (g)	2,041^{ab}	2,190^{a}	1,996^{b}	2,208^{A}	1,943^{B}	48.8	*	**	NS
Daily feed intake (g/bird/d)	82.8^{Bb}	87.4^{Aa}	81.5^{B}	88.0^{A}	79.9^{B}	1.18	**	**	NS
Feed conversion rate	2.283	2.240	2.296	2.235	2.311	0.06	NS	NS	NS
Mortality (%)	1.72	2.01	1.44	1.72	1.72	0.03	NS	NS	NS

[1]CON = Control (untreated); [2]SAL = *in ovo* injected with physiological saline (mock-treated); [3]GOS = *in ovo* injected with GOS (prebiotic-treated); [4]TN – thermoneutral conditions; [5]HS – heat stress conditions (on days 36 - 50).
SEM = standard error mean.
Significance: NS = P > 0.05; * P< 0.05; ** P<0.01.
a,b Means within a row lacking a common superscript differ ($P < 0.05$).
A,B Means within a row lacking a common superscript differ (P < 0.01).

Breast Muscle Weight, pH, and Color

At slaughter (50 d), eviscerated carcass yield did not exhibit significant differences among the experimental groups (TN: C: 67.4 %, S: 67.5 %, GOS: 66.5 %, P > 0.05, and HS: C: 70.1 %, S: 69.8 %, GOS: 71.5 %, P > 0.05).

Results regarding the effects of *in ovo* injection of GOS in response to heat stress on carcass traits and physico-chemical properties of breast muscle are reported in Table 7. Carcass traits, such as breast weight and breast yield, are of economic importance in

chicken meat production. The GOS treatment had no influence on carcass weight (ranging from 1603.9g to 1659.1g), being similar ($P > 0.05$) between experimental groups. Breast weight was affected by prebiotic treatment; in particular, it was higher ($P < 0.05$) in C group compared to GOS (341.6 vs 315.6 g, respectively); as a consequence, the breast yield was higher ($P < 0.05$) in C group in comparison with GOS (21.1 vs 19.7 %, respectively). In a previous study, conducted on Ross 308 broiler chickens *in ovo* injected with GOS and subjected to heat stress, Tavaniello *et al*. (2020) did not find any significant effect of GOS on breast muscle weight. In previous studies, Maiorano *et al*. (2017) and Tavaniello *et al*. (2018) found a positive effect of GOS *in ovo* injection on carcass and breast weight of Ross 308 broiler chickens as compared to control group.

As expected, heat stress, applied for the last 14 days of the rearing cycle, had a detrimental effect on carcass weight, resulted in lower weight ($P < 0.01$) in the HS group in comparison with TN (1555.9 vs 1706.7 g, respectively). As a consequence, breast weight was significantly lower ($P < 0.01$) in HS group compared to C (316.2 vs 347.2 g, respectively), which is consistent with the previous study in Ross 308 broiler chickens subjected to chronic heat stress conditions (30° C for 8h) for the last 10 days of the rearing cycle (Tavaniello *et al*., 2020). Similar results were found by Goo *et al*. (2019) and Ma *et al*. (2021). Another study also showed that chronic heat stress reduced the proportion of pectoral muscles in broilers (Zhang *et al*., 2012; Cramer *et al*., 2018). In contrast, in a study conducted on China local slow-growing chickens (Beijing You chicken, BJY), Lu *et al*. (2007) did not find any detrimental effect of constant high ambient temperature (34 °C, from 5 to 8 weeks of age) on carcass and breast weight. On the other hand, heat stress stimulates the hypothalamic–pituitary–adrenal axis in poultry and increases in circulating corticosterone hormone (Sapolsky *et al*., 2000), this would

likely increase catabolism of skeletal muscle contributing to reduced body growth (Scanes, 2016; Beckford *et al*., 2020). However, it suggests that slow-growing broilers from different regions may respond differently to ambient temperature. In fact, heat-resistance depends on the genetic adaptation of the chickens. Native chickens from tropical and sub-tropical regions are more tolerant to high ambient temperatures than fast-growing lines. Since they are smaller and lighter and have not been subjected to selective pressure for meat-related traits, they have retained their genetic adaptation to handle high temperatures (reviewed in Pietrzak *et al*., 2020). A significant interaction ($P < 0.01$) between treatment and temperature was observed for carcass weight (C: TN = 1772 g, HS = 1491 g; S: TN = 1685 g, HS = 1634 g; GOS: TN = 1667 g, HS = 1545 g), highlighting the mitigating effect of GOS under heat stress conditions. In a hot environment, when the air temperature is above the upper critical temperature, the ability to dissipate heat is limited. The thermoregulation characteristics of poultry differ from those of mammals due to their high rate of metabolism associated with more intensive heat production and low heat dissipation capacity caused by their feathers and lack of sweat glands (Babinszky *et al*., 2011). In order to maintain thermal equilibrium, animals reduced their feed intake and thus reduced the increase in heat. In addition, high ambient temperature will cause rising hyperthermia in the body, reducing the activity of the appetite centre in the medulla oblongata (Babinszky *et al*., 2011). High ambient temperatures may also reduce the digestibility of nutrients in poultry, which may be due to reduced trypsin, chymotrypsin, and amylase activities (Hai *et al*., 2000). Therefore, it is the higher temperature that triggers the reduction of feed intake. In order to reduce heat production, domestic animals also reduce physical activity (Collin *et al*., 2001) and spend less time eating (Brown-Brandl *et al*., 2001). The rate of feed refusal during heat stress increases

with age in broilers (Gonzalez-Esquerra and Leeson, 2005). Besides, under severe heat stress (34-35 °C), the feed intake of laying hens can be reduced by about 30-50% (Babinszky *et al*., 2011). In a recent study, broilers subjected to heat stress had significantly reduced feed intake (16.4%), reduced body weight (32.6%), and increased feed conversion rate (25.6%) (Sohail *et al*., 2012). In a recent study (Deng *et al*., 2012), a 12-day heat stress period resulted in a 28.58 g/day reduction in feed intake.

Ultimate muscle pH was affected by prebiotic treatment, it was higher ($P < 0.05$) in GOS group in comparison with C, while S group presented intermediate values ($P > 0.05$). Heat stress had no effect on ultimate pH. The obtained values are within the normal range, with absence of meat defects. These results contrast with our previous findings (Tavaniello *et al*., 2020), where it was found that *in ovo* delivery of GOS did not affect ($P > 0.05$) ultimate pH (pH24) of PM, while heat stress had a significant influence on it. A higher meat pH in high ambient temperature was also observed by Lu *et al*. (2007) and Goo *et al*. (2019), while Awad *et al*. (2020) reported lower meat pH in chickens reared under heat stress. However, the variability of the effects reported for GOS, or HS may be explained by different genetic backgrounds, slaughtering age and the duration of the heat stress treatments applied. The pH value is one of the most important physical parameters of meat. It has a central role in determining the protein behaviour both in fresh and processed meat products, in fact it is used as a predictor of meat technological and sensory qualities (Fletcher, 1999; Van Laack *et al*., 2000). However, the ultimate pH values found in the present study are the normal values for breast muscles in broiler chickens (Maiorano *et al*., 2012).

Meat color is the first sensory aspect for consumers to evaluate the quality of chicken, which is generally reflected by parameter values such as brightness, redness and

yellowness and is mainly affected by pH, myoglobin as well as deposited fat (Han *et al*., 2012). Close relationship between the pH of the muscles and the color of the meat is recognized. Color parameters were partially affected by GOS. In particular, meat from C ($P < 0.05$) and S ($P < 0.01$) chickens were lighter than that from GOS group; whereas meat from the latter group showed a higher ($P < 0.05$) redness index (a*) compared to S one. No significant effect of the treatment on yellowness (b*) was found. The observed color coordinates fit within the range which is accepted for good chicken meat appearance. even if lightness was slightly higher than that reported for normal meat. Meat with L* values (degree of paleness) higher than 54 is considered light and tends to be pale, soft, exudative meat (Woelfel *et al*., 2002). However, it possible to exclude the presence of defects in meat because pH values were in the range for normal meat (5.74 – 5.83). Otherwise, in our previous study (Tavaniello *et al*., 2020), it was observed that the breast muscle of Ross 308 chicken from GOS and S groups was lighter than that of group C ($P <0.01$), and the yellowness index (b *) in group C was higher than that of group S. It has been reported that slow-growing genotypes have meats with a lower redness index and a higher yellowness index than genotypes with rapid growth rates (Fanatico *et al*., 2005, 2007); therefore, the values found in the present study can be considered normal and within the acceptable range for commercial meats. Heat stresses reduce ($P < 0.01$) the redness of meat compared with thermoneutral conditions. No effect was evidenced from the heat stress on the lightness and yellowness of meat. Similarly, Zhang *et al*. (2012) found that heat-exposed Arbor Acres broilers had higher L*, and lower a* than the birds in standard temperature group. The results of decreased a* value indicated that there is more oxidized myoglobin in the heat-exposed birds' muscle (Mancini and Hunt, 2005). On the contrary, nevertheless, other studies on Cobb (Goo *et al*., 2019) and local slow-

growing chickens (Lu *et al*., 2007) reported no effect of heat stress on meat color. In our previous study conducted on Ross 308 broilers heat stress increased the yellowness and reduced lightness in fast-growing genotypes (Tavaniello *et al*., 2020) compared with thermoneutral conditions. It indicates that for different poultry genotypes, heat stress may cause changes at different levels in meat color.

