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Director: Prof. Valentino Bontempo

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The role of some additives in enhancing feed efficiency and health in monogastric animals

Marcello Comi

Tutor: **Prof. Valentino Bontempo**

Coordinator: **Prof. Giovanni Savoini**

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CHAPTER 1

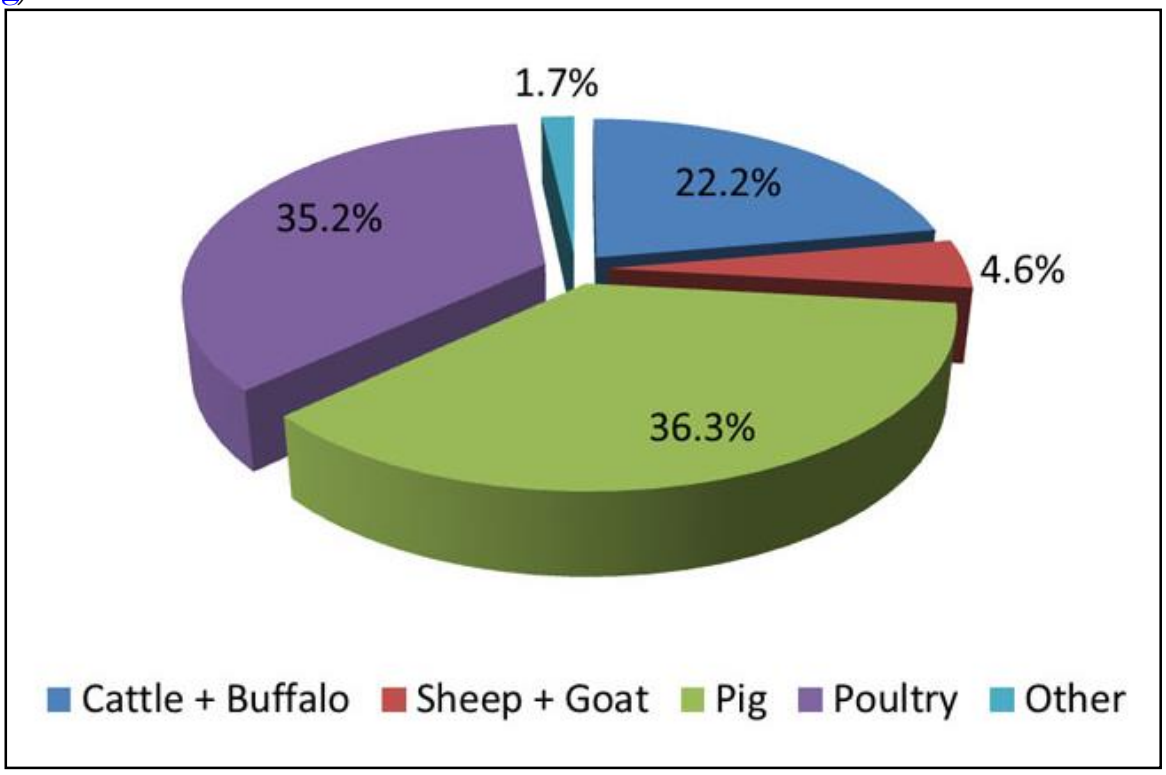
1 Foreword

1.1 World meat production: an overview

1.1.1 Looking towards 2030-2050

The most common sources of meat come from domesticated animals such as cattle, pigs, poultry and less commonly buffalo, sheep and goats. For thousands of years poultry supplied meat and eggs, while cattle, sheep and goats provided meat and milk and pigs provided meat as the main sources of animal protein for humans. The meat derived from cattle is known as beef, meat derived from pigs as pork and from chickens as poultry. World meat production has been estimated to double by 2050 in developed countries providing many opportunities for livestock farmers and meat producers. However such an expected increase in meat production brings a big challenge as regards the safe processing and marketing of hygienic meat and meat products. (http://www.fao.org/ag/againfo/themes/en/meat/home.html; http://www.fao.org/ag/againfo/themes/en/meat/backgr_sources.html)

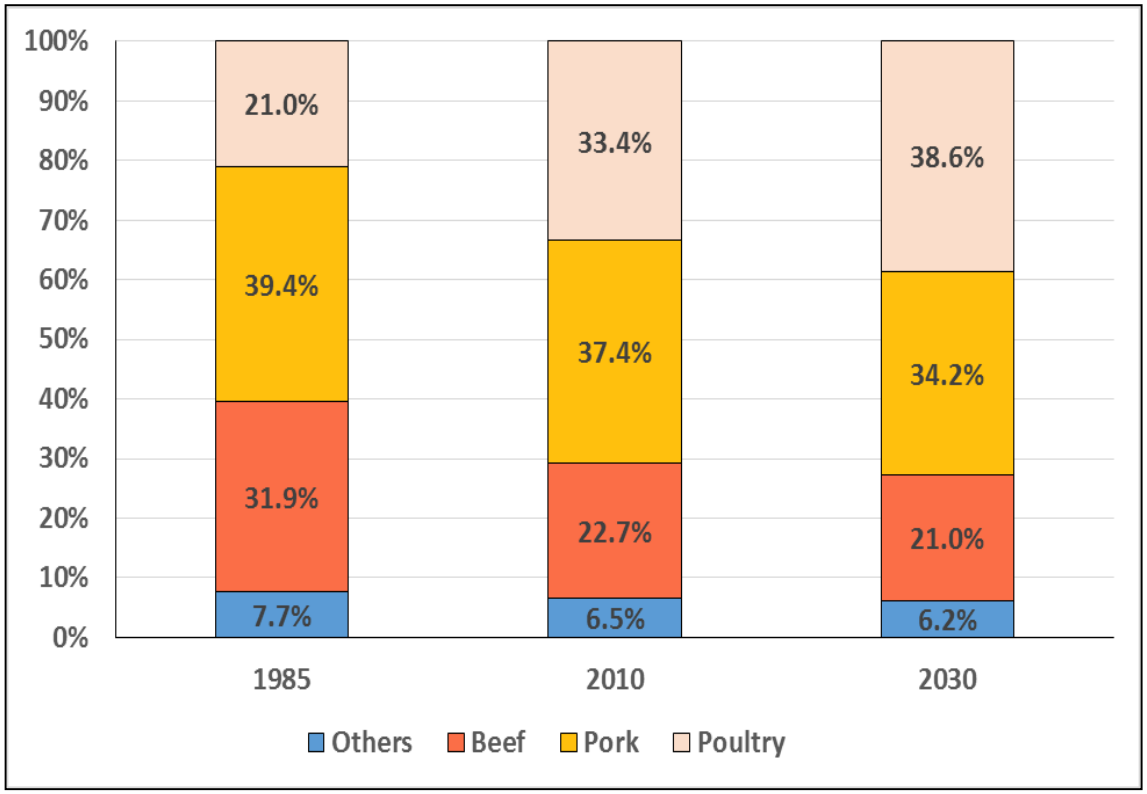
Figure 1: source of the world's meet supply (http://www.fao.org/ag/againfo/themes/images/meat/backgr_sources_data.jpg)



Nowadays there is a growing demand for high value animal protein and, as the worlds' livestock sector is increasing at a remarkable rate due to a combination of population growth, rising incomes and urbanisation, this in turn put additional pressure on the livestock industry to meet the demand. With an increase meat production perspective on annual basis from 218 million tonnes in 1997-1999 to 376 million tonnes by 2030, there is a strong positive relationship between the level of income and the consumption of animal protein, with the consumption of meat, milk and eggs increasing at the expense of staple foods. (Bruinsma et al., 2015; http://www.who.int/nutrition/topics/3_foodconsumption/en/index4.html).

Looking at 2030, the consumption of livestock products will continue to increase in developing countries. However, future growth rate in consumption of meat and milk could be slower than in the past, as there is less opportunity for further increases in major consuming countries. Moreover, the scope for increased demand is limited in developed countries due to the slow rate of population growth and the fact that there is already a high consumption of livestock products. There is also a concern for health and food safety, with a focus on animal fats and the discovery of new diseases such as bovine spongiform encephalopathy (BSE) and variant Creutzfeldt-Jakob disease. Although in developing countries the annual meat consumption per person more than doubled between 1964-66 and 1997-99, there were significant differences between countries (Roppa, 2012).

Figure 2: global mix of animal protein consumption is changing towards cheaper, low-fat meats until 2030 (Roppa L., 2012)



There has only been a 1.3 percent rise per year in the total meat consumption in the industrial countries in the last 10 years; however, the demand for meat in developing countries has grown rapidly at 5.6 percent each year in the last 20 years. This rate is estimated to slow by half in the next 20 years, partly due to slower population growth and partly because of the same reason that happened to some developed countries such as China and Brazil; they have now reached reasonably high levels of consumption and therefore have less chance for further rises.

Table 1: Projected meat production (million tonnes) needed for meeting projected consumption growth pattern (Roppa L., 2012).

Projected meat production (million tonnes) needed for meeting projected consumption growth pattern			
	2010	**2030**	**% Growth**
Beef	64.9	83.6	28.8
Pork	107.9	136.1	26.1
Poultry	97.5	153.8	57.7
sheep & goat	12.8	18.2	42.2
other meats	5.6	6.6	17.8
total meat	288.7	398.3	38.0
human population (bilion)	6.85	8.2	19.7

1.1.2 Environment and health problems

Commercial and industrial systems bring their own environmental issues, which differ to those of extensive systems. Problems with waste disposal and pollution can be found in areas with a high concentration of animals, particularly urban areas. Transporting animals to distant markets along with higher animal densities, can lead to the frustration of natural animal behaviour therefore causing distress. A third problem is that with more trade in livestock products and feedstuffs; the more chance there is of disease transmission, both within and across natural boundaries. This could apply to livestock diseases limited such as foot-and-mouth, and to those that may affect both livestock and humans, such as avian flu. There is still a major threat from infectious diseases like rinderpest or foot-and-mouth in developing countries and there can be more widespread infections, even to developed countries from the increase in trade. Programmes to eradicate these diseases are moving away from countrywide control strategies to more focused and flexible approaches, in the hope that they build a more cost effective form of control. Antibiotics are commonly used amongst industrial livestock enterprises, which have resulted in antibiotic resistance among bacteria, including those that cause human diseases and there is also resistance emerging within livestock parasites to antihelminthics. A final health problem is the use of growth hormones in these industrial enterprises to speed fattening and increase the efficiency of conversion into meat. Although negative impacts on human health have never been proved, this has become a cause for public concern, which in turn has led to restrictions of use within the E.U. (Bruinsma et al., 2015).

1.1.3 Pork industry: present and future trends

The European Union is the biggest world exporter and second largest world producer of pig meat (http://ec.europa.eu/agriculture/pigmeat/index_en.htm). Due to a delay in the adjustment of pig production, slaughtering's in the E.U continued to rise in the second quarter of 2015 even with the decrease of pig meat prices. Even though it was slower than 2014, it reached +3.9% for the whole first half of the year and almost all the member states increased production. Spain had the biggest growth recorded (+9.1% or 265 000 tonnes in first half of 2015) based on a strong increase of breeding sows (+5% or 105 000 heads).

Increased production was recorded also in the other major pig meat producers: Denmark, Germany, the Netherlands, Poland, Belgium, France and the United Kingdom.

The first signs of a decline in the reproductive herd can be seen as an effect of the less than average pig meat prices. There was a slight decrease in the number of sows and gilts not yet covered in the main producing member states (-0.7% and -1.7%) compared to June 2014 (as shown in the June 2015 livestock survey). Whilst this change is reasonably small, there is a similarity between this and the increase of breeding sows seen in the December survey 2014.

Meanwhile, the number of piglets recorded is still on the rise (+1.1%), which would explain the current depressed piglet price. These findings might limit the overall 2015 annual increase in meat production to around 2.7% year-on-year and in 2016 to 0.5%.

There was a surge in pig meat exports in the first six months of 2015 (+15%), which can be explained by increased production, lower E.U. meat prices, a depreciated euro and a high demand from Asia. Shipments to China, Philippines,

Georgia, Balkan countries and South Korea where the main increases were recorded and to Japan, where the smaller quantities were exported can be explained by its good inventories and the fact that the US has started recovering from the 2014 PEDv episode and is regaining its market share.