Water-holding capacity is of great importance, since the content and distribution of water within muscles may affect the visual appearance as well as the tenderness and juiciness of meat. It has been reported that acute and chronic heat stresses cause the poor water-holding capacity. Heat stress caused a high metabolic rate rigor mortis, resulting in pronounced protein denaturation affecting the protein's ability to bind water and results in poor water-holding capacity, and consequently in higher drip loss and cooking loss (reviewed in Zhang *et al*., 2012). Neither treatment nor temperature had effect on WCH, cooking loss and WBSF which were similar ($P > 0.05$) among experimental groups. Similar results were found by Tavaniello *et al*. (2020). On the contrary, Zhang *et al*. (2012) found that broilers in constant high temperature group have higher meat cooking losses than those in the other group, which is similar to the result of Sandercock *et al*. (2001), who showed a higher drip loss for breast meat of heat-stress birds (32.5°C and RH of 67.1%). Cramer *et al*. (2018) did not find any significant effect of heat stress and probiotic feeding on color characteristics, WHC, and shear force of broiler breast muscle, but observed a lower cooking loss in heat-stressed chickens compared to those reared under thermoneutral conditions. Other previous works reported a decrease in the WHC of meat from broilers exposed to heat stress (Lu *et al*., 2007) or no significant effect of heat stress on drip loss (Goo *et al*., 2019; Awad *et al*., 2020) but a higher shear force of broiler breast muscle reared in HS condition (Awad *et al*., 2020).

Table 7. Carcass traits and physic-chemical properties of breast muscle of slow-growing broiler chickens injected *in ovo* with GOS in response to heat stress.

	Treatment (Tr)			Temperature (T)		SEM	Significance		
	CON[1]	SAL[2]	GOS[3]	TN[4]	HS[5]		Tr	T	TrxT
Carcass weight (g)	1626.8	1659.1	1603.9	1706.7	1555.9	14.93	NS	**	**
Breast weight (g)	341.6[a]	338.0	315.6[b]	347.3	316.2	4.20	*	**	NS
Breast yield (g)	21.2[a]	20.4	19.7[b]	20.3	20.4	0.21	*	NS	NS
pH24	5.74[B]	5.77	5.83[A]	5.78	5.79	0.01	**	NS	NS
Colour 24h									
L*	54.11[a]	54.93[A]	52.32[Bb]	53.34	54.17	0.28	**	NS	NS
a*	1.55	1.31[B]	2.09[A]	1.93	1.39	0.09	**	**	NS
b*	16.75	16.75	16.28	16.73	16.46	0.29	NS	NS	NS
WHC (%)	11.98	12.03	12.02	12.16	11.86	0.11	NS	NS	NS
Cooking loss (%)	22.22	22.27	22.31	22.27	22.27	0.12	NS	NS	NS
WBSF[3] (Kg/cm^2)	1.57	1.57	1.65	1.62	1.57	0.05	NS	NS	NS

[1]CON = Control (untreated); [2]SAL = *in ovo* injected with physiological saline (mock-treated); [3]GOS = *in ovo* injected with GOS (prebiotic-treated); [4]TN – thermoneutral conditions; [5]HS – heat stress conditions (on days 36 - 50).
[3]WBSF = Warner-Bratzler shear force.
SEM = standard error mean.
Significance: NS = P > 0.05; * P < 0.05; ** P < 0.01.
a,b Means within a row lacking a common superscript differ (P < 0.05).
A,B Means within a row lacking a common superscript differ (P < 0.01).

Nutrient content, cholesterol content, intramuscular collagen properties

In Table 8, are reported results concerning the proximate composition, cholesterol content and intramuscular collagen properties of breast muscle. Treatment with prebiotics had no effect (P > 0.05) on the nutritional parameters analysed, in accordance with the results of Tavaniello *et al*. (2020). To our knowledge, limited information is available in literature

on the effect of prebiotics on nutritional properties of chicken meat.

Cholesterol content has become an important component in composition studies on meat and poultry products, since it is a nutritionally important component of meat. Cholesterol and its metabolites, and immediate biosynthetic precursors of cholesterol play essential roles in cellular membrane physiology, dietary nutrient absorption, reproductive biology, stress responses, salt and water balance, and calcium metabolism (Dinh *et al*., 2011). The cholesterol values found in the present study (ranging from 37.07 to 40.05 mg/100g) were not affected ($P > 0.05$) by GOS, which was similar to the results in Ross 308 broiler chicken from Tavaniello *et al*. (2020). Similarly, Tavaniello *et al*. (2018) did not found any significant effect of different prebiotics, *in ovo* injected, on muscle cholesterol content. On the contrary, Pilarski *et al*. (2005) reported that *in ovo* application of fructooligosaccharides in Hybro G broiler breeder eggs caused a decrease of breast muscle cholesterol in comparison with the control group. Cholesterol content in broiler meat can be altered by various factors (composition of diet, age, sex; Wang *et al*., 2006) as well as the use of different methodologies for cholesterol quantification or for sampling (Bragagnolo and Rodriguez-Amaya, 2002).

Collagen is a protein abundant in connective tissue and is a factor contributing to the variation in meat tenderness and texture (Purslow, 2005) of different species, including birds (Baeza *et al*., 1998; Maiorano *et al*., 2011, 2012; Tavaniello *et al*., 2014; Sirri *et al*., 2016). 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 (McCormick, 1999; Maiorano *et al*., 2015). In the current study, intramuscular collagen (IMC) concentration (ranging from 16.67 to 17.56 μg/mg of lyophilized muscular tissue) and collagen maturity

(ranging from 0.067 to 0.070 mol HLP/mol of collagen) were not influenced ($P > 0.05$) by *in ovo* GOS administration. These values are higher than those reported by Tavaniello *et al*. (2020) on fast-growing broiler chickens ranging from 0.039 to 0.042 mol of HLP/mol of collagen. These differences due to the higher maturity of collagen related to the slow-growing rate of the chicken strain used in this trial (Hubbard) compared with chicken (Ross 308) used in the work of Tavaniello and co-authors a fast-growing rate strain. In fact, studies document growth rate-dependent shifts in muscle collagen amount and/or crosslinking (Harper, 1999; Maiorano *et al*., 2001; McCormick, 1994). During rapid growth (e.g., fast-growing genotypes), newly synthesized collagen dilutes older collagen and is less crosslinked than the pre-existing collagen (Etherington, 1987), with a positive effect on meat tenderness (McCormick, 1994; Maiorano *et al*., 2009).

Regarding the effect of heat stress on the above-mentioned meat quality parameters, heat stressed chickens provided a meat with higher moisture ($P < 0.01$) and lower protein ($P < 0.05$) content compared with TN group; lipid and ash content were not affected by temperature. Zhang *et al*. (2012) reported a higher moisture and fat content and lower protein content in breast meat from Arbor Acres broilers exposed to constant high temperature compared to standard temperature group. Tavaniello *et al*. (2020) found a higher lipid content and a lower total collagen amount in Ross 308 broiler chickens exposed to heat stress. It has been reported that the lack of homeostasis and heat stress itself will accelerate the production of free radicals in the body, leading to a higher risk of oxidation. Excessive production of reactive oxygen species (ROS) caused by heat stress can cause a variety of metabolic changes, which will not only cause oxidative damage to multiple organs of chickens but also oxidative damage to chicken skeletal muscles (Lin *et al*., 2006; Mujahid *et al*., 2007). This, in turn, will negatively affect the protein function

and oxidative stability of chicken skeletal muscle (Zhang *et al*., 2012). A study found that broilers respond to high-temperature threats by reducing protein synthesis and increasing protein breakdown (Lin *et al*., 2006). In fact, it has been reported that high ambient temperature significantly decreased body protein content, protein gain, the protein retains and intake (reviewed by Zhang *et al*., 2012). Furthermore, strong interactions between the *in ovo* treatment and temperature were observed in case of protein ($P < 0.01$). In particular, it was found that *in ovo* delivery of GOS decreased protein content (C: TN = 24.60%, HS = 24.86%; S: TN = 24.56%, HS = 24.32%; GOS: TN = 25.03%, HS = 23.99%) in heat stress condition.

As mentioned before, total lipid content was not affected by heat stress; however, other studies found heat stress results in the lower body and muscle tissue proteins but higher fat levels (Gonzalez-Esquerra and Leeson, 2005; Aksit *et al*., 2006). Exposure to high ambient temperatures reduces basal metabolism and physical activity, thereby reducing metabolic heat production (Geraert *et al*., 1996). This may be the cause of increased abdominal, subcutaneous and intermuscular fat deposition (Ain Baziz *et al*., 1996; Geraert *et al*., 1996). Increasing evidence indicates that much of the variation in response to heat stress is apparently genetically based (Mack *et al*., 2013; Soleimani *et al*., 2011; Felver-Gant *et al*., 2012). By comparing the results of the present study with those conducted on Ross 308 broiler chickens (Tavaniello *et al*., 2020), we can assume that slow-growing chickens may not reduce basal metabolism and physical activity due to high temperatures and may be more able to adapt to changes caused by heat stress as compared to fast-growing chickens. It is consistent with Lu *et al*. (2007) who found slow-growing chickens showed higher resistance to high ambient temperature. In addition, significant interactions ($P < 0.001$) were found for lipid content (C: TN = 2.45%, HS =

2.44%; S: TN = 2.27%, HS = 2.86%; GOS: TN = 2.51%, HS = 2.08%), GOS reduced lipid content in HS condition.

Table 8. Proximate composition, cholesterol content and intramuscular collagen properties of slow-growing broiler chickens injected *in ovo* with GOS in response to heat stress.

	Treatment (Tr)			Temperature (T)		SEM	Significance		
	CON[1]	SAL[2]	GOS[3]	TN[4]	HS[5]		Tr	T	TrxT
Moisture (%)	72.43	72.64	72.51	72.29	72.76	0.07	NS	***	NS
Protein (%)	24.74	24.44	24.51	24.73	24.40	0.07	NS	*	**
Lipid (%)	2.44	2.57	2.29	2.41	2.46	0.05	NS	NS	***
Ash (%)	0.81	0.83	0.83	0.81	0.84	0.01	NS	NS	NS
Cholesterol (mg/100g)	37.07	40.05	38.04	39.23	37.60	0.60	NS	NS	NS
Collagen (µg/g)	17.56	16.67	17.02	17.07	17.08	0.24	NS	NS	NS
HLP[3] (mol/mol of collagen)	0.070	0.067	0.069	0.069	0.068	0.001	NS	NS	NS

[1]CON = Control (untreated); [2]SAL = *in ovo* injected with physiological saline (mock-treated); [3]GOS = *in ovo* injected with GOS (prebiotic-treated); [4]TN – thermoneutral conditions; [5]HS – heat stress conditions (on days 36 - 50).
SEM = standard error mean.
Significance: NS = P > 0.05; * P < 0.05; ** P < 0.01; *** P < 0.001.

Fatty acids profile

In terms of human health, fatty acids (FA) composition of meat products is a very important parameter of meat quality. Broiler fat is characterized by a significant amount of monounsaturated fatty acids (MUFA), and, in comparison with red meat, substantial amounts of polyunsaturated fats, especially the n-6 linoleic acid and arachidonic acid. Moreover, it may represent an important source of long-chain n-3 fatty acids (Hibbeln *et al*., 2006; Attia *et al*., 2017). The FA profile of breast meat is shown in Table 9. Taking

into account the general FA profile, the most abundant fatty acids were saturated fatty acids (SFA; ranging from 35.57 to 36.76 %) and polyunsaturated fatty acids (PUFA; ranging from 35.10 to 37.25 %), followed by MUFA (ranging from 27.10 to 28.11 %).

Total SFA content and the single SFA was not significantly affected by the prebiotic treatment ($P > 0.05$). Among the individual SFA, the most abundant was the palmitic acid (C16:0; ranging from 26.89 to 27.14 %), followed by stearic acid (C18:0; ranging from 7.76 to 8.65%); while others SFA (C14: 0, C15: 0, C17: 0, C20: 0, C22: 0 and C24: 0) were all less than 0.5%. Similar results were obtained by Tavaniello *et al*. (2020) in a study conducted on fast-growing chickens stimulated *in ovo* with GOS, authors did not find any effect on the concentrations of total SFA and individual SFA (except C22:0) of breast muscle, and palmitic and stearic acids were the most abundant SFA. Palmitic acid is thought to increase cholesterol levels together with lauric and myristic acid, while stearic acid has little or no effect (Zock *et al*., 1994). In fact, stearic acid is generally considered to be a neutral fatty acid because it has been shown to have no net impact on the plasmatic level of either LDL or HDL cholesterol in humans (Yu *et al*., 1995).

Considering the total and individual MUFA, both the amounts were not affected ($P > 0.05$) by GOS. The most abundant MUFA was the oleic acid (C18:1; ranging from 24.92 to 25.51 %; $P > 0.05$). Likewise, Tavaniello *et al*. (2020) did not found any significant effect of GOS *in ovo* injection on fast-growing chickens. They found a lower content of oleic acid (ranging from 18.06 to 19.06 %), which leads to lower MUFA (ranging from 20.18 to 22.23 %). Another study found that the concentration of oleic acid of breast muscle ranged from 21.79 to 30.43 % among fast-growing chickens (Cobb 700), middle-growing strains (Naked neck Kabir) and slow-growing strain (Brown Classic Lohman) (Sirri *et al*., 2011). The reason for variation in the concentration could be related to the

dietary compositions and genetic background. As well known, from the nutritional point of view, oleic acid plays a key role in human diet in reducing lipaemia and consequently the risk of stroke (D'Alessandro *et al*., 2012).

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 22.51 to 23.93 %, $P > 0.05$), followed by arachidonic (C 20:4 n-6; ranging from 7.04 to 7.71 %, $P > 0.05$) and α-linolenic acid (ALA, C18:3 n-3, ranging from 1.13 to 1.22 %, $P > 0.05$). Otherwise, in fast-growing chickens (Tavaniello *et al*., 2020), treatment significantly affected the total PUFA content, which was slightly lower ($P = 0.077$) in GOS group compared to Saline group, with intermediate values ($P > 0.05$) for C group. The same trend was found for n-6 PUFA ($P = 0.062$). Furthermore, Tavaniello *et al*. (2018), found that GOS *in ovo* injected increased the content of SFA and PUFA, and reduced MUFA content in breast muscle of chickens. However, it must be taken into account that FA composition of meat greatly depends on diet composition, on the production of short-chain FAs and their amount and on different genotypes.

In ovo prebiotic supplementation had no effects on all calculated nutritional ratios (n6/n3, P/S, AI, and TI) considered in the current research as also found in fast-growing chickens. The values of the n-6/n-3 ratio observed in this study are little bit higher than those found for fast-growing chickens (Tavaniello *et al*., 2020) and are distant from the ideal value of 1 and the maximum value of 4 (Wood *et al*., 2003). Generally, poultry is characterized by the highest n-6/n-3 ratio compared to other types of meat, essentially due to the higher amount of n-6 FA than muscles of the other species (Rule *et al*., 2002; Wood *et al*., 2003). In fact, linoleic acid is the predominant essential FA in poultry and as a result the n-6

PUFA are the primary products found in tissue lipids. Consequently, the n-6/n-3 ratio in poultry meat is at a distance from the ideal value of 1 and above the recommended maximum of 4.

The P/S ratio is known to be a measure of the propensity of the diet to influence the occurrence of coronary disease (Wood *et al*., 2003). From a nutritional point of view, a higher P/S ratio is recommended since dietary intake of unsaturated fatty acids has been shown to reduce the risk of cardiovascular disease and may reduce the incidence of certain cancers, asthma and diabetes in other cases (Milicevic *et al*., 2014); it should be increased to above 0.4 (Wood *et al*., 2003). The values of P/S ratio obtained in the present study, ranging from 0.98 to 1.05, are little bit lower than those reported for fast-growing chickens (ranging from 1.03 to 1.17; Tavaniello *et al*., 2020). From a nutritional point of view, a higher P/S ratio is recommended; indeed, it should be increased to above 0.4. The P/S values obtained, are than bovine meat (0.1), pigs (0.58) (Wood *et al*., 2003) and lambs (0.15-0.32, Wood *et al*., 2003, Maiorano *et al*., 2016).