Exports to Hong Kong from the E.U are deteriorating as they favour direct exports to China leading to 172,000 tonnes (+52%) in the first six months of 2015 and a publication of Rabobank noted that, China may need to import an extra 600 000 tonnes in 2015 because of its shrinking pig numbers.

Smallholdings are in decline whilst large-scale farms continue to thrive, this may present an opportunity for further exports to China. However, in the past there have been examples of domestic prices rising, limiting consumption and the growth of imports. In addition, it is important to monitor the exchange rate between the euro and the yuan and the demand the Chinese consumer has on the meat sector.

With a continuous strong global demand, higher competition on prices and exchange rates on the world market and a lower supply in Brazil, EU exports are expected to grow by 7.5% to 2 million tonnes in 2015.

As Brazil and America become more competitive and more available, EU exports may continue to grow at a slower pace in 2016. However, even the sanitary and economic import ban were to be lifted on Russia, it is unlikely that EU exports would resume there because of the economic situation (http://ec.europa.eu/agriculture/markets-and-prices/short-term-outlook/pdf/2015-11_en.pdf). After the Russian ban, the EU was able to find alternative markets in the pig sector namely due to low supply in the U.S. however, the EU cannot slow production because of high investments in the breeding sector. The decrease in piglet prices shows that the EU market cannot absorb the additional quantities. Based on the forecast of slaughter and weights, pork production is estimated to increase to a record amount of 23 million metric

tons in 2015 and the only way to relieve the market is to increase exports. EU exports are expected to grow to a record amount of 2.35 million metric tonnes, due to Chinas' growth market, although with unrest within the sector, margins will be tight. As forecasted by FAS Posts the total EU sow stock will be cut by about 135,000 head in 2015. Increased efficiency will result in only a slight reduction in the pig crop and slaughter.
(http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Livestock%20and%20Products%20Annual_The%20Hague_EU-28_9-9-2015.pdf)
On average swine feed prices are predicted to remain the same throughout 2015/2016 as in the previous season. Slaughter weights are forecast to stay at 89 kg according to the stagnant feed costs and as previously mentioned, pork production is calculated to increase to a record volume of 23 million metric tonnes, mostly coming from Spain, Germany, Poland, the UK, the Benelux countries and Hungary. However, a significant increase is not anticipated in any of the EU Member States.
(http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Livestock%20and%20Products%20Annual_The%20Hague_EU-28_9-9-2015.pdf)

Table 2: EU-28 pig meat market balance ('000 tonnes carcass weight equivalent) *(Short Term Outlook for EU arable crops, dairy and meat markets – Winter 2015: http://ec.europa.eu/agriculture/markets-and-prices/short-term-outlook/index_en.htm).*

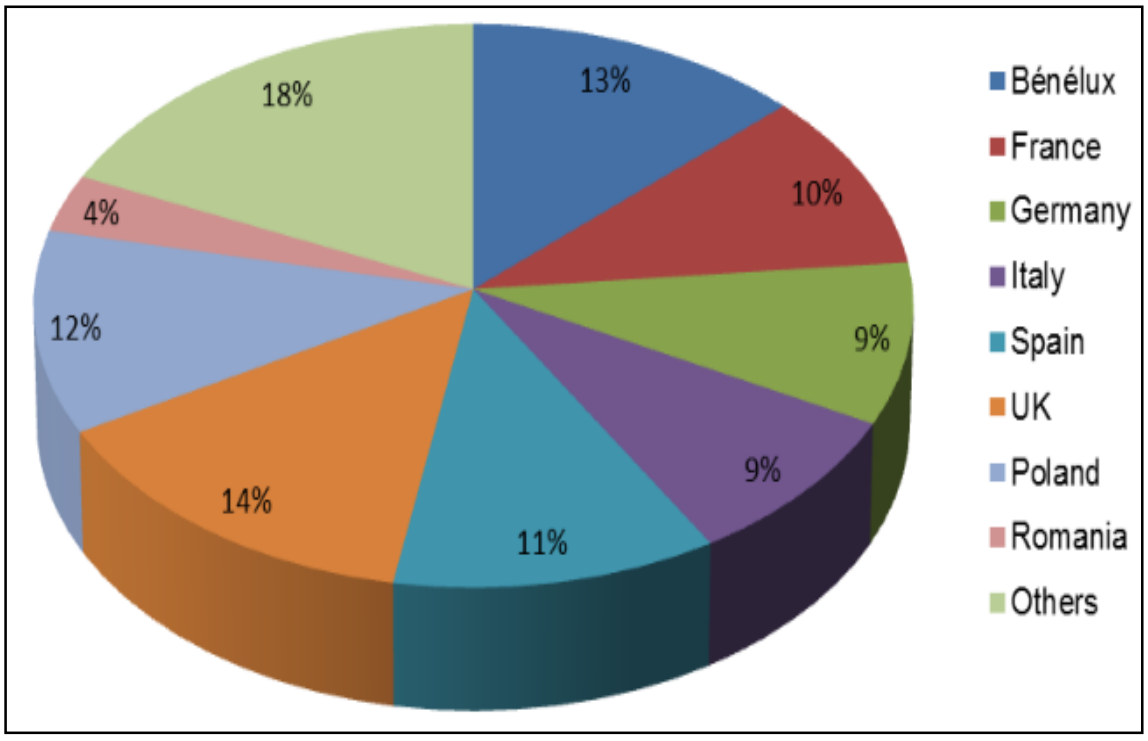
	EU-28						% variation				
	2011	2012	2013	2014e	2015f	2016f	12/11	13/12	14/13	15/14	16/15
Gross Indigenous Production	**23 058**	**22 554**	**22 385**	**22 834**	**23 441**	**23 557**	**-2.2**	**-0.8**	**2.0**	**2.7**	**0.5**
Live Imports	0	0	0	0	0	0					
Live Exports	62	36	26	36	23	24	-42.4	-27.3	36.2	-35.0	5.0
Net Production	**22 995**	**22 519**	**22 359**	**22 799**	**23 418**	**23 533**	**-2.1**	**-0.7**	**2.0**	**2.7**	**0.5**
EU-15	19 438	19 127	19 055	19 334	19 846	19 925	-1.6	-0.4	1.5	2.6	0.4
EU-N13	3 558	3 391	3 304	3 465	3 572	3 608	-4.7	-2.6	4.9	3.1	1.0
Meat Imports	**18**	**19**	**16**	**15**	**15**	**15**	**9.9**	**-19.4**	**-7.6**	**2.0**	**4.0**
Meat Exports	**2 151**	**2 154**	**2 201**	**1 918**	**2 062**	**2 124**	**0.1**	**2.2**	**-12.9**	**7.5**	**3.0**
Consumption	**20 862**	**20 384**	**20 173**	**20 895**	**21 371**	**21 424**	**-2.3**	**-1.0**	**3.6**	**2.3**	**0.3**
***Per Capita* Consumption[1] (kg)**	**32.2**	**31.4**	**31.0**	**32.0**	**32.7**	**32.7**	**-2.5**	**-1.2**	**3.2**	**2.0**	**0.0**
Share in total meat cons. (%)	**49.8**	**49.2**	**49.2**	**49.4**	**49.4**	**49.3**					
Self-sufficiency rate (%)	**111**	**111**	**111**	**109**	**110**	**110**					

[1] In retail weight. Coefficient to transform carcass weight into retail weight is 0.78 for pig meat.

1.1.4 Poultry industry: expansion to continue in EU production

As well as the world's second largest pig meat producers, the EU is also one of the top producers in poultry meat and a net exporter for poultry products. The market organisation for the poultry sector has been improved in recent years, to safeguard the development of the sector, the quality of the products and consumers protection while harmonizing the entire market. (http://ec.europa.eu/agriculture/poultry/index_en.htm)

Figure 3: EU-28 main broiler meat producing countries (http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Poultry%20and%20Products%20Annual_Paris_EU-28_9-29-2014.pdf).



There is a domestic demand for Eu-28 broiler meat slowly increasing, as it is cheaper and more convenient, therefore making it less affected by economic

crises such as recession. This has resulted in the sector continuing to grow throughout 2014 and 2015. The only country, which is likely to decrease, is France due to the removal of EU-28 export subsidies.

In 2012 and 2013 the broiler production costs were affected by the surge in increase of the global grain prices, fortunately they were able to transfer these costs onto their domestic customers and maintain their operational margins or lower them slightly.

Operating margins and competiveness is likely to increase, even if retail prices fall as a result of the plummet of grain prices in 2013 and 2014 combined with the price weakness predicted in 2015.

Brazil and Thailand are currently the largest suppliers of broiler meat to the EU-28, when the EU-28 market to Thai uncooked broiler meat opened on 1st July 2012, there was a significant increase in exports of Thai broiler parts due to the fact they were of a quality, better suited to the needs of the EU importers. (http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Poultry%20and%20Products%20Annual_Paris_EU-28_9-29-2014.pdf).

It was also able to fill the gap that Brazil had created by shifting to non- EU markets, which led to Brazil decreasing exports in the EU by 25,000 tonnes. This economic downturn benefitted the poultry sector due to a reduction in beef sales, as people leaned towards cheaper meat. (http://ec.europa.eu/agriculture/markets-and-prices/short-term-outlook/pdf/2015-11_en.pdf).

Similar to the pig sector, feed prices will continue to be affordable in 2015-2016 due to good crop harvest and worldwide support for more growth in the production of poultry meat. Production increased by 3.6 % in the first half of 2015, compared to the same period in 2014, mainly in the EU-N13 (+10.3%). In comparison to this, Germany is decreasing the amount of slaughters over the same period (-0.4%). The increase in poultry meat production is estimated to

reach approximately 2.6 percent more throughout 2015, than in 2014 (13.6 million tonnes), this could continue into 2016 albeit at a reduced pace.

To compensate for the loss of the Russian market, Ghana, Saudi Arabia, Benin and The Philippines all contributed and therefore during the first six months of 2015, the EU poultry meat exports expanded by +2%. There is visibility of expanding exports to china and the Ukraine has reached 27,500 tonnes in the first half of the year, whilst exports to Hong Kong are decreasing

The poultry tariff rate quotas attributed to Ukraine within the framework of the bilateral free trade agreement with the EU explain the sudden surge of their exports to the EU (+13 500 tonnes), though still far below their poultry imports from the Union.

During 2015, exports may reach 1.4 million tonnes, a growth of 3.5 percent, this being driven by the Asian and African countries demand. In spite of the fact that South Africa has taken measures on anti-dumping against some EU companies and is supporting its domestic industry, the EU has not stopped shipping large amounts there (85,000 tonnes in the first half of 2015).

Competition is set to come from the US and Brazil in 2016 on the global market, which could limit the growth of the EUs exports to 25,000 tonnes.

Poultry meat prices have recovered since a drop in April and were within the band of 190-195 Eur/100kg c.w. although are expected to decrease in September and October as with the nature of the season.

After the strong increase in 2014, EU per capita consumption is expected to increase at a slower pace to 22.5 kg and 22.7 kg c.w. in 2015 and 2016 respectively (http://ec.europa.eu/agriculture/markets-and-prices/short-term-outlook/pdf/2015-11_en.pdf).

Table 2: EU-28 poultry meat maret balance ('000 tonnes carcass weight equivalent) (Short Term Outlook for EU arable crops, dairy and meat markets – Winter 2015: http://ec.europa.eu/agriculture/markets-and-prices/short-term-outlook/index_en.htm).

	EU-28						% variation				
	2011	2012	2013	2014e	2015f	2016f	12/11	13/12	14/13	15/14	16/15
Gross Indigenous Production	**12 359**	**12 691**	**12 798**	**13 268**	**13 614**	**13 766**	***2.7***	***0.8***	***3.7***	***2.6***	***1.1***
Live Imports	1	1	1	1	1	1					
Live Exports	9	10	10	11	10	10	*16.9*	*2.4*	*5.7*	*-4.0*	*0.0*
Net Production	**12 351**	**12 683**	**12 789**	**13 259**	**13 605**	**13 757**	***2.7***	***0.8***	***3.7***	***2.6***	***1.1***
EU-15	9 690	9 821	9 835	10 083	10 180	10 207	*1.3*	*0.1*	*2.5*	*1.0*	*0.3*
EU-N13	2 661	2 862	2 954	3 176	3 425	3 550	*7.5*	*3.2*	*7.5*	*7.8*	*3.6*
Meat Imports	**831**	**841**	**791**	**816**	**828**	**849**	***1.3***	***-5.9***	***3.1***	***1.5***	***2.5***
Meat Exports	**1 290**	**1 313**	**1 300**	**1 350**	**1 397**	**1 425**	***1.8***	***-1.0***	***3.8***	***3.5***	***2.0***
Consumption	**11 892**	**12 210**	**12 280**	**12 725**	**13 036**	**13 180**	***2.7***	***0.6***	***3.6***	***2.4***	***1.1***
***Per Capita* Consumption[1] (kg)**	**20.7**	**21.2**	**21.3**	**22.0**	**22.5**	**22.7**	***2.5***	***0.4***	***3.3***	***2.2***	***0.9***
Share in total meat cons. (%)	***28.4***	***29.5***	***29.9***	***30.1***	***30.2***	***30.3***					
Self-sufficiency rate (%)	**104**	**104**	**104**	**104**	**104**	**104**					

[1] In retail weight. Coefficient to transform carcass weight into retail weight is 0.88 for poultry meat.

With the above mentioned indications about market trends and production of meat in the World and European is interesting to evaluate what are the strategies to improve the production of poultry and pigs, the two categories of production that, given the projections, will increase in the next future.

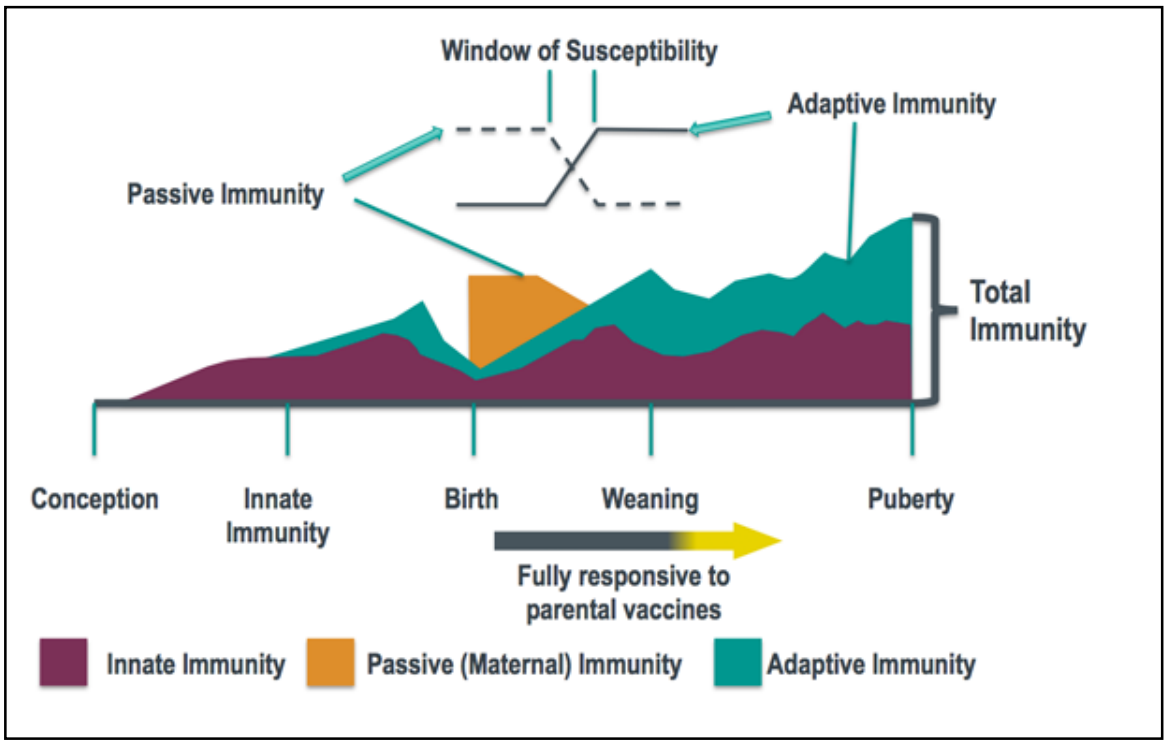
The aim of the present work is to study the supplementation of dietary feed additives to improve the welfare and the feed efficiency both in poultry and swine in order to further develop the corresponding livestock production sectors.

1.2 Gut health and immune system in pigs

The gut and immune system form a complex integrated structure that has evolved to provide effective digestion and defence against ingested toxins and pathogenic bacteria (Cummings et al., 2004). There is huge variation between what is considered as a normal healthy gut and immune function; therefore, it is difficult to clarify the benefits to individuals' aspects within a normal range, even though it is possible to measure the different aspects of digestion and immunity. However, it is still important to set specifications on ideal function for use by the consumer, industry and those concerned with the public health. There is a large market for gut functional foods worldwide, which is derived from the digestive tract, being the most frequent object of functional, and health claims (Cummings et al., 2004).

At birth, the piglet is extremely immunodeficent and heavily relies upon a supply of both specific and non-specific immune factors present in maternal colostrum and milk for immune protection, development and survival (Stokes et al., 2004). Directly after birth the piglet takes up macromolecules from the intestinal lumen in a non-selective way (Stokes et al., 2004). Maternal immunoglobulin cannot be transported through a pig's placenta, which means that newborn piglets acquire this from colostrum during the first 24 to 48 hours of life. The T-cell component of the intestinal mucosa in newborn piglets is extremely poorly developed along with the mucosal immune system, in the course of the first few weeks of life, it endures a rapid period of expansion and specialisation. Therefore it is essential that the mucosal T-cells are rigidly monitored and active responses to potential pathogens are expressed, also there should be control and maintenance of mucosal tolerance (Lallès et al., 2007).

Figure 4: time line of immunity in pigs (http://www.circumvent-g2.com/immunology.aspx)



The functional immaturity of the neonatal cellular and secretory immune systems is such that new-borns pigs are only able to generate limited T and B cell responses when challenged with pathogens, thus contributing to their immuno-compromised state (Butler et al., 2002; Stokes et al., 2004).

There are several organs such as bone marrow, spleen, thymus and mesenteric lymph nodes and several cell types such as lymphocytes that are specific in recognising foreign antigens and phagocytes that, together, make up a young pigs immune system. There are two branches in the immune defence system- the innate or the acquired. The innate immune system is believed to have developed before the adaptive immune system and therefore has become the first line of defence against a pathogenic/antigenic challenge.

It is necessary to have the development of immunocompetence for optimum growth and performance, although, in relation to exposure to the range of

antigens associated with pathogens, commensal bacteria and food, reacting to antigens must be considered when defining immunocompetence. This should incorporate the ability to generate tolerance to food and commensal bacterial antigens as well active immune responses to pathogens (Bailey et al., 2001). A wide range of evidence suggests immune development and function is impacted upon by early nutrition and environment, the so-called hygiene hypothesis (Braun-Fahrländer et al., 2002; Kalliomäki et al., 2003; Stokes et al., 2004).

The various mechanisms that protect the pig from infectious agents can be considered in six groups:

- **Complement system** - This is a non-specific protective mechanism that acts on any foreign cells or viruses that do not possess certain pig proteins on their surface. It consists of a number of chemicals found in the plasma, which act together as a cascade to remove or destroy organisms.
- **Chemical factors** - These include non-specific enzymes (such as lysozyme in saliva) and acids, which may be found in mucus, saliva and gastric juices. These block or kill pathogens.
- **Mechanical factors** - These include the skin, mucus, sweat, lining of the nose, mouth, oesophagus, intestine, colon, vagina, flow of urine and the passage of faeces.
- **Macrophage cells** - These are found throughout the body in tissues and in the blood stream where they are called monocytes. They engulf and digest bacteria. They also have an important role in controlling viral and fungal diseases. The cells are of two types called leucocytes and monocytes.
- **Specific acquired immunity** - This is of two types; that which is activated by cells and called cell mediated immunity and antibodies present in the blood called humoral immunity. Cell mediated immunity

arises when T type lymphocytes come into contact with antigens and they are stimulated to produce antibodies. It takes 7-14 days for these to develop. Humoral immunity is produced from B lymphocytes which have met the antigen previously and their response is immediate. Some lymphocytes also kill other cells that contain antigens or they may act immediately against antigens.

- **Immunoglobulins** - Specific antibodies of which there are different types namely immunoglobulins, IgG, IgM and IgA. They are found in blood, in milk and particularly in colostrum. All internal surfaces of the body also contain them.

(http://www.thepigsite.com/pighealth/article/5/immune-system/)

1.2.1 Antibodies response

Lymphocytes arise continuously from progenitor cells in the bone marrow. Most functions of the immune system can be described by grouping lymphocytes into three basic types:

1. B cells
2. Cytoxic T cells (TC cells)
3. Helper T cells (TH cells)

Cell surface receptors that are able to bind antigens are carried by all three lymphocytes and because one cell recognises one antigen, it is able to control the affinity of the immune system. All antigen receptors are glycoproteins and systems are able to allow only one type of receptor to integrate within each cell. Depending on the cytokines they produce, T helper cells in the immune system of weaned piglets and their atopic type of immune responsiveness have been titled T helper (TH)1 or TH2 (Mosmann et al., 1986; Del Prete et al., 1991).

1.2.2 Inflammatory response

Inflammatory and immune processes are mediated and controlled by a diverse range of molecules follow as: proteins; the pro-inflammatory cytokines, tumour necrosis factor (TNF) and interleukins (1L)-1 and -6; derivatives from membrane phospholipids; the eicosanoids prostaglandins (PG), leukotrienes (LT); diacylglycerol and ceramide; miscellaneous compounds such as CAMP, inositol phosphates and reactive oxygen species (Grimble, 1998). Even though the pro-inflammatory cytokines are overall products of the immune system, endothelial cells and fibroblasts also have the ability for production. A deluge of cytolune production is activated by TNF that acts as a trigger; the molecule is released quickly as a reaction to inflammatory and infective agents. It then incites the production of a large amount of other cytokines, including IL-1 and IL-6 with which it has many actions in common (Akira et al., 1990). These include generation of a fever, reactive oxygen species and acute-phase protein production, muscle proteolysis, hyperglycaemia, hyperlipidaemia, up-regulation of adhesion molecules and changes in the plasma concentrations of cations (Tracey and Cerami, 1993; Grimble, 1996). TNF, by induction of the chemokine IL-8, may also prolong the inflammatory process (Standiford et al., 1990a,b). Immunological events can be regulated by TNF, by the induction of IL-1, which then stimulates IL-2 and IL-4 production, these last two cytokines end in higher lymphocyte proliferation and the switching of immunoglobulin classes (Chrétien et al., 1990). TNF is also vital for killing fungi and several viruses as well as being an early effector in the inflammatory and immune processes (Ito and O'Malley, 1987). However, any unnecessary or excessive TNF production has close links with endotoxic shock, sepsis and adult respiratory distress syndrome (Tracey and Cerami, 1993) and a range of disorders such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and atherosclerosis (Grimble, 1996). This

cytokine can also augment human immunodeficiency virus replication (Shreck et al., 1991)

1.3 Major sources of disease in piglets and feed additives promoting health condition

It is well known that weaning, as a stress process, and post weaning anorexia or under nutrition are major causes of disease (Pluske et al., 1997; Lallès et al., 2004). Changes in gastric function throughout weaning have received little attention comparatively to the intestine. The intestine is an essential component of the gastrointestinal tract (GIT) and is a main digestion site, place to absorb nutrients and for hydro-mineral exchange homeostasis, it harbours a complex microbiota and a highly evolved mucosal immune system. GIT disorders in pigs from post weaning can be a result of alterations in GIT architecture and function and also from changing the adapting enteric microbiota (Konstantinov et al., 2004b) and immune responses (Stokes et al., 2004; Bailey et al., 2005). GIT physiology, microbiology and immunology all impact on the gut health balance. By changing the weaner diet it is possible to manipulate the composition of the microbiota and its metabolic activities to the advantage of the piglet. Whilst the issues on GIT physiology have been clarified, not all the influences on local immune system are understood (Lallès et al., 2007).