The atherogenic index (AI) and the thrombogenic index (TI) represent criteria for evaluating the level and interrelation through which some FA may have atherogenic or thrombogenic properties, respectively. The low AI (ranging from 0.44 to 0.45) and TI (ranging from 0.82 to 0.85) values found in the current study revealed a good nutritional quality of the meat. Both indices were lower than the recommended limit of 1 (Garaffo *et al*., 2011) with important health benefits.

The temperature had marginal effects on FA composition of breast meat, total contents of SFA, MUFA, and PUFA were similar ($P > 0.05$) among groups. Also, the composition of the single FA was partially affected by temperature; in particular, linoleic (C 18:2 n-6), eicosatrienoic (C 20:3 n-3) and docosanoic acid (C 22:1) were higher in HS group

compared to TN one ($P < 0.05$). Similarly, the composition of the single FA was affected by temperature in fast-growing chicken (Tavaniello *et al*., 2020); in particular, long-chain PUFA of both n-3 (C22:5, C22:6) and n-6 (C20:4, C22:2, C22:4) series were higher in HS group compared to TN one. As a result, the total content of n-3 PUFA was higher in HS group. Other statistically significant differences (C14:0, C17:0, C20:1) were found in fast-growing chickens, which were present in a very small amount (less than 1%) (Tavaniello *et al*., 2020). To our knowledge the information regarding the effect of heat stress on FA composition of chicken meat is limited. In a study conducted on French local broiler chicken, Ain Baziz *et al*. (1996) found that meat from heat-exposed birds (32°C from 4 to 7 weeks old) had the same FA profile than that of control chickens with ad libitum feeding, while in pair-feeding conditions, heat-exposed birds showed a higher SFA and lower PUFA contents compared to control chickens. It should be stated that both genotypes, diet composition and heat stress patterns may change the FA profile. It was found that compared with control chickens, the heat-exposed poultry showed lower MUFA content, which was consistent with Tekce *et al*. (2020) and Zhao *et al*. (2019), although this difference was not significant. In another study conducted on Cobb 500, Jahromi *et al*. (2015) found that meat from heat-exposed birds (35°C) showed greater MUFA content but lower PUFA content and PUFA/SFA ratio compared to control chickens; while, when birds were fed with basal diet plus 0.1 % probiotic mixture raised in 35 °C meat showed lower total SFA and higher total MUFA compared with control one. The nutritional ratios (n6/n3, P/S, AI, and TI) were not affected ($P > 0.05$) by temperature both in slow-growing and fast-growing chickens. In slow-growing chickens, interactions between *in ovo* treatment and temperature were observed only in case of margaric, (C 17:0), stearic (C 18:0), arachidic acid (C 20:0) and n-6/n-3 ratio. Among

them, GOS in HS birds decreased stearic acid (C: TN =7.37%, HS: 9.92%; SAL: TN =9.73%, HS: 6.62%; GOS: TN =6.84%, HS: 8.67%) and increased n-6/n-3 ratio (C: TN =8.89; HS: 8.18; SAL: TN =7.61, HS: 8.61; GOS: TN =7.73, HS: 8.72). It was found that in fast-growing chickens, *in ovo* delivery of GOS mitigate the changes in FA due to heat stress, such as decreased SFA content and increased MUFA content, as well as having positive effect on all nutritional indices (Tavaniello *et al*., 2020). The comparison of the results between fast- growing and slow-growing chickens allow to affirm that heat stress influence production performance and meat quality in fast-growing chicken, while slow-growing chickens are more tenacious to heat stress. In terms of interaction, *in ovo* injection with GOS is more valuable in fast-growing broilers, especially in mitigating the detrimental effects on fatty acid composition by heat stress.

Table 9. Fatty acid composition (% of total fatty acids) and nutritional indices in breast muscle of slow-growing broiler chickens injected *in ovo* with GOS in response to heat stress.

Item3	Treatment (Tr)			Temperature (T)		SEM	Significance		
	CON[1]	SAL[2]	GOS[3]	TN[4]	HS[5]		Tr	T	TrxT
Fatty acids									
C14:0	0.39	0.44	0.36	0.38	0.41	0.02	NS	NS	NS
C14:1	0.06	0.05	0.05	0.05	0.05	0.00	NS	NS	NS
C15:0	0.08	0.08	0.07	0.08	0.07	0.00	NS	NS	NS
C16:0	27.14	27.07	26.89	27.32	26.75	0.22	NS	NS	NS
C16:1	1.93	2.23	1.90	2.02	2.02	0.08	NS	NS	NS
C17:0	0.17	0.16	0.16	0.16	0.17	0.01	NS	NS	*
C18:0	8.65	8.17	7.76	7.98	8.41	0.41	NS	NS	*
C18:1	25.11	25.51	24.92	25.81	24.55	0.49	NS	NS	NS
C18:2 n-6	23.93	22.51	23.78	22.34	24.47	0.43	NS	*	NS
C18:3 n-6	0.13	0.13	0.12	0.13	0.12	0.00	NS	NS	NS
C18:3 n-3	1.22	1.15	1.13	1.14	1.19	0.03	NS	NS	NS
C20:0	0.11	0.11	0.11	0.11	0.11	0.01	NS	NS	*
C20:1	0.20	0.20	0.20	0.20	0.20	0.00	NS	NS	NS
C20:2n-6	0.64	0.67	0.68	0.63	0.69	0.02	NS	NS	NS
C20:3 n-6	0.92	0.97	1.00	0.95	0.97	0.03	NS	*	NS
C20:3 n-3	0.09	0.08	0.11	0.07	0.11	0.01	NS	NS	NS
C20:4 n-6	7.29	7.04	7.71	7.70	7.00	0.23	NS	NS	NS
C20:5 n-3	0.29	0.33	0.32	0.34	0.29	0.01	NS	NS	NS
C22:0	0.16	0.17	0.17	0.17	0.16	0.01	NS	NS	NS
C22:1	0.02	0.03	0.03	0.02	0.04	0.01	NS	*	NS
C22:2n-6	0.05	0.02	0.03	0.03	0.04	0.01	NS	NS	NS
C22:4n-6	0.41	0.42	0.47	0.44	0.42	0.02	NS	NS	NS
C22:5 n-3	1.01	1.07	1.13	1.11	1.03	0.04	NS	NS	NS
C22:6 n-3	0.69	0.71	0.77	0.77	0.68	0.03	NS	NS	NS
C24:0	0.07	0.11	0.06	0.05	0.11	0.02	NS	NS	NS
Total									
ΣSFA	36.76	36.32	35.57	36.25	36.18	0.46	NS	NS	NS
ΣMUFA	27.31	28.03	27.10	28.11	26.85	0.56	NS	NS	NS
ΣPUFA	36.67	35.10	37.25	35.66	37.02	0.45	NS	NS	NS
Σn-6	33.37	31.77	33.78	32.23	33.72	0.44	NS	NS	NS
Σn-3	3.94	4.01	4.15	4.07	4.00	0.07	NS	NS	NS
Nutritional Indices[6]									
n-6/n-3	8.53	8.11	8.22	8.07	8.50	0.13	NS	NS	*
P/S	1.01	0.98	1.05	0.99	1.03	0.02	NS	NS	NS
AI	0.44	0.45	0.44	0.45	0.44	0.01	NS	NS	NS
TI	0.86	0.86	0.82	0.85	0.84	0.02	NS	NS	NS

[1]CON = Control (untreated); [2]SAL = *in ovo* injected with physiological saline (mock-treated); [3]GOS = *in ovo* injected with GOS (prebiotic-treated); [4]TN – thermoneutral conditions; [5]HS – heat stress conditions (on days 36 - 50).[6]P/S = PUFA/SFA; AI= Atherogenic index; TI= Thrombogenic index. SEM = standard error mean. Significance: NS = $P > 0.05$; *$P < 0.05$.

7.3 Conclusion

In conclusion, *in ovo* injection with GOS had no negative effects on in vivo performance and meat quality traits. As expected, thermal challenge applied for the last 14 days of the finisher feeding phase, had a dampening effect on growth performance. However, under heat stress conditions GOS: i) countered the negative effect of heat stress on carcass weight; ii) decreased lipid and protein content and increased the n6/n3 ratio in meat from heat stressed chickens. Considering that this is the first study carried out with the aforementioned genotype, further investigations are needed to better understand the activity of these substances on animal metabolism.