A very serious economic problem for the global pig industry is post-weaning diarrhoea. A condition in weaned pigs that can be identified by the discharge of watery faeces during the first two weeks after weaning. It can cause widespread morbidity or mortality in the most serious cases and although it is a multi-factorial disease, the exact pathogenesis is not understood. Usually, it is linked with faecal shedding of large numbers of β-hemolytic enterotoxigenic E. coli

serotypes, that are found in the small intestine after weaning. Often this type of diarrhoea is referred to as post weaning colibacillosis. There are also other pathogenic types of non-enterotoxigenic E.coli that are sometimes connected to post weaning diarrhoea as well as numerous types of bacterial pilus (fimbrial) adhesins involved in attachment to the intestinal mucosa (Fairbrother et al., 2005; Pluske, 2013).

Neonatal and early-weaned piglets can get diarrhoea due to enterotoxigenic *Escherichia coli* (ETEC) which is another problem in the pig farming industry, although pigs that are over 8 weeks old have been found to be resistant to infection. For the disease to occur each strain of ETEC must have adhesins and enterotoxins which are referred to as virulence determinants (Francis, 2002). Toxins produced by ETEC strains that cause diarrhoea in pigs include heat labile enterotoxin (LT), heat stable enterotoxin type A (STa); heat stable enterotoxin type B (STb); Shiga toxin type 2e (Stx2e), and enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) (Wilson and Francis, 1986; Imberechts wt al., 1997; Choi et al., 2001). The virulence determinants usually occur in patterns with specific serogroups and fimbriae. Table 3 shows the correlation between the ages of pigs that are commonly infected with ETEC of each type and the serogroups. The gene for STb can be found in nearly all strains of ETEC isolated from weaned pigs with diarrhoea. The strong presence of this gene in post weaning diarrhoea supports the idea that STb plays a crucial role in pathogenesis that cannot be copied by other enterotoxins. However, STb is not an isolated gene in ETEC strains, which implies that other enterotoxins are also pivotal in post weaning diarrhoeal disease; they also possess genes for STa, LT or both.

Table 3. Characteristics of enterotoxigenic *Escherichia coli* (ETEC) strains associated with infections in pigs of various ages (Francis, 2002)

	ETEC characteristics			Age group affected	
Serogroup	Fimbriae	Toxins	Hemolysins	Neonatal	Weaned
O8	K99	STa	–	Yes	No
O9	K99, 987P	STa	–	Yes	No
O20	987P	STa	–	Yes	No
O101	K99	STa	–	Yes	No
O141	987P	STa	–	Yes	Yes
O8	K88	LT, STb± STa	+	Yes	Yes
O149	K88	LT, STb± STa	+	Yes	Yes
O157	K88	LT, STb± STa	+	Yes	Yes
O138	F18ab, F18ac	STa, STb ± Stx2e	+	No	Yes
O139	F18ab	STa, STb ± Stx2e	+	No	Yes
O141	F18ac	STa, STb ± Stx2e	+	No	Yes
O157	F18ac	STa, STb ± Stx2e	+	No	Yes

European-wide directives have been put in place to create a sustainable production of pigs without the use of production enhancers and chemotherapeutics. It is now only possible to have economically viable pig production when the physiological mechanisms of defence against pathogens and tolerance against nutrients and commensal bacteria in the intestinal immune system are taken into account (Stokes et al., 2004)

1.3.1 Impact of bioactive substances on the gastrointestinal tract

Research has been made into gaining the best diet composition for protein, AA and energy, because of the ban on in-feed antibiotics. This has also led to an evaluation of other substances such as Organic acid and compounds from plant origins with known antimicrobial properties. (Lallès et al., 2009).

1.3.2 Spray-dried animal plasma (SDP)

SDP is sourced from animal blood after exclusion of cells, concentration and spray drying. There are three types of SDP products, from porcine (SDPP), bovine (SDBP) and unknown or mixed animal origin (SDAP) that are available commercially. After a temporary ban in Europe due to the bovine spongiform encephalopathy crisis, feeding SDPP is now permitted for pigs, although it is vital that the collection and processing of the blood is done in a hygienic manner and poor quality SDP containing high levels of bacteria should be irradiated or have a formaldehyde treatment of SDP for improved growth (DeRouchey et al., 2004). The spray drying process has proved to be effective in inactivating pseudirabies and porcine respiratory and reproductive syndrome viruses (Polo et al. 2005), this helps to alleviate the transmission of infectious diseases (van Dijk et al, 2002).

When porcine and bovine SDP are incorporated into weaning diets, they can stimulate growth performance and feed intake (Coffey and Cromwell, 2001; van Dijk et al. 2001) and also it has shown that SDPP was more effective than SDBP (Pierce et al. 2005).

This observation could suggest some degree of specificity in the effect of IgG in SDPP against porcine pathogens. Pierce et al. (2005) showed that it was the immunoglobulin-rich fraction that was responsible for the beneficial effects of SDP. This has a larger effect on piglets weaned at 22 days when compared to 32 days (Torrallardona et al., 2002) and are even greater in poorer health conditions. Therefore showing that the protective effect of SDP could be via the immune system or directly against pathogens (Coffey and Cromwell, 1995; Bergstrom et al., 1997; Lallès et al., 2009).

1.3.3 Specific amino acids

Amino acids are not only a building block for peptides and proteins, they are also believed to have therapeutic effects on the GIT and the whole organism (Kim et al., 2007). Certain amino acids can aid health by improving gut tissue anabolism, reducing the impact of stress and modulating local immunology. When growth is constrained by infection, inflammation or stress, amino acids could prove beneficial (Gruys et al., 1998).

Biologically active AA can be added to the diet in case of a deficiency during disease and experimental results suggest that the profile of AA required for the immune system differs substantially from that for growth (Reeds et al., 1994). To induce significant bioactivity for therapeutic purposes it is necessary to have surplus administration of dietary amino acids (Massey et al., 1998). Glutamine, tryptophan, arginine, cysteine and threonine were the five amino acids that showed consistent bioactivity in regards to physiology, immunology, endocrinology and metabolism (Lallès et al., 2009).

1.3.4 Bovine colostrum

Studies showed that bovine colostrum, when included in weaning diets led to increased growth and reduced the number of days to slaughter (Pluske et al., 1999). Such an effect on growth was associated with a drastic enhancement of feed intake post-weaning when bovine colostrum was provided at 40 g/kg starter feed to pigs (Le Hue¨ rou-Luron et al., 2004; Boudry et al., 2008) and results showed that the colostrum had a positive effect for up to five weeks after the end of the supplementation (Le Huërou-Luron et al., 2004). A different study found when the pigs were pair-fed the colostrum-supplemented and the

control diets, the only significant effects of colostrum supplementation were a decreased gastric pH at 1 and 2 weeks post-weaning and an increased duodenal lactobacilli to coliform ratio caused by numerically lower coliform counts (Huguet et al., 2006). This evidence shows that primarily colostrum supplementation acts by stimulation the appetite post weaning.

Intestinal explants incubated in media with or without colostrum revealed that it may stimulate the expression of genes involved in epithelial cell migration along the crypt–villous axis and genes bearing anti-apoptotic properties (Huguet et al., 2007). On the contrary, bovine colostrum was administered to pigs (0,1 or 5g/pig per day for 21 days after weaning), there was no effect on growth or feed intake (Boudry et al, 2007a and 2007b). The effects of bovine colostrum on immunity were unclear in these latter studies (Lallès et al., 2009).

1.3.5 Plant extracts

Herbs are well known for their antiseptic attributes and anti-bacterial activities, so that they have been used in humans for therapeutic use for thousands of years (Cowan, 1999). In last years, a lot of research has been developed in this area because of their possible alternative to the use of antibiotics in animal production (Dorman, 1999; Bozin et al., 2006 Essential oils – occurrence and composition Volatile or essential oils, distilled from non-woody parts of herbs, contain principally terpenoids and minor constituents including various aliphatic hydrocarbons, acids, alcohols, aldehydes and other compounds (Dorman, 1999; Bozin et al., 2006). Oregano oil (Origanum vulgare) and thyme oil (Thymus vulgaris) contain mainly carvacrol and thymol (Peñalver et al., 2005; Bozin et al., 2006). Eugenol dominates in oils from clove (Syzygium aromaticum) and cinnamon (Cinnamomum zeylanicum) (Dusˇan et al., 2006). Cinnamaldehyde is

the main component of C. verum or C. cassia (Ooi et al., 2006). Essential oils themselves make 1.5% to 4.5% of the plant (Bozin et al., 2006; Hazzit et al.,2006; Yang et al., 2007). Alternative environmental conditions and climates make the composition of the oils extremely variable within plants and they can also be influenced in the final composition by the distillation method used (Yang et al., 2007; Lallès et al., 2009).

Plant extracts do not harm animals or people as they contain stable determined contents of main active ingredients. To use them in animals feed as supplements they have to follow strict guidelines, they are normally fed to healthy animals by the farmer to add nutritional value on a fixed period for example throughout all of the production period.

Windisch et al. (2008) states that particularly in the EU, it is important that feed additives demonstrate identity and traceability for the commercial product and how valid the results for nutritional effects and the interactions with other feed additives. It is also required that feed additives can show safety for animal, operator, environment and consumer.

1.3.6 Nucleotides

Nucleotides play an important part in most biochemical processes. They are intracellular compounds with low molecular weight and are present in all foods of animal and vegetable origin as free nucleotides and nucleic acids. Meat has a high content of nucleic acids while milk and eggs have lower levels. The RNA and DNA concentrations in these products and all foods largely depend on their cell density (Gil, A. 2002). In relation to feed ingredients often used in diets for piglets, nucleotides are naturally present but in sows' milk there are considerably less concentrations (Mateo et al. 2004a)

Dietary nucleotides are a group of bioactive agents which are supposed to diminish challenges in the ileum and of piglets related to weaning (Andre´ s-Elias et al., 2007). Nucleotides used in dietary supplementation can have advantageous effects on gastrointestinal, hepatic and immunological functions, even though they are synthesized endogenously (Carver and walker, 1995; Sauer et al, 2010; Sauer et al., 2011; Sauer et al., 2012).

Walker (1996) states that the previous modulator effects of nucleotides on the intestinal and immune tissues should be treated as separate issues, although now they are considered to be closely linked. The main reason is that intestinal epithelial cells can produce immunomodulatory molecules like cytokines, therefore regulating the immune response. Secondly, a noteworthy amount of the intestinal cells are of immune origin (Walker, 1996; Gil, 2002).

Dietary nucleotides could be used as an alternative to antibiotics when feeding young animals because they can aid maintenance in intestinal health. Research has been done to support this, albeit in human nutrition and results showed that if nucleotides are included in parenteral formulas and formulas for infant milk, intestinal health can be improved and it can aid in the development of an infants' immune system (Mateo et al., 2004b).

Nucleotides could aid in the prevention of post weaning diarrhoea in supplemented piglets, as shown by a decrease in the number of parenteral antibiotic treatments needed (Martinez-Puig et al., 2007).

1.4 Gut health and immune system in poultry

The greatest element for the total cost in the production of meat and eggs in the poultry industry is feed and globally, the foremost ingredients used for poultry diets is corn and soybean, although in some parts of the world, poultry diets commonly have high levels of wheat (Leeson, 2004). Feed is seen as the biggest form of exposure for birds to various factors through the gastrointestinal tract (Yegani et al., 2008).

1.4.1 Gastrointestinal tract development in poultry

The weight of the embryonic small intestine increases much faster than body weight as incubation progresses and during the last 3 days of incubation, the ratio of small intestinal weight to body weight increases from approximately 1% on d 17 of incubation to 3.5% at hatch (Uni et al., 2003). Then, in the post hatch period, the small intestine will gain weight at a much quicker rate than the rest of body mass, although, increases in intestinal weight and length are different in the duodenum, jejunum, and ileum. Intestinal development after hatch is also rapid with respect to enzymatic and absorptive activities (Uni et al., 1999; Sklan, 2001). Throughout the two weeks post hatch, the small intestine of a newly hatched chick undergoes morphological, biochemical and molecular modifications, but the more significant changes happen in the first 24 hours post hatch. The chicken small intestine matures in a manner similar to neonatal mammals (Geyra et al., 2001; Yegani et al., 2008).

With the intake of exogenous feed comes the rapid development of the GI tract and associated organs. It is crucial that the timing and form of nutrients to chicks after hatch is correct for the intestines to develop. If feed is available from an early stage, it is shown to encourage growth and development of the intestinal

tract and can also augment the uptake of yolk by the small intestines posthatch. (Uni, et al., 1998; Geyra et al., 2001; Noy and Sklan, 2001; Noy et al., 2001; Potturi et al., 2005; Yegani et al., 2008). Research has also shown that giving nutrients to the growing embryo, through in-ovo feeding, can heighten the development of the intestinal tract and when feed is delayed, birds have shown to have slower intestinal development and low performance (Corless and Sell, 1999; Vieira and Moran, 1999; Geyra et al., 2001; Bigot et al., 2003; Maiorka et al., 2003; Patterson et al., 2005; Yegani et al., 2008).

1.4.2 Avian gut immune system

Birds and mammals have similar organisation and mechanisms of immunity, which is precisely influenced by factors such as genetics, nutrition, environments and physiological factors. (Qureshi et al., 1998; Sharma, 2003). The immune system of birds is made up of numerous cells and soluble factors that need to work together in order to have a protective immune response, thus making it very complex. Poultry must have a properly functioning immune system as commercial flocks are reared in intense conditions. In these conditions the poultry become vulnerable to the spread of disease outbreaks and infectious agents that can occur rapidly (Sharma, 2003). Lymphoid organs are predominantly the main structural category of the avian immune system. The primary lymphoid organs are believed to be the bursa of Fabricius (a site of Blymphocyte development and differentiation) and the thymus (a site of T-lymphocyte development and differentiation) as noted by Qureshi et al (1998). Once the immune cells are fully functional they leave the primary lymphoid organs and populate secondary lymphoid organs, which can be distinguished by aggregates of lymphocytes and antigen-presenting cells that are scattered

throughout the body. Some examples of secondary lymphoid organs are the Spleen, bone marrow, gland of Harder, bronchial-associated (BALT), and gut-associated (GALT) lymphoid tissues. In chickens, GALT includes the bursa of Fabricius, cecal tonsils, Peyer's patches, and lymphoid aggregates in the urodeum and proctodeum (Befus et al., 1980; Yegani et al., 2008).

1.4.3 Microflora of the gastrointestinal tract (git)

Bacteria are the main microorganisms in the GI tract microflora but there is also a mixture of fungi, and protozoa as well (Gabriel et al., 2006). The chemical composition of the digesta can determine the composition of the microbial community in the GI tract due to the fact that each bacterial species has different substrate preferences and growth requirements (Apajalahti et al., 2004). Moreover Apajalahtis' (2004) research suggests that broiler chicken diets containing corn, sorghum, barley, oats, or rye had various effects as corn- and sorghum-based diets increased numbers of *Enterococcus*, barley-based diet increased numbers of *Lactobacillus*, oats-based diet enhanced growth of *Escherichia* and *Lactococcus*, and rye-based diet increased the number of *Streptococcus* in broiler chickens. Added to this, evidence suggests that throughout different parts of the GI tract there is momentous diversity in bacterial populations and from the proximal to distal GI tract, there is a dense population (Richards et al. 2005). As chickens mature, the unique microbial profile in each area of the GI tract becomes increasingly complex (Gong et al., 2002a,b; Wielen et al., 2002; Hagen et al., 2003; Lu et al., 2003; Amit-Romach et al., 2004). It is now possible to gain new information on locating different bacterial populations in intestinal contents and mucosal samples when compared with routine culturing methods, due to advances in ribosomal DNA based molecular techniques. What's more is that

these techniques can also be used to monitor the effect of diets and other variables on the microbial communities of the GI tract in commercial environments (Apajalahti et al., 1998, 2001; Gong et al., 2002b; Knarreborg et al., 2002; Van der Wielen et al., 2002; Amit-Romach et al., 2004; Yegani et al., 2008).

1.4.4 Factors affecting intestinal health

The digestive tract of the chicken is an area, which has plenty of potential exposure to pathogens as birds can ingest non-nutrients and potentially threatening organisms as well as nutrients and beneficial organisms. Within the lumen, there is usually feed, constituents, resident and transient microbial populations, endogenous nutrients, and secretions from the GI tract and its accessory organs such as the liver, gall bladder, and pancreas. The nutrients must be able to cross the intestinal wall into the body by the GI tract, but the GI tract must also stop any deleterious components of the diet from crossing the intestinal barrier (Korver, 2006). Therefore, it becomes a selective barrier between the tissues of the bird and its luminal environment that is composed of physical, chemical, immunological, and microbiological components. The chicken gut is a delicate balance amongst components and this can be affected in a negative manner by numerous factors associated with diet, infectious disease agents, environment, and management practices (Yegani et al., 2008).

1.4.4.1 Diet: Physical Texture and Form of Feed.

According to Brunsgaard, (1998) and Engberg et al (2004) the morphological and physiological attributes of the intestinal tract can be affected by the physical

form of cereal components, however the research in this area has been found to be inconsistent. It is possible that when compared to coarsely ground feed, finely ground feed could increase mortality linked with necrotic enteritis. Research by Branton et al. (1987) showed that of finely ground wheat increased mortality to 28.9%, while coarsely ground wheat had a lower result of mortality at 18.1%, in both tests; mortalities were linked with a mixture of necrotic enteritis and coccidiosis.

Research has also shown that dietary whole wheat can affect gut performance in broilers during the development of the GI tract, particularly the gizzard and can increase uptake of dietary nutrients from the lower digestive tract (Hetland et al., 2002; Yasar, 2003; Engberg et al., 2004; Taylor and Jones, 2004; Yegani et al., 2008).

1.4.4.2 Diet: Non-starch Polysaccharides and Enzymes

The predominant group of anti-nutritional compounds in feed ingredients is the non-starch polysaccharides (NSP) and cereals that are used in poultry diets all have different levels of NSP such as β-glucans and arabinoxylans (Iji, 1999). Resistance to the birds' digestive enzymes and that they can create a viscous environment in the intestinal lumen are common attributes to different NSP and lead to the excretion of sticky droppings (Choct and Annison, 1992a,b). NSP can lower the digesta passage rate and availabiltity of nutrients due to the high viscosity of the intestinal contents, which, in turn, lead to digestive and health problems. Increased digesta retention time facilitates bacterial colonization and activity in the small intestine (Waldenstedt et al., 2000). High levels of NSP can be found in barley, wheat, rye and oats and according to Bedford and Schulze (1998) can mean increased digesta viscosity, decreased passage rate, digestive enzymatic activities and nutrient digestibility, depressed feed conversion

efficiency, and growth rate of the birds. However, certain exogenous enzymes are able to breakdown NSP and lower digesta viscosity, which therefore heightens digesta passage rate, and improve bird performance (Almirall et al., 1995; Bustany, 1996; Choct et al., 1996, 1999; Jorgensen et al., 1996; Leeson et al., 2000; Mathlouthi et al., 2003; Wu et al., 2004a; Yegani et al., 2008). The glycanases (xylanases and β-glucanases) are the most commonly used enzymes in poultry feed industry. They split the non-starch polysaccharides (NSP) in cereal grains such as wheat and barley and microbial phytase, which targets phytate complexes in plant-derived ingredients (Wu et al., 2004b). The predominant reason to add exogenous enzymes to poultry diets is to improve the utilisation of nutrients in raw materials. This can be done using one or more of the following techniques: firstly, by degradation of specific bonds in ingredients not usually degraded by endogenous digestive enzymes; secondly, by degradation of anti-nutritive factors that lower the availability of nutrients; thirdly, by increased accessibility of nutrients to endogenous digestive enzymes, and/or finally, with the supplementation of the enzyme capacity of young animals (Wu et al., 2004b).

1.4.4.3 Diet: Type of added fat

The most common fats used in the feed industry are tallow and poultry fat and the most widely used vegetable oils are soybean oil, palm oil, and maize oil. There are numerous studies, examining the results of adding different fats and oils on fat digestion, Apparent metabolisable energy (AME) and growth performance in broiler chickens (Zumbado et al., 1999; Dei et al., 2006; Wongsuthavas et al., 2007). Two studies were conducted by Pesti et al (2002), to look at the effect of eight types of fat (feed grade poultry grease, pet grade poultry grease, restaurant grease, white grease, animal/vegetable oil blend, palm oil, yellow grease and soybean oil) on the AME and growth performance of

broilers. During the first experiment, the poultry were given a basal diet with 60g/kg of different fat sources. The diets with the lower AME values were those containing poultry grease in feed and pet grade and palm oil and those that contained soybean oil, yellow grease and animal/vegetable oil blend had higher AME values, which were then followed by white grease and restaurant grease. Experiment 2 consisted of the birds being supplemented with 30 or 60g/kg of each fat source in the basal diet. This time results showed that the different fat sources did not affect performance, although there was significantly less feed per gain of broilers fed on diets with 60g/kg of fat than those with 30g/kg of fat. Another experiment in the form of a 7 week trial this time conducted by Firman et al. (2008) where they studied the performance of broilers fed diets supplemented with 30 g/kg of soybean oil, yellow grease, poultry fat, tallow, vegetable and animal fat blend, lard and palm oil. Results showed there was no difference between the fat sources in broiler performance. As noted previously, animal fats have a high proportion of long chain saturated fatty acids that are poorly digested compared to vegetable oils which are high in unsaturated fatty acids, however, if the two are blended together, this would be a viable alternative for poultry feed industry (Wiseman and Lessire, 1987; Scaife et al. 1994; Wiseman et al., 1998; Danicke et al., 2000). Research has been done on the subject of blending saturated and unsaturated fatty acids (U:S); Ketels and De Groote (1989), reported that increasing U:S ratios increased the utilisation of saturated fatty acids, but there was no effect on unsaturated fatty acids . Leeson and Summers (2005) advise a ratio of 3:1 (U:S) should be used for optimum fat digestibility in birds of all ages. Furthermore, it has been well noted that, blending animal fats with vegetable oils, can produce a synergistic effect, leading to an improved utilisation of saturated fats (Lall and Slinger, 1973; Sibbald, 1978). Sibbald (1978) reported that the AME of mixtures of soybean oil and tallow was higher than the sum of the means of its components. Similarly,

Muztar et al. (1981) found that the AME of blends of tallow and rapeseed soapstocks was higher (by over 4%) than the calculated AME of its components. Danicke et al. (2000) used a blend of beef tallow and soybean oil in a rye based diet of 100g/kg fat for broilers (0:100, 20:80, 40:60, 60:40, 80:20 and 100:0) this gave U:S ratios of 5.47, 3.23, 2.11, 1.45, 1.00 and 0.69, respectively. The results showed that when the amount of tallow was increased, there was higher feed per gain however, weight gain and AME was decreased. Preston et al (2001) conducted another study into the effects of blending animal fats and vegetable oils. Broilers were fed on wheat based diets with a blend of tallow and soybean oil of 60 g/kg of fat (a ratio of 2:1) and recorded that fat type had an impact on the digestibility of fat which were 0.85, 0.76 and 0.69, however, there was no real effect on bird performance

1.4.4.3.1 Digestion and absorption of lipids

In the newly hatched chick, energy metabolism changes from yolk-based lipid supply to a predominantly exogenous carbohydrate feed source as early as one day posthatch (Kuenzel and Kuenzel, 1977). Feed intake increases during the growing period and changes occur in the gastrointestinal tract (GIT) and its secretions match these developments (Noi et al., 1995).