8. RESEARCH N. 2

Enterprise activity (Gesco Consorzio Cooperativo a r.l. - Amadori group): survey on the chicken breast meat quality characteristics from intensive farming

The second part of the thesis work was carried out at the GESCO laboratories Amadori group in the Teramo site according to the activity foreseen by the PON PhD scholarship (National Operational Program for Research and Innovation 2014-2020 CCI 2014IT16M2OP005, European Social Fund, Action I.1 "Innovative Doctorates with Industrial Characterization"; CUP H39H18000160006), in a non-continuous six-month period interspersed with periods of inactivity due to Covid-19. Unfortunately, this period of interruption did not allow for in-depth and complete analysis, but in any case, is reported a preliminary description of two commercial lines Vegetale® (fast growing) and Campese® (slow growing) brand Amadori. The pre-established activity and rigorously subject for many of its aspects to corporate privacy, has specifically provided for the evaluation of slow-growing commercial hybrid chicken breast qualitative characteristics of different sex (Trial 1) and slow-growing and fast-growing commercial hybrids of different slaughter ages (Trials 2 and 3).

8.1 Materials and Methods

8.1.1 Trial 1

The first trial evaluated the effect of sex on slow growing chickens (Red 75 - Campese® brand) meat quality.

Animals (13 animals/m^2 inside) were raised according to the recommendations of the European Union directive 86/609/EEC in a Southern Italy's farm (Foggia, Puglia Region, Italy). Animals were raised semi-extensively with outdoor access from 28^{th} day of age and with unlimited daily go out according to the season. The temperature inside breeding was between 18-26 °C.

Birds were fed *ad libitum* with a commercial diet antibiotics and GMOs free producted from feed mill Amadori, in agree with their age. Water was supplied on *ad libitum* basis. The diet was based on three different feeds administered in three different growth periods: starter 0-28 day of age, grower I 29-45 day of age, and finisher 46-56 or slaughter age. The components are reported in Table 10, as in the original label. The diet components percentages and the specific ages of administration cannot be reported because subjected to company patent.

Table 10. Components of commercial diet for chicken Red 75.

Starter (0-28 Day)	**Grower I** (29-45 Day)	**Finisher** (46-slaugheter age)
Corn	Corn	Corn
Soybean	Soybean	Soybean
Wheat	Wheat	Wheat
Wheat flour	Wheat grains	Wheat grains
Peas	Wheat flour	Wheat flour
Decorated sunflower seed flour	Decorated sunflower seed flour	Peas
Bicalcic phosphate	Peas	Soybean oil
Soybean oil	Soybean oil	Calcium carbonate
Calcium carbonate	Calcium carbonate	Bicalcic phosphate
Sodium chloride	Bicalcic phosphate	Sodium chloride
Sodium bicarbonate	Sodium chloride	Sodium bicarbonate
	Sodium bicarbonate	

Animals are taken from the farm and transported to the closest slaughterhouse for reduce the transport stress.

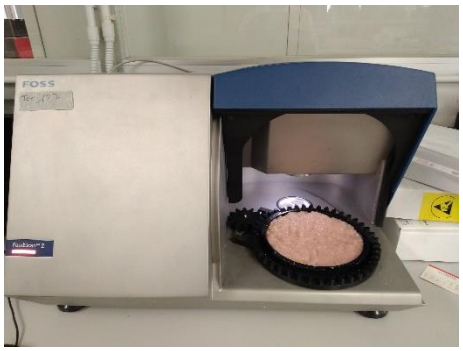
Animals were slaughtered at 57^{th} day of age in Amadori's slaughterhouse (Mosciano Sant'Angelo, Teramo, Abruzzo, Italy) after storing with carbon dioxide. Among the slaughtered chickens, the breast muscles of 10 males and 15 females were randomly selected and weighed.

The pH and colour were measured on the right pectoral muscle at 24h *post-mortem*. The pH was measured using an over-the-counter pH meter (Crison MicropH2001) equipped with a penetration glass electrode (Figure 7).

Figure 7. pH measurement with over-the-counter pH meter penetration glass electrode.

The colour measurement occurred following homogenization of the right pectoral muscle and subsequent detection of the three colour coordinates (lightness, L*; redness, a*; yellowness, b*). The instrument has been calibrated by white reflecting plate, with three repetitions. The sample was ground and placed on transparent plate; the measurement was carried out three consecutive times. At the same time, the proximate composition, intramuscular collagen, and fatty acid composition were detected by FOSS FoodScan2 (Near-Infrared Spectrophotometer with Colour Module Software vv 8.9.9.1) (Figure 8). The instrument has been calibrated differently according to the measured parameter: a) for the meat proximate composition by inserting in the instrument the data obtained from the wet analysis (AOAC, 1990) of three sample for group (three repetition for each sample); b) for intramuscular collagen and fatty acid composition every week the manufacturer of the instrument sends the data of the analysis carried out by them on fresh chicken meat samples, in a wet way, to be inserted into the instrument for indirect calibration.

Figure 8. Measurement with FOSS FoodScan2 (Near-Infrared Spectrophotometer).



Data were analysed by one way analysis of variance (SPSS Inc. 2010).

8.1.2 Trial 2

In this second trial was evaluated the effect of slaughter age (57 *versus* 69 days) on males slow-growing chicken (Red 75 – Campese® brand) meat quality. In particular, the comparison was between the ten males of the first trial with males Red 75 slaughtered at 69 days. Birds slaughtered at 69 days were raised in the same farm conditions of the birds of 57 days. In particular, were raised (13 animals/m^2 inside) according to the recommendations of the European Union directive 86/609/EEC in a Southern Italy's farm (Foggia, Puglia Region, Italy). Animals were raised semi-extensively with outdoor access from 28th day of age and with unlimited daily go out according to the season. The temperature inside breeding was between 18-26 °C.

Birds were fed *ad libitum* with a commercial diet antibiotics and GMOs free produced from feed mill Amadori, in agree with their age. Water was supplied on *ad libitum* basis. The diet was based on three different feeds administered in three different growth periods: starter 0-28 day of age, grower I 29-45 day of age, and finisher 46-56 or

slaughter age. The components are reported in Table 10, as in the original label. The diet components percentages and the specific ages of administration cannot be reported because subjected to company patent.

Animals are taken from the farm and transported to the closest slaughterhouse for reduce the transport stress.

Animals were slaughtered in Amadori's slaughterhouse (Mosciano Sant'Angelo, Teramo, Abruzzo, Italy) after storing with carbon dioxide. Among the slaughtered chickens, the breast muscles were collected randomly from 10 males of 57 days and 15 males of 69 days and weighed.

The pH and colour were measured on the right breast muscle at 24h *post-mortem* using the same procedure of the trial 1. The proximate composition, intramuscular collagen, and fatty acid composition of breast muscle, were also measured with the same procedure showed in the trial 1.

Data were analysed by one way analysis of variance (SPSS Inc. 2010).

8.1.3 Trial 3

The third trial evaluated the effect of age on fast-growing (Ross 308 – Vegetale® brand) chickens' quality. The animals (33 Kg/m^2 - animals weight) were reared according to the recommendations of the European Union directive 86/609/EEC in a Central Italy's farm (Pescara, Abruzzo, Italy). Animals were raised intensively with 6 hours of light per day of which for continuous. The temperature inside breeding was between 18-26 °C.

Animals were fed *ad libitum* with a commercial diet antibiotics and GMOs free, in line with their age and with free access to water (Table 11). Three different feeds were

administered in four different growth periods: starter 0-12 day of age, grower I 13-21 day of age, grower II 22-40 day of age, finisher from 40 day of age until slaughter age. The diet components percentages and the specific ages of administration cannot be reported because under company patent. Anyway, the constituents are reported, as in the original label, from the highest to the lowest in quantity.

Table 11. Components of the commercial diet for chickens Ross 308.

Starter (0-12 Day)	**Grower I** (13-21 Day)	**Grower II** (22-40 Day)	**Finisher** (40-slaugheter age)
Corn	Corn	Corn	Corn
Soybean	Soybean	Soybean	Soybean
Wheat	Wheat	Wheat	Wheat
Soybean flour feed	Peas	Peas	Wheat flour
Hydrolyzed yeast	Animal fats	Animal fats	Peas
Soy protein	Bicalcic phosphate	Bicalcic	Sorghum
Wheat flour	Calcium carbonate	Calcium	Animal fats
Peas	Sodium chloride	Sodium chloride	Sunflower flour
Corn gluten			Bicalcic phosphate
Animal fats			Calcium carbonate
Bicalcic phosphate			Sodium chloride
Calcium carbonate			Sodium bicarbonate
Sodium chloride			

Animals are taken from the farm and transported to the closest slaughterhouse for reduce the transport stress.