As the gastrointestinal tract of the newly hatched chick is in a process of development and maturation, with rapid development especially evident in the duodenum, jejunum and pancreas, it has been noted that the time at which maximum specific activities of digestive enzymes in the pancreas and intestinal brush border can vary. Most often it occurs at or shortly after hatch and certain activities of these enzymes decrease with age. In the early part of the post hatch period, total digestive enzyme activity increases due to the pancreas and intestines gaining weight rapidly, although sometimes the increase in total

enzyme activity may not be able to keep up with an increase of feed intake. Research on the uses of nutrients on digestion are somewhat varied, there is evidence to suggest that as well as some lipids, the use of carbohydrates and protein is not as efficient in the first week or two after hatch as it is in older chickens (Jin et al., 1998). Ingested lipid undergo intestinal emulsification, digestion, micellar solubilisation, cell membrane permeation, intracellular esterification and incorporation into lipoproteins before release to the interstitial fluid. Essential for both emulsification and formation in the intestine are bile salts secretion and this is known to be influenced by the quality and quantity of dietary lipids as well as other emulsifiers. Changes in pancreatic lipase content and secretion can be caused by modifications of dietary lipid contents. Colipase secretion by the pancreas seems dependent on both lipid and protein intakes. Bile salts can have an important role as moderators for intracellular processes, which in turn are crucial for lipid absorption and increase in rate as the rate for lipid intake increases. Newly hatched chicks do not have the full development needed to be able to digest lipids. When bile salts, lipase or phospholipids are added to chick diets, animal fat digestion is improved confirming that lipid digestive processes are not completely functional in newly hatched chicks. During the first weeks after hatch the lipase concentration in pancreatic tissue and intestinal contents of poultry increases significantly and comparable changes with age have also been noted in bile salt secretion. The development in enterocytes of fatty acid-binding protein activity seems to parallel the development in lipase activity and bile salt secretion in poultry (Krogdahal, 1985).

1.4.4.3.2 Strategies to improve fat digestion

During the first weeks post hatch, young birds have a poor ability to digest and absorb fat although it is unclear if it is because of a deficiency in lipase, bile or both (Carew et al., 1972; Wiseman and Salvador, 1991). The digestion of fats is a process that requires sufficient quantities of bile salts, which are essential for emulsification, and enzyme lipase and when trying to improve fat digestion, the supplementation of enzyme and emulsifiers must be considered.

1.4.4.3.3 Emulsifiers

Emulsifiers commonly used in the feed industry are classed in two groups: natural and synthetic emulsifiers. Natural emulsifiers are those such as bile and phospholipids that are produced in the body of an animal or, those from foods such as soy lecithin (Soares and Lopez-Bote, 2002). Synthetic emulsifiers are modified emulsifiers such as lysolecithin or lysophosphatidylcholine (Zhang et al., 2011). It is essential that fats are emulsified before they can be digested by lipolytic enzymes as they do not solubilise in the gastrointestinal tract. Certain attributes of the fat contribute to the ease of emulsification such as chain length, position of fatty acids on triglycerides and fat saturation (Gu and Li, 2003) which shows that emulsifiers can improve the utilisation of lipids, particularly of animal fats. They also help to overcome the insufficiency of recirculation and naturally low bile production in young birds.

1.4.4.3.4 Lecithin

Lecithin is a by-product from the processing of soybean oil, and has shown to improve fat digestion in diets for young pigs (Overland et al., 1993; Soares and Lopez-Bote, 2002) and broilers (Polin, 1980). As a phospholipid it is a combination of surface-active agents, consisting of a hydrophobic portion with affinity for fats, and a hydrophobic portion with an affinity for water (Gu and Li, 2003). A study by Polin (1980) supplemented a diet containing 40 g/kg tallow with 0.2, 2 and 20 g/kg lecithin and discovered that the absorption of tallow was increased in broilers fed 20 g/kg lecithin compared to those fed 0.2 and 2 g/kg. Azman and Ciftci (2004) investigated the impact of replacing dietary fat with lecithin on broiler performance. During the study, broiler starters were fed diets containing 40 g/kg soybean oil (control) and, 40 g/kg soybean oil and soy-lecithin mixtures (in 75:25 and 50:50 proportions) or 40 g/kg of beef tallow and soy lecithin mixtures (in 50:50 proportions) from 5 to 21 days of age. From days 22 to 35, birds were fed grower diets containing 60 g/kg soybean oil or tallow mixed with the same proportion of soy lecithin as in the starter diets. The results showed that feeding tallow and lecithin combination has a higher feed per gain than a soybean oil and lecithin combination; however, there were no differences in feed per gain.

1.4.4.3.5 Commercial synthetic emulsifiers

It is not economically viable to use bile supplements or natural emulsifiers as a way of improving the utilisation of fat, therefore the use of synthetic emulsifiers are economically justified because they are cheaper and there are many products available commercially. Synthetic products include blends of hydrolysed lecithin,

lysophosphatidylcholine and glycerol polyethylene glycol ricinolate. Lysophosphatidylcholine is a strong biosurfactant and the mono-acyl derivative of phosphatidylcholine, which is produced by the action of enzyme phosphorlipase. A2-Lysophospholipids have one fatty acid residue per molecule, making them more hydrophilic than phospholipids, they can also form spherical micelles in aqueous solutions which can enhance emulsification in the gastrointestinal tract (Vasanthakumari et al., 2011). According to Melegy et al. (2010) lysophospholipids are also able to create small micelles and are more competent than bile and soy lecithin.

An evaluation was completed by Zhang et al. (2011) on the competence of commercial commercial lysophosphatidylcholine and their performance and AME of broilers with diets consisting of three fat sources; soybean oil, tallow and poultry fat. With 30 g/kg in starter diets and at 40 g/kg in grower diets. It was concluded that using lysophosphatidylcholine as a supplement on birds throughout the starter phase led to an increase in weight gain when fed all three fat sources, yet in the grower phase, no differences were noted. The AME results showed that in both phases AME increased with the supplementation of lysophosphatidylcholine, the chicks fed on diets with poultry fat and emulsifier were much higher than those that were fed diets with supplements of soybean oil or tallow plus emulsifier (13.02, 12.93 and 12.77 MJ/kg, respectively). Roy et al. (2010) investigated the effects of glycerol polyethylene glycol ricinolate (an ester of ethylene oxide) and palm oil as an emulsifier. The starter phase considered 35g/kg and the grower phase 28g/kg supplemented with three levels of glycerol polyethylene glycol ricinolate (0, 10 and 20 g/kg). Results showed that compared to the 0 and 20g/kg additions, the addition of 10g/kg of emulsifier lead to increased live weight but lower feed per gain.

1.4.4.4 Infectious Agents

As the GI tract is used to breakdown food materials into simple components which are transported and absorbed to use as aids in production, maintenance and growth, thus safeguarding the bird. Any physical chemical or biological hindrance of these processes can in end in enteric disease (Dekich, 1998). The cause of an enteric disease is complicated because mixtures of viruses, bacteria and other infectious or indeed non-infectious agents could be involved (Reynolds, 2003; Yegani et al., 2008).

1.4.4.4.1 Bacterial Infections

Poor feed conversion efficiency and a lower rate of body weight can be caused by low damage to the intestinal tract by pathogenic bacteria, however, if the damage by bacterial infections is more severe, it can lead to overt disease and high mortality (Porter, 1998). One of the most severe diseases of the chicken intestine is noted by Long et al. (1974) as the lesions of necrotic enteritis (NE) and is caused by clostridium perfringens. Necrotic enteritis, caused by Clostridium perfringens, has been reported in most areas of the world where poultry are produced (see review by McDevitt et al., 2006). Breeder farms, processing plants, grow out houses and hatcheries can all be contaminated by clostridium perfringens as it is widespread (Craven et al., 2001, 2003).

Another disease of economic importance to the poultry industry that has been studied for more than a century is Coccidiosis (Williams, 2005), a widespread disease common in countries that produce poultry on a commercial basis (McDougald et al., 1997). Coccidiosis is dependent on the survival of oocytes of the parasite in the bedding or soil which then spreads from bird to bird and to the whole flock (McDougald, 1998). Mucosal damage promotes the formation

and development of C. perfringens, making subclinical coccidiosis a crucial factor in the development of necrotic enteritis in broiler chickens (Baba et al., 1992b; McDougald, 2003). It has also been shown that certain indigenous bacterial species such as Streptococcus fecalis, E. coli, Lactobacillus species, and Bacteroides species may play a role in pathology of cecal coccidiosis (Yegani et al., 2008).

1.4.4.4.2 Antibiotic Growth Promoter (AGP)

Results from studies have been shown that AGP improved animal performance and health status (Coates et al., 1955; Miles et al., 2006), some author removal from poultry diets will likely result in changes to the microbial composition of the intestinal tract, which may in turn have consequences for commercial poultry flocks (Knarreborg et al., 2002; Bedford, 2000). In contrast to this, there is growing evidence on the economic feasibility of including antibiotics in poultry diets. Graham et al (2007) noted a study done into the effects of removing antibiotics that are used for growth promotion in commercial broiler chickens, using large scale empirical data collected by the US broiler industry. The economic analysis showed that using AGP in poultry production can be associated with economic losses to producers (WHO, 2003) and that weight gained as a result of AGP is not enough to compensate for the cost of antibiotics. A study in Denmark found that removing AGP had minor economic effects linked to decreased feed efficiency, there were no changes in mortality or weight gain when AGP was removed, therefore savings in the cost of AGP are justified (WHO, 2003). However, it is suggested that there has been a significant increase in the use of therapeutic antibiotics for food animals in Europe even with the efforts to improve practices in animal

production, due to an increase in animal infections because of the removal of antibiotics (Casewell et al., 2003). In countries that have stopped using AGP, the amount of NE cases in poultry flocks has risen (Immerseel et al., 2004). There is a lot of pressure on the poultry industry to discover feasible alternatives to treat NE associated problems, due the removal of these compounds from poultry diets in Europe and reduction or removal in North America, as well as restrictions and customer preferences (Yegani et al., 2008).

1.4.4.4.3 Polyphenols

With reference to the aforementioned removal of antibiotics as additives has accelerated research into alternative options (Hughes et al. 2005). Collett (2004) believes that a multifactorial approach is required to overcome the challenges to an on-farm situation. Whilst it is accepted that the use of feed additives to manipulate gut function and microbial habitat in animals is an important tool to improve feed efficiency and growth (Hughes et al. 2005), any viable alternative to antibiotics needs to have a beneficial sustainable effect on animal production (e.g., feed efficiency, body weight gain) and health. It should be safe for both animals and humans, application and storage should be easy, and it should have positive economic effects (Collett, 2004).

Plant extracts have been discovered to have antimicrobial effects on certain pathogens (Tepe et al., 2004; Papadopoulou et al., 2005) and phenolic compounds are also recognised as having effects on health due to their antioxidant, anti-carcinogenic, anti-inflammatory, and antimicrobial activities. Selma et al (2009) suggests that these effects could be explained by their bioactive metabolites and to the modulation of the intestinal bacterial population.

Specifically, some phenolic compounds such as resveratrol, hydroxytyrosol, quercetin, and several phenolic acids have been reported to inhibit various pathogenic microorganisms (Aziz et al., 1998).

Available data on the absorption and digestibility of polyphenols suggest relatively low bioavailability of polymeric proanthocyanidins in rats (Donovan et al., 2002) and chickens (Brenes et al., 2008). Selma et al. (2009) also found that extremely high levels of unabsorbed phenolics stay in the gut, this along with the metabolites could be important to the maintenance of the intestinal environment by modulation of the microbiota. There are various phenolic compounds that are classed as potential antibacterial compounds that can restrain pathogenic bacteria in the gut. Evidence of the antimicrobial effect of grape seed extract has been observed in vitro (Rodríguez-Vaquero et al., 2007; Gañán et al., 2009; Hervert-Hernández et al., 2009) and in vivo in rats (Dolara et al., 2005) and chickens (McDougald et al., 2008). Finally, there is enough data to reinforce antimicrobial activity of polyphenols, although there is little evidence on the possible stimulatory role of phenolic compounds (Viveros et al., 2011).