Animals were slaughtered in Amadori's slaughterhouse (Mosciano Sant'Angelo, Teramo, Abruzzo, Italy) after storing with carbon dioxide. Among the slaughtered chickens, the breast muscles were collected randomly from 15 males of 39 days and 15 males of 52 days and weighed.

The pH and colour were measured on the right pectoral breast muscle at 24h *post-mortem* using the same procedure of the trial 1. The proximate composition, intramuscular collagen, and fatty acid composition of breast muscle, were also measured with the same procedure showed in the trial 1.

Data were analysed by one way analysis of variance (SPSS Inc. 2010).

8.2 Results

8.2.1 Trial 1

The first trial compared breast muscle characteristics of slow-growing birds with same age but different sex. Breast muscle weight and its physical-chemical characteristics are reported in Table 12.

The statistical analysis indicated that sex did not affect the breast weight (339.78 and 324.35 g for males and females, respectively; $P>0.05$). On the contrary, the pH_{24} value of meat was higher in females compared to males (5.88 *versus* 5.73; $P<0.01$).

This study highlighted the effect of sex on the meat colour showing a significant variation between sexes. In particular, the lightness was higher in males compared to females (48.50 *versus* 40.07; $P<0.001$), unlike the red and yellow indices which were higher in females compared to males ($P<0.001$) (Table 12).

Table 12. Effect of sex on weight and physical-chemical characteristics of slow-growing chickens breast muscle.

	Sex		SEM	Significance
	Males	Females		
Breast weight (g)	339.78	324.35	15.71	NS
pH_{24}	5.73	5.88	0.05	**
Colour 24 h				
L*	48.50	40.07	0.65	***
a*	5.05	6.62	0.17	***
b*	12.84	14.83	0.32	***

SEM = Standard error mean.
Significance: NS = P > 0.05; ** P < 0.01; *** P < 0.001.

Protein and lipid content was similar between males and females (P>0.05), unlike the moisture and ash content which were significantly affected by the factor considered. In particular, the moisture content was higher in males (+ 1.3%) compared to females (P<0.001), while the ash content was higher in females compared to males (P<0.001) (Table 13).

Collagen content (Table 13) was higher (P<0.001) in males compared to females (0.32 *versus* 0.23 %).

Table 13. Effect of sex on proximate composition and intramuscular collagen content of slow-growing chickens breast muscle.

	Sex		SEM	Significance
	Males	Females		
Moisture (%)	74.88	73.58	0.16	***
Protein (%)	23.82	23.92	0.12	NS
Lipid (%)	2.00	1.88	0.05	NS
Ash (%)	0.96	1.27	0.02	***
Collagen (%)	0.32	0.23	0.02	***

SEM = Standard error mean.
Significance: NS = P > 0.05; *** P < 0.001.

The total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids content is shown in Table 14. In the study, the effect of this factor denoted higher SFA and PUFA content in females compared to males (P<0.001), unlike for MUFA content which was higher in males compared to females (P<0.001).

Table 14. Effect of sex on total saturated fatty acid content (SFA). monounsaturated (MUFA) and polyunsaturated (PUFA) of slow-growing chickens breast muscle.

	Sex		SEM	Significance
	Males	Females		
ΣSFA (%)	25.23	28.14	0.51	***
ΣMUFA (%)	44.57	36.57	0.52	***
ΣPUFA (%)	31.01	36.58	0.23	***

SEM = Standard error mean.
Significance: *** P < 0.001.

8.2.2 Trial 2

Breast muscle weight and its physical-chemical characteristics are reported in Table 15. The breast weight was significantly (P<0.001) higher in chickens slaughtered at 69 days (425.77 g) compared to birds with 57 days of age (339.78 g). The pH_{24} (5.73) was not affected by the age (P>0.05).

The meat colour was affected by the age considered: the lightness was higher (P<0.001) in chickens slaughtered at 57 days (48.50) compared to 69 days (40.87). Opposite trend to the lightness was found for the red and yellow indices, which have higher values in older chickens (P<0.001 and P<0.01, respectively).

Table 15. Effect of age on physical-chemical characteristics of slow-growing chickens breast muscle.

	Age		SEM	Significance
	57 days	69 days		
Breast weight (g)	339.78	425.77	15.95	***
pH_{24}	5.73	5.73	0.04	NS
Colour 24 h				
L*	48.50	40.87	0.67	***
a*	5.05	6.35	0.17	***
b*	12.84	13.73	0.27	**

SEM = Standard error mean.
Significance: NS = P > 0.05; ** P < 0.01; *** P < 0.001.

Proximate composition and intramuscular collagen content of breast muscle are presented in Table 16. The lipid and protein content were not influenced by the age (P>0.05), unlike the moisture and ash content. Specifically, the moisture value decreases with animals' increasing age (74.88% versus 73.14% at the 57^{th} and 69^{th} day, respectively; P<0.001), while the value of the ashes had a contrary trend (0.96% versus 1.31% at 57^{th} and 69^{th} day, respectively; P<0.05). Collagen content was significantly higher (P<0.05) in 69-day-old chickens (0.37 %) compared to in 57-day-old ones (0.32 %).

Table 16. Effect of age on proximate composition and intramuscular collagen content of slow-growing chickens breast muscle.

	Age		SEM	Significance
	57 days	69 days		
Moisture (%)	74.88	73.14	0.18	***
Protein (%)	23.82	23.81	0.12	NS
Lipid (%)	2.0	1.76	0.14	NS
Ash (%)	0.96	1.31	0.02	***
Collagen (%)	0.32	0.37	0.02	*

SEM = Standard error mean.
Significance: NS = P > 0.05; * P<0.005; *** P < 0.001.

The fatty acid profile was affected by the broilers' slaughter age (Table 17). SFA and PUFA content was higher in 69-day-old chickens (compared to 57-day-old chickens (P<0.01 and P>0.001, respectively), while MUFA content decreased (-7.92%; P<0.001) with increasing animal's age.

Table 17. Effect of age on total saturated fatty acid content (SFA). monounsaturated (MUFA) and polyunsaturated (PUFA) of slow-growing chickens breast muscle.

	Age		SEM	Significance
	57 days	69 days		
ΣSFA (%)	25.23	27.09	0.50	**
ΣMUFA (%)	44.57	36.65	0.62	***
ΣPUFA (%)	31.01	35.45	0.30	***

SEM = Standard error mean.
Significance: ** P < 0.01; *** P < 0.001.

8.2.3 Trial 3

This trial underlines the meat qualitative characteristics in fast-growing male chicken's (Ross 308) slaughtered at two different ages (Table 18). As expected, the breast weight of the 52-day-old animals was higher (P < 0.001) compared to younger animals (967.01 *versus* 725.38 g, respectively). The pH_{24} was not affected (P > 0.05) from slaughter age. The colour coordinates were influenced by the factor considered; the lightness was higher in 52-day-old chickens than in 39-day-old chickens (66.27 *versus* 64.53; P<0.01). The red and yellow indices show lower values with increasing age (P<0.01 and P<0.001, respectively).

Table 18. Effect of age on weight and physical-chemical characteristics of fast-growing chickens breast muscle.

	Age		SEM	Significance
	39 days	52 days		
Breast weight (g)	725.38	967.01	27.43	***
pH_{24}	5.96	5.91	0.02	NS
Colour 24 h				
L*	64.53	66.27	0.32	**
a*	10.67	9.14	0.26	**
b*	19.13	17.21	0.22	***

SEM = Standard error mean.
Significance: NS = P > 0.05; ** P < 0.01; *** P < 0.001.

The meat proximate composition and collagen content are reported in Table 19. Regarding meat proximate composition, only ash content was influenced by slaughter age, it which was significantly higher (P<0.01) in chickens slaughtered at 52 days compared to younger chickens. Collagen content increased with age (0.45 *versus* 0.48 % for 39 d and 52 d, respectively; P = 0.055).

Table 19. Effect of age on proximate composition and intramuscular collagen content of fast-growing chickens breast muscle.

	Age		SEM	Significance
	39 days	52 days		
Moisture (%)	75.03	74.81	0.14	NS
Protein (%)	21.50	21.69	0.12	NS
Lipid (%)	2.27	2.29	0.09	NS
Ash (%)	1.10	1.14	0.01	**
Collagen (%)	0.45	0.48	0.01	P = 0.055

SEM = Standard error mean.
Significance: NS = P > 0.05; ** P < 0.01.

Fatty acid profile is presented in Table 20. The SFA and MUFA total content was not affected (P>0.05) by slaughter age, unlike the PUFA total content which was significantly higher (P<0.05) in 39-day-old chickens compared to 52-day-old chickens (36.49 *versus* 35.41 %).

Table 20. Effect of age on total saturated fatty acid content (SFA). monounsaturated (MUFA) and polyunsaturated (PUFA) of fast-growing chickens breast muscle.

	Age		SEM	Significance
	39 days	52 days		
ΣSFA (%)	26.61	27.06	0.25	NS
ΣMUFA (%)	39.97	41.09	0.33	NS
ΣPUFA (%)	36.49	35.41	0.27	*

SEM = Standard error mean.
Significance: NS = P > 0.05; * P < 0.05.

8.3 Discussion

This second part of the thesis concerned a survey on the physical-chemical quality of some typologies chicken meat placed on the market by a leading Italian company in the agri-food sector, Amadori. In this regard, chicken breast muscle samples obtained from slow- and fast growing commercial genetic types were analysed, taking into consideration the effect of sex (Trial 1) or the age of slaughter (Trials 2 and 3).

8.3.1 Trial 1

Most poultry meat derives from intensive poultry production systems (95%) and a small portion (5%) from the extensive rearing systems (ERS) such as organic, free-range, and

low-input production systems (Dal Bosco *et al*., 2021). Recently, chicken meat production has diversified because consumers' demands for welfare and housing conditions are more oriented on less intensive systems or organic. Obviously, these farming systems are less standardizable than the intensive ones and are very different between the countries of the European Union. The differences are due to several factors, such as the type of climate, the cost of labour and feed, the availability of bird genotypes and land resources, and willingness of consumers to pay for premium products.

For less intensive systems medium- and slow-growing chicken types have been recommended. In fact, this could be an alternative at organic production typically requires a long growing period, for which fast-growing genotypes are not suitable due to their short growing period. Medium- and slow-growing chickens are more suitable at semi-extensive rearing system because of their higher vitality, disease resistance, and adaptability to outdoor conditions compared fast-growing chickens (Sirri *et al*., 2011).

Although the males had heavier breast muscle (+ 4.5%) than the females, the differences were not significant. This not very large weight difference could be due to the slaughter age (57 days) which if it had been greater would have probably increased the difference between the weights of the breasts, to the advantage of the males.

The pH value declines *post-mortem*, and this process is very important in the conversion of muscle to meat as it affects meat quality characteristics (colour, water-holding capacity, and tenderness; Chodova *et al*., 2021). The statistical analysis indicated that females showed higher pH value. For chicken meat, previous study has shown that pH_{24} values below 5.7 indicate poor quality meat for protein damage (Van Laack *et al*., 2000). Values of pH obtained for meat from males and females of the present study are comparable with those reported in previous studies on different broiler strains (Fletcher,

1999; López *et al*., 2011; Sirri *et al*, 2011) and are not below at the optimal values of a good quality meat.

Among the physical traits of meat, colour is most important, in fact it is the first characteristic that directs the consumer's purchase. Colour is affected by numerous factors, including the presence and concentrations of heme pigments, genetics, and diet (Batkowska *et al*., 2015). In this study evidenced an evident effect of sex on the meat colour. Meat from males was more lightness, but less redness and yellowness. The differences in lightness and yellowness of meat between males and females could be due to the greater amount of subcutaneous fat in females (Chodová *et al*., 2021). A similar trend for lightness and yellowness was reported by Chodová *et al*. (2021) in medium- and slow-growing chickens; while López *et al*. (2011) found a higher b* value in females compared to males at 42 days of age and did not underlining effects for the other indices. The colour values found in the present study are in agree with the data reported in literature for slow- and fast-growing chickens (Fanatico *et al*., 2007; Chodová *et al*., 2021).

In literature is reported the effect of sex on meat nutritional values: the moisture and protein content in males' chickens is generally higher compared to females, and the males have a lower lipid value compared to females; while the ash content is similar in both sexes (in Demby and Cunningham, 1980; Baeza *et al*., 2010). The findings of the present research are not completely in agreement with the data reported in literature. In particular, the protein and lipid content were similar between the two sexes, while the moisture and ash content were respectively higher and lower in males compared to females. These findings agree with the results of Baeza *et al*. (2010) for moisture, lipid and protein

content in chickens' breasts slaughtered at 120 days of age and in agreement with Chodová *et al*. (2021) relatively to ash content.

The intramuscular collagen content is an important parameter in meat quality as it influences its tenderness (Maiorano *et al*., 2007). A higher collagen content, as well as the stability of its fibers, can affect the toughness and quality of the meat (McCormick, 1999; Maiorano *et al*., 2007): in fact, with a high content the meat is hard, while with a low content it is more tender. In this study, the collagen content was higher in males compared to females indicating that meat from male may be less tender. The collagen content found in this study is like values reported in literature (Coró *et al*., 2003; Cygan-Szczegielniak *et al*., 2019). The intramuscular collagen amount and the stability of its fibers can depend on many factors, such as diet, race, growth rate, animal age and exercise (McCormick, 1999; Maiorano *et al*., 2007; Janicki and Buzała, 2013).

In general, chicken meat has higher PUFA content compared to pork or bovine meat, with a beneficial role for human health, e.g., protective against coronary heart disease (Peña-Saldarriaga *et al*., 2020). In literature (Wood and Enser, 1997) has been very studied how the diet and farming condition affect fatty acid profile; however, are few data available on the effect of sex in slow-growing chickens. In the present study, the effect of this factor has evidenced higher SFA and PUFA content in females compared with males, unlike for MUFA which was higher in males compared to females. The values found are in agree with the data reported in literature, with a prevalence of monounsaturated and polyunsaturated fatty acids compared to saturated fatty acids (Tavaniello *et al*., 2019). Partially different results were achieved by Baeza *et al*. (2010), the authors found in females' meat (breast and thighs) a higher percentage of MUFA and a lower percentage of PUFA compared to males, both of animals slaughtered at 84 days and at 120 days;

they also observed a greater quantity of SFA in 120-day-old males compared to females of the same age. The fatty acid composition is affected from the diet and the slaughter age (Poureslami *et al*., 2010). This latter factor can be explain the differences between the results of the present study and those obtained by Baeza *et al*. (2010).

8.3.2 Trial 2

The purpose of poultry industry is to combine consumer expectations with a high production rate. The age of slaughter and the genotype are among the most important factors affecting *in vivo* and carcass traits chickens' performances as well as meat quality, thus influencing the organoleptic and technological properties (Połtowicz and Doktor, 2012). Therefore, it is interesting evaluate the slaughter age which reflects the best quality characteristics and optimal production times. Considering that there is little information on the effect of the slaughter age on slow-growing chickens meat quality, this second trial assessed the effect of the slaughter age on slow-growing male broilers, which are known to have longer rearing times than fast-growing chickens. In fact, slow-growing chickens show a daily weight gain of up to 20 g and a live weight of 2.2–2.5 kg in 56–81 d (Dal Bosco *et al*., 2012).

The first parameter to evaluate the production performance is the breast muscle weight which. As expected, was greater in chickens slaughtered at 69 days (+ 20.2%) compared to those with 57 days of age.

The ultimate pH was not affected by age and the values found, similar between the two age groups, are in agree with the data presented by Połtowicz and Doktor (2012) though authors show a pH_{24} value higher in older chickens (5.83 and 6.10 for 56 and 70 days of

age, respectively). In accordance with the results of this study, Uhlířová *et al*. (2018) reported no slaughter age effect on pH of geese meat.

The younger bird's meat was found lightness (L*) and less yellowness (b*) and redness (a*). The lightness trend of younger birds reflects data present in literature (Połtowicz and Doktor, 2012). Probably the decrease in lightness with increasing slaughter age is due to a decrease in myoglobin (Wideman *et al*., 2016). The values are in agree with the data reported in literature for slow-growing chickens, although no effects of the factor considered are reported for a* and b* descriptors (Połtowicz and Doktor, 2012).

Literature reported the slaughter age effects on the meat's nutrients content (Evaris *et al*., 2019). In the present study, however, the lipid content and protein content were not influenced by the slaughter age, unlike the moisture and ash content. With the age increase, the meat resulted with less moisture and more ashes. There are no recent data in literature on the effect of the slaughter age on chicken meat proximate composition, therefore it is not possible to make a direct comparison. However, the values of meat composition found are in agree with the data reported in literature for slow-growing chickens (Devatkal *et al*., 2019).