1.5 References

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CHAPTER 2

2 Objectives

There is a wide interest in developing management and feeding strategies to stimulate gut development and health in monogastric animals. The ultimate aim of these strategies is to improve productivity, while minimizing the use of antibiotics and rather expensive feed ingredients: indeed, under practical conditions, animals don't achieve the maximum of their growth performance potential. Large amounts of research have been conducted evaluating the impact of a wide range of feed ingredients and feed additives on various aspects of gut health and development in monogastric animals.

The main objective of this thesis was to improve our knowledge on the properties of new additives as feeding strategy, in order to increase general health in piglets around weaning and poultry, with the aim to substitute antibiotics growth promoters. Four different trials were designed to study different strategies: in the first study proposed, effects of the inclusion of Nucleotides in post-weaning piglet diets on growing performance and intestinal proinflammatory interleukines gene expressionwere investigated; the aim of the second trial was to evaluate the effects of a novel synthetic emulsifier product on growth performance of chickens for fattening and weaned piglets; third study was performed to evaluate influence of a novel synthetic emulsifier product on growth performance, plasma lipid profile and related genes expression of broiler chicks; finally, the fourth study was carried out to determine in vitro antimicrobial activity of a polyphenol-rich olive extract and application on growth performances and meat quality in broiler chickens.

CHAPTER 3

3 Effects of the inclusion of Nucleotides in post-weaning piglet diets on growing performance and intestinal proinflammatory interleukines gene expression

3.1 Abstract

Dietary nucleotides in piglets could help to cope with the stressful phase of weaning by the positive effects on immune response and intestinal health status with a lower incidence of enteric diseases and a consequent increased performance, especially with pathogens presence in the gut. The aim of the study was to evaluate the effects of nucleotides inclusion in the diet of piglets on ileal proinflammatory interleukin gene expression.

Thirty-six weanling piglets (28 d of age, 7.85 ± 0.25 kg L.W.) were used in 28 days study. Piglets were allotted to 2 homogeneous groups (C, T) and fed the basal diet supplemented with nucleotides (UMP 88.05%, GMP 5.51%, AMP 3.82%, CMP 1.94% and IMP 0.68%; 0.8 g/piglet/day in 2.1 ml water solution) or saline. On day 14 both experimental groups were challenged with 1x109 CFU/g E. coli 0149:F4(K88). Growth performance and faecal score were evaluated weekly, while blood samples for immunological serum parameters, Fe and Vitamin B12 serum content were collected on days 0, 13, 18 and 26. Proinflammatory IL1a, IL1b, IL6, IL10, and TNF, TLR2 and TLR4 gene expression in ileal Peyer patches were evaluated at slaughtering after individual tissue sample collection by RT-PCR and β-actin as housekeeping gene.

Growth performances were not affected by dietary treatment, while faecal score was ameliorated in T piglets after one week on trial ($P < 0.05$). Haptoglobin serum content was decreased in treated pigs ($P<0.01$). Dietary treatment did not affect serum Fe content, while vitamin B12 level was higher in nucleotide-fed animals ($P < 0.05$). At slaughtering IL6, IL10, TNF, TLR2 and TLR4 gene expression were decreased in nucleotide-fed pigs ($P < 0.01$).

By these findings, dietary nucleotides supplementation in postweaning piglets can positively affect gut health status, ameliorating inflammatory response and digestibility of nutrients in microbial stress conditions.

3.2 Introduction

The achieving of adequate piglet performance during the post-weaning process is a primary economic target as a crucial phase in the current pig production system. Weaning is the most significant event in the life of pigs as they are abruptly forced to adapt to nutritional, immunological and psychological disruptions (Kim et al., 2012). Early weaning is commercially advantageous, but is generally accepted as a stressful event often associated with a period of underfeeding (Le Dividich and Herpin, 1994), that may cause a strong gut modification and decreased length of intestinal villi. Consequently, reduced digestion and absorption of nutrient can take place causing poor performance and often diarrhoea (Nabuurs, 1991). Innate and adaptive immune system of weaned pigs are yet to be fully developed and specialized, while passive immunity from the sows' secretions are depleted at weaning (King and Pluske, 2003; Gallois et al., 2009). Nevertheless, pigs at weaning remain susceptible to a number of bacterial and viral diseases but the most significant diseases that at least partly associated with the dietary components at weaning are the pathogenic bacteria-originated diseases, which can cause diarrhoea after weaning. These diseases include post-weaning colibacillosis caused by serotypes of enterotoxigenic Escherichia coli (ETEC) (Kim et al., 2012).

The concept of functional feeds represents an emerging new paradigm to develop diets for pigs. Functional feeds extend beyond the satisfying basic nutritional requirements to improve growth and feed utilisation also the general health and stress resistance of the animals. Functional feeds would help to reduce the cost of medication and production and simultaneously improve consumers' perception (Hoffmann, 2007).

Unconventional management, feeding practices, and additives that act as alternatives to antibiotics have been tested (Turner et al., 2001; Stein, 2002; Mateo et al., 2004). The adoption of new systems for pig production involving immune competent, healthy and fast growing animals is fundamental step to adjust the current production system to the legislative system (Superchi et al., 2011).

The dietary inclusion of additives that may act as alternatives to antibiotics have been suggested as a way to breed pigs without using in-feed antibiotics (Turner et al., 2001). Research in human nutrition has demonstrated that the inclusion of nucleotides in parenteral formulas and infant milk formulas improve intestinal health and the development of the immune system in infants. Because of their role in maintaining intestinal health, dietary nucleotides may act as an alternative to antibiotics in the feeding of young animal (Mateo et al., 2004). In this case, a dietary nucleotides supplementation could help piglets to cope this transition phase due to their important role in cell division, cell growth and modulation of the immune system and may help maintain intestinal health and reduce the incidence of enteric disease (Mateo et al., 2004). The major nucleotides in colostrum and milk of sow include adenosine 5'monophosfate (AMP), cytidine 5'monophosphate (CMP), guanosine 5'monophosphate (GMP), inosine 5'monophosphate (IMP) and uridine 5'monophosphate (UMP) (Mateo et al., 2004). At birth, mammals have an immature immune system, which is why they receive a large amount of nucleotides from mother's milk, which help to mature the immune system. This may explain why the milk of mammals is particularly rich in nucleotides (Xavier Córdoba, et al. 2008). Nucleotides in milk may originate from two different sources (i.e., dietary sources or nucleotides synthesized de novo). The decreased concentration of nucleotides in milk during the latter stages of lactation may reflect decreased de novo synthesis because the diet was not changed and the feed intake of the sows was constant during this

period. This decrease in the de novo synthesis may be a response to a reduced need for nucleotides by the nursing piglets. However, the decrease in milk nucleotide concentration could also be a response to an increased utilization of nucleotides within the mammary gland, where they are used as a substrate for the synthesis of DNA (Voet and Voet, 1995). Because the concentration of DNA within the mammary gland increases from d 5 to 21 of lactation (Kim et al., 1999), more nucleotides are needed for DNA synthesis during this period, which might be the reason why the concentration of nucleotides in milk decreased (Mateo et al.2004). It was recently reported that weanling pigs ingest lower quantities of nucleotides when they are fed post-weaning starter diets compared to the intake from sow milk. If these levels could be increased in solid feed offered to pigs, then it might help with the transition at weaning (Mateo et al., 2004). Some authors suggested that dietary supplementation with nucleotides may exert beneficial effects on gastrointestinal, hepatic and immunological functions (Carver and Walker, 1995; Sauer et al., 2011). Additionally, in in vivo studies with monogastrics, e.g., pigs, enounced that the affinity to diarrhoeal infections was reduced (Martinez-Puig et al., 2007; Sauer et al., 2012).

Thus, the objective of the present trial was to investigate the effect of a supplementation of nucleotides in post-weaning piglet on growing performance and ileal proinflammatory interleukin gene expression.

3.3 Materials and methods

3.3.1 Animals, housing, diet

The experiment was conducted according to protocols approved by the University Animal Care and Use Committee. The study was performed at Animal Production Research and Teaching Centre of the Polo Veterinario di

Lodi. Thirty-six piglets (TOPIGS 40 x TOPIGS FOMEVA), males and females, 28 d of age (7.85 ± 0.25 kg) and initially homogeneous for weight were selected for this study. Piglets were allocated into 18 pens (2 piglets/pen), and randomly distributed into two experimental groups (C and T). All animals were identified by ear tags with unique numbers. Piglets were allotted in boxes on plastic slatted floor, located in in the same room, equally disposed and fed manually every day. Pigs were kept at a controlled temperature (28°Cat the beginning and 24°Cat the end of the experiment, with a 1°C decrease every 3 d). Before starting the experimental period, all the animals of the two treatment groups were vaccinated for Porcine Circovirus (PCV) Type 2 and Mycoplasma hyopneumoniae. All piglets were in excellent health and physiological conditions.

All piglets were fed a basal diet formulated to meet or exceed nutrient requirements (NRC, 2012) (Table 1). Feed was offered ad libitum dry diet by the same staff every day, and piglets had free access to drinking water.

Control piglets were oral administrated 2.1 ml of saline solution and Nucleotides animals were oral administered with 0.8g/head/day of nucleotides in 2.1 ml water solution every morning at 8.00 am. Nucleotides composition is shown in table 2.

3.3.2 Challenge

After a 2 weeks adaptation to basal diet all piglets were orally challenged with *E. coli* 0149:F4(K88) $1x10^9$ CFU/g. The ETEC K88 strain O149 was provided by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Reggio Emilia, Italy. The *E. coli* solution was grown in Luria broth (LB) medium containing 1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0. Tryptone and yeast extract were from Oxoid (Basingstoke, England). After overnight incubation at 37°C with shaking, bacteria were diluted 1:100 in fresh LB.

Following incubation, the bacterial cells were harvested by centrifugation at 3,000 × g for 10 min at 4°C, washed in PBS, and re-suspended in PBS. Bacteria grown to mid-log phase (about 0.3 OD600) were used for all experiments. Bacterial concentration was determined by densitometry and confirmed by serial dilution followed by viable plate counts on LB agar.

3.3.3 Measurements

Individual body weight (BW) was recorded weekly from starting trial by electronic scale (Ohaus ES100L, Pine Brook, New Jersey; sensitivity ± 0.02 kg). Feed consumption was measured daily on a pen basis. Faecal Score was recorded once a week on pen basis by subjective four-point scale where 1=firm and 4=watery (Wellock et al., 2007). On day 0, 13, 18 and 26 blood samples were collected from jugular vein into 10ml vacuum tubes with ethylenediaminetetraacetic acid (VT100STK, 0.1ml EDTA) as anticoagulant from the same piglet in each pen sampled for faecal microbial assays, and subsequently analysed for white blood cells (WBC), red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cells distribution width (RDW), platelet (PLT), mean platelet volume (MPV), plateletcrit (PCT), platelet distribution width (PDW). Moreover neutrophil, lymphocyte, monocyte, eosinophil and basophil plasma content were determined on the same samples. On day 28 all experimental piglets of each experimental group were slaughtered and pen dressing percentage calculated. Approximately 10mg of ileal Peyer's patches tissue from 10 representatives piglets of each group was sampled and immediately stored in 1.5mL cryovials with 0.9ml RNAlater solution (Invitrogen,

Life Technologies Ltd, Paisley, UK), and frozen at -20°C. Collected samples from ileal Peyer's patches were subsequently analysed for interleukin 1α (IL1α), interleukin 1β (IL1β), interleukin (IL6), interleukin 10 (IL10), peroxisome proliferator-activated receptor γ (PPARγ), tumor necrosis factor α (TNFα), toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4), and β-actin as housekeeping gene. Total RNA was double extracted with TRIzol Reagent® (Invitrogen, Life Technologies Ltd, Paisley, UK), purified with a commercial kit (Macherey-Nagel, Oensingen, Switzerland), and quantified using a Nanodrop (Thermo Scientific, Waltham, MA, USA). Specific mRNAs were amplified and quantified using the iScriptTM One Step RT-PCR for Probes reagent (Bio-Rad, CA, USA), according to the manufacturer's instructions. RT Q-PCR analysis was performed with CFX384 Real-Time System (Bio-Rad, CA, USA). Thermal protocol was: 50 °C for 10 minutes for reverse transcription and then 95 °C for 10 seconds/60°C 30seconds for 40 cycles. Proper amplification was checked by performing a melting curve of each PCR product at the end of the last cycle of amplification. Primers and probes for real-time qPCR were purchased from Applied Biosystems (Carlsbad, California, USA) except the set for β-actin quantification (forward primer 5'-ACTCGATCATGAAGTGCGAC-3', reverse primer 5'-GTGATCTCCTTCTGCATCCTG-3', Taqman probe 5'-CGTGTTGGCGTAGAGGTCCTTCC-3'), which were designed with IDT software available online, optimized to work in a one-step protocol and were synthesized by Eurofin MWG Operon (Huntsville, AL, USA). The relative expression levels were determined by normalizing the Ct of the indicated mRNA with the Ct of *S. scrofa* β-actin, here used as the housekeeping gene for normalization. Ct values of β-actin were comparable in all samples from different animal groups treated with different diets.