The data in literature show that the collagen content, which gives meat toughness, changes with increasing age: the meat becomes harder due to the transformation of the meat cross-links protein (Bailey *et al*., 1971; Shimokomaki *et al*., 1972). In agreement with Coró *et al*. (2003), the collagen content was higher in older birds compared to younger ones.

The age's effect on fatty acid profile has been reported in previous study on chickens (Popova *et al*., 2016). The authors reported that MUFAs decreased with age in both breast and thigh muscles. This corresponded to the significant decrease in oleic acid in

the older chickens and the lower desaturase activity. From a nutritional point of view, oleic acid plays a key role in human diet as involved in reducing lipaemia and consequently the risk of stroke (D'Alessandro *et al*., 2012). Moreover, they found an increase of PUFA content with the age increase. The findings of the present study are partially in agreement with Popova *et al*. (2016); in fact, it was found with the increase of the slaughter age increased the SFA and PUFA content, while that of MUFA decreased. It is well known that the higher intake of saturated fatty acid (SFA) in the human diet increases the risk of the development of coronary heart disease, atherosclerosis, and cancer (Mensink and Katan, 1992), whereas monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA), especially n-3, have a number of associated health benefits. The beneficial effects of PUFA have been associated to a protection against cardiovascular diseases (Hooper *et al*., 2015), whereas for MUFA to a beneficial effect on insulin sensitivity and cholesterolemia (Nettleton *et al*., 2017).

8.3.3 Trial 3

Nowadays, the production of chicken meat is derived from fast-growing genotypes reared indoors under controlled climatic conditions (photoperiod, light intensity, temperature) and balanced diets that ensure a high intensity of growth and a low feed conversion ratio. Thanks to the advances in genomics and improving of the nutrition, birds reach the market weight of 2 kg like as early as 5 to 6 weeks of age (Devatkal *et al*., 2019). However, fast-growing chickens due to this excessive genetic selection can be susceptible to health problems or sudden death syndrome, as well as lower meat quality, histological and biochemical modifications (Petracci and Cavani, 2012) and increased risk of recent muscle abnormalities, as giant muscle fibers, wooden breast, white striping, and poor

cohesiveness of breast meat (Petracci and Cavani, 2012; Soglia *et al*., 2016; Ismail and Joo, 2017; Baldi *et al*., 2019).

In this third study was evaluated the effect of the slaughter age (39 *versus* 52 days) on meat quality of fast-growing male chickens (Ross 308).

As expected, oldest birds (52 day) showed heavier breast muscle weight compared to younger animals (39 days). The weights of the breast muscles registered in the birds at 39 and 52 days of age are higher compared with those reported in other studies for Ross birds slaughtered respectively on day 42 of age (616 g: Tavaniello *et al*., 2020) and on day 51 of age (888 g: Sirri *et al*., 2016).

Slaughter age did not affect significantly the pH_{24} values. Comparable breast muscle pH values between the younger and older broilers (28 and 41 d of age), of different genetic lines, were found by Janisch *et al*. (2011). Conflicting results were reported by Glamoclija *et al*. (2015), who observed lower pH values in different strain elderly chickens (42 *versus* 50 days). However, the values found in the present study, ranging from 5.96 to 5.91, indicate a normal chicken meat.

On the contrary, meat colour was influenced by slaughter age. Meat from older birds was more lightness but less redness and yellowness. Similar trend for L* and a* descriptors were reported by Janisch *et al*. (2011), while Abdullah *et al*. (2010) found a darker colour meat in the younger birds (32 days) compared with older ones (41 days).

Moisture, protein and lipid content were not affected from age; while breast meat from birds slaughtered at 52 days had a higher ash content compared with breast meat from younger birds. This result contrasts with the data reported by Abdullah *et al*. (2010) on Hubbard and Lohman chickens, the authors do not underline the influence of slaughter age on the proximate composition, except for the protein content that results higher in

young chickens. It is probably that the difference in age between birds slaughtered at 39 and 52 days of age might not be big enough to influence breast meat composition of those birds. The meat proximate composition values obtained in the present study are comparable with the results reported in literature for commercial hybrids (Souza *et al*., 2011; Puvača *et al*., 2015).

Regarding intramuscular collagen, in agreement with the results obtained in the trial 2 and with literature (Coró *et al*., 2003), the intramuscular collagen content increased with the age indicating that meat from chickens 52 days is less tender compared to birds of 39 days.

The fatty acid profile was partially affected by slaughter age. SFA and MUFA content was not significantly affected, on the contrary PUFA content that was greater in 39-day-old chickens (+ 1.08%) compared to 52-day-old chickens. There are not many data in literature regarding the effect of age on the fatty acid profile in similar commercial conditions to the present study. A similar work, only for genotype used, is presented by Dal Bosco *et al*. (2014) which reported, in contrast this trial, a significant change in SFA and MUFA value for Ross 308 chickens aged 70 and 81 days, and no influence on the PUFA content. It should be underlined that the results reported by Dal Bosco *et al*. (2014) are not completely comparable to the results of the present study, because the authors used a semi-extensive breeding technique and female animals with different diets, characteristics that certainly influence the fatty acid profile. In any case, the observed values are comparable with the data reported in literature (Baeza *et al*., 2010; Tavaniello *et al*., 2019).

8.4 Conclusions

The findings of these three trials indicated that the slow-growing genotype, such as the one considered in the first and second study (Red 75 - Campese® brand) is very adaptable to the semi-extensive production. This is confirmed from the good breast muscle weights reached both males and females slaughtered at 57th day of age and from males slaughtered at 69th day of age. As expected, the males had heavier breast muscles compared to females (Trial 1) when slaughtered at 57 days, even if the differences were not statistically significant. Meat physical-chemical characteristics were affected by sex. Although the pH meat value was higher in females compared to males, probably indicating a slightly stress before slaughter, it is still resulted in both sexes optimal for a good quality meat. Meat from males had higher L* (lightness) and lower a* (redness) and b* (yellowness). The differences in lightness and yellowness of meat between males and females could be due to the greater amount of subcutaneous fat in females. Meat proximate composition was affected slightly by sex. Meat from males had lower ash content but higher moisture and collagen content. These results indicate that meat from males could be less tender but with a greater juiciness. Sex dramatically affected the fatty acid composition. Meat from females exhibited higher proportions of SFA and PUFA, but lower MUFA content.

As regards the effects of age, studied in the bird Red 75 slaughtered at 57 and 69 days (Test 2), obviously the greater age of slaughter implied a greater breast muscle weight. This result, although positive, may not necessarily be advantageous for the industry sector as it must be considered from a cost-benefit perspective. The younger animals showed a greater meat lightness and lower redness and yellowness, as well as more

moisture and less ash and collagen content. These results indicate that meat from younger birds could be more tender and with a greater juiciness. The fatty acid composition was strong influenced also by the age factor, with the increase of the slaughter age increased the SFA and PUFA content, while that of MUFA decreased.

Likewise in the slow genotype and as expected, in Ross 308 birds (Trial 3) a greater breast weight was shown as the slaughter age increased. Considering instead the meat colour, the trend observed in these animals appear different to that observed in slow-growing bird slaughtered at different age (Trial 2); in fact, meat from older animals was more lightness but less redness and yellowness compared with those of younger birds. As for the meat proximate composition, slaughter age affected only the ash content, resulting greater for older animals than the younger; similarly, the collagen content was higher in older animals. These findings were also confirmed in the slow-growing genotype. Finally, considering the fatty acid profile, only the PUFA content was influenced by age, resulting higher in young animals, this finding contrast with that previously observed in slow growing animals which showed a higher value for older animals.

In conclusion, the Campese® and Vegetable® line meat, despite being different from a physical-chemical and nutritional point of view between males and females and between animals of different ages, was of good quality reflecting the range of qualitative values reported in the literature. The slow growing genotype is used for a niche production and therefore more expensive for industry; differently, the fast-growing genotype (Ross 308, Vegetale® Amadori line) is well suited to an intensive production that allows poultry industry to tolerate the higher costs of semi-extensive production, since the products obtained from intensive rearing are mostly destined for large-scale distribution. Despite this, it is interesting to evaluate the slaughter age because on this basis the animals are

selected for the final product required: for example, rotisserie chickens are slaughtered at a lower age (39 days) than heavy chickens (52 days) mainly used to produce processed products. Unfortunately, due to the blocks imposed by the COVID-19 pandemic was not possible complete the laboratory analyses, therefore further studies are necessary to better explain the different trends found in some parameters, as for example the fatty acid profile.

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