3.4 Statistical analysis

Growing performance, faecal score, haematological parameters, Fe, Vitamin B12, and haptoglobin serum content were analysed by a multivariate ANOVA for repeated measurements of SAS version 9.2 (SAS Institute Inc., Cary, NC, USA.). Statistical analysis included in the model the effect of treatment, time and relative interaction. Gene expression levels and slaughtering performance were analysed by a General Linear Model (GLM). The pen was considered the experimental unit for growth performance, while the piglet represented the experimental unit for blood parameters and gene expression. The least square treatments means and standard errors are presented in tabular form, and the significance was declared at $P \leq 0.05$.

3.5 Results

3.5.1 Growth performance and fecal score

Results on growth performance are presented in table 3. No effect was observed before *E. coli* challenge (day 14). After challenge, control animals showed a higher ADG compared to treated animals during third week (14-21 d) (P= 0.0242). After *E. coli* challenge at day 14 no more significant effect was found, including carcass BW and dressing percentage. Faecal score results are presented in Table 4. No main effect was showed in the model, After 7 days was detected, in T animals, a significant effect of increase of faeces consistency against C animals (P=0.005). No other difference in faecal score was detected during rest of the trial especially after experimental infection with *E. coli* at day 14.

3.5.2 Blood parameters and interleukins gene expression

Results of haemachrome are presented in Table 5. Treated animals showed a significant effect of interaction between treatment and time on monocytes count during all period of the trial (P=0.0137); especially at day 26 treated group showed a significant decrease on monocytes count (P=0.01). Similarly Basophils count showed a significant effect of treatment in the model (P=0.05); at day 26 treated animals significantly reduced value of Basophils value compared to control animals. No other parameters showed difference during before and after challenge periods. Results of haematological parameters are reported in Table 6. Treatment effect resulted in Haptoglobin content in all period of trial (P= 0.0035); Animals administrated with nucleotides showed a significant reduction in haptoglobin content at day 18 and 26 (P=0.02 and P=0.03). Vitamin B12 showed a significant treatment effect, like Haptoglobin, for the total period of trial (P= 0.03). No effect resulted in IgA, IgG and Fe blood content. Results of interleukins gene expression are reported in Table 7. Gene expression of IL1a and IL1b and PPARG did not show any effect of influence of the treatment. Pigs administrated with nucleotides showed high significant effect of reduction of gene expression of IL6, IL10, TNF, TLR2 and TLR4 ($P \leq 0.01$).

3.6 Discussion

The modulatotion effects of nucleotides on the intestinal and immune tissues have been traditionally considered as separate issues. However, nowadays it is well known that both are closely related, not only because a significant proportion of intestinal cells are of immune origin but also because intestinal epithelial cells can produce immunomodulatory molecules, such as cytokines, which regulate the immune response (Walker, 1996).

Nucleotides and their related metabolic products play key roles in many biological processes. Nucleotides can be synthesized endogenously and thus are not considered essential nutrients, but they can be considered as "conditionally essential" for some beneficial effects (Carver, 1999). Moreover, these nutrients may become essential when the endogenous supply is insufficient for normal function, even though their absence from the diet does not lead to a classic clinical deficiency syndrome (Superchi et al., 2011). Most dietary nucleotides are rapidly metabolized and excreted, however some are incorporated into tissues, particularly at younger ages and during fasting condition as in the few days starting the post-weaning phase. Under limited nucleotides intake or rapid growth or certain disease conditions, dietary nucleotides may spare the cost of *de novo* nucleotides synthesis and optimize the function of rapidly dividing tissues such as those of the gastrointestinal and immune systems (Carver, 1999). For these reasons dietary nucleotides supplementation could help immune system of post-weaning piglets maintaining the intestinal health and reducing the incidence of enteric disease (Mateo et al., 2004).

One of most important function of nucleotides is the development of an articulate intestinal functional role during a stress period in which infectious diseases may frequently occur together with low rates of growth (Domeneghini et al., 2004).

In the present study, the administration of dietary nucleotide in post-weaning piglets did not affect growth performance of treated animals according to Martinez-Puig et al. (2007) and Adrés-Elias et al. (2007). Obtained results on BW are in contrast with Superchi et al. (2011), but the duration of treatment seems to be an important factor affecting the results on performance. In fact, nucleotides effectiveness has been proved to be depending both on dosage applied and duration of treatment (Moore et al., 2011): longer periods of administration could exacerbate more positive effects on growth rates than a short-time supplementation (Mateo, 2005).

Anyway, the most interesting result obtained from this trial can be associated to the improvement of immune response of treated animals than control, especially when challenged with *E. coli*. Decreased serum level of such an acute phase protein as haptoglobin is an index of lower severity of inflammation processes (Petersen et al., 2003; Pineiro et al., 2007).

As a result, haptoglobin reduces oxidative damage associated with hemolysis by binding free hemoglobin released by damaged erythrocytes, and jointly with hemopexin and transferrin helps to improve the harmful effects of free iron and to restrict the availability of free iron to invading bacteria (Cray et al., 2009). Our finding of lower Hp serum content in nucleotide-fed animals when challenged than respective control groups confirm findings by others authors (Eurell et al., 1992; Petersen et al., 2003; Pineiro et al., 2007) who evidenced as the use of dietary nucleotides has positive effects on serum acute phase proteins content, especially haptoglobin.

Serum level of Vitamin B12 was significantly higher in T group of piglets which indicate that normal absorption occurred in ileum due to nucleotide supplementation may increase VH: LPD in the ileum, which may be a result of improved intestinal health (Moore et al., 2009).

In addition, the different serum level of Vitamin B12 between Treated and control groups can work as an index of good nutrient absorption: effect of treatment was found in the model. Although no effect was showed in singular measure but all numerical values of Treated group resulted higher than control group. \It means that nutrient absorption can be better in treated animals especially after challenge where values detected in Control group is similar to Treated animals after challenge.

If some serum parameters can show positive results on inflammatory response at circulating blood levels, the most interesting results obtained in the present trial are relative to the gene expression levels on proinflammatory ileal cytokines.

Many researchers have evaluated proinflammatory cytokines in the past years, but considering the concentration in circulating peripheral blood (Zelickova et al., 2008; Borghetti et al., 2011; Superchi et al., 2011). Now no literature is available on interleukins gene expression on intestinal tissue, especially on ileal Peyer's patches as one of most inductive tissue in the gut (Pié et al., 2004).

IL-10 plays a central role in the maintenance of normal intestinal immune homeostasis, as demonstrated by the intestinal inflammation observed in IL-10 knockout mice (Dvoraket al., 2007). On the other hand, TLR4 recognises lipopolysaccharide (LPS) a constituent of the cell wall of Gram-negative bacteria, while TLR2 reacts with a wider spectrum of bacterial products such as lipoproteins, peptidoglycans and lipoteichoic acid which can be found in both Gram-positive and Gram-negative bacteria (Hakansson et al., 2011).

In this study, a significantly decrease in the expression level of IL6, IL 10, TNF alpha, TLR2 and TLR4 were found in T group compared C group due to *E. coli* challenge. However, nucleotides diet in T group of piglets help to an increase in growth rate of the non-pathogenic bacteria and increased production of their metabolites may have provided an environment that restricted the proliferation

of the pathogenic strains (Mateo et al., 2004) resulting decreased level of ileal proinflammatory cytokines expression occurred.

Dietary nucleotides have been shown to enhance the production and the gene expression of IL-6 and IL-8 by foetal small intestinal explants when challenged with IL-1 beta, the response being nucleotide concentration dependent. (Gill, 2002).

Decreased proinflammatory TLR2 and 4 gene expression levels in Treated animals due to challenged with *E. coli* than the control group, confirms a lower inflammatory response in nucleotide-fed group that should reserve more energy for nutrients absorption by shifting the amount of energy necessary to contrast higher inflammation to metabolic processes of digestion (Superchi et al., 2011). This mechanism seems to work even better when a pathogen contamination is present, thus the administration of dietary nucleotides can be successful in practical farm condition when pathogen microbial presence could be high.

The extent of the damaged mucosa was greater in controls and these animals were more susceptible to the lethal effects of the endotoxin lipopolysaccharide from *E. coli*, which suggests that dietary nucleotides may block the bacterial translocation by preventing endotoxin-induced mucosal damage (Kulkarni et al, 1989; Carver & Walker, 1995; Gil, 2001; Gil, 2002).

3.7 Conclusions

Dietary nucleotides supplementation in post-weaning piglets associated with stress inducted with challenge of *E. coli*, can positively affect gut health status and enhancing inflammatory response. It would be interesting to investigate observed properties in field conditions characterized by high pathogenic pressure.

3.8 References

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3.9 Tables

Table 1: Ingredient and composition of the diet administered to animals (as fed basis)

Components	**(% as fed)**
Barley flaked	21.35
Micronized wheat	18.20
Micronized barley	16.00
Soybean protein concentrate	9.00
Soybean meal (48%CP)	8.20
Flaked Corn	6.00
Micronized Corn meal	5.00
Lactose	5.00
Minvit premix **	4.00
Soybean oil	3.60
Potato protein concentrate	2.00
Calcium carbonate	0.90
Monocalcium Phosphate	0.40
Sodium cloride	0.30
Flavor	0.05
Total	**100**

Composition[***]	(% as fed)
CP	18.56
CF	3.17
Ca	0.69
P	0.62
Ca/P ratio	1:1
ME (Kcal/kg)	3,370.00

[**]Provided per kg of complete diet: vitamin A, 10,000 IU; vitamin D3, 1,000 IU; vitamin E, 50 mg; vitamin B1 1.0 mg; vitamin B2 3.0 mg; vitamin B6 3.0 mg; vitamin B12, 0.03 mg; riboflavin, 9 mg; pantothenic acid, 14 mg; nicotinic acid, 15 mg; biotin, 0.06 mg; vitamin PP, 0.35 mg; folic acid, 0.97 mg; vitamin K3, 3 mg; choline, 300 mg; Fe, 100 mg; Cu, 20 mg; Co, 0.75 mg; Zn, 100 mg; Mn, 10 mg; I, 0.85 mg; Se, 0.4 mg; ethoxyquin, 150 mg.

[***]CP: Crude Protein; CF: Crude Fiber; Ca: Calcium; P: Phosphorus; ME: Metabolized Energy

Table 2: Composition of nucleotide compound orally administered to post-weaning piglets

Nucleotides*	**%**
UMP	88.05
IMP	0.68
GMP	5.51
CMP	1.94
AMP	3.82

*UMP: uridine monophosphate IMP: inosine monophosphate; GMP: guanosine monophosphate; CMP: cytidine monophosphate; AMP: adenosine monophosphate

Table 3 Effect of dietary nucleotides on growth performance of piglets

Item	Time	Treatment		SEM	*P*-value		
		C	T		Treatment	Time	Treatment*time
WEIGHT							
kg	0 d	7.86	7.83	0.35	0.707	<.0001	0.953
	7 d	8.57	8.57	0.35			
	14 d	11.03	11.47	0.35			
	21 d	14.68	14.61	0.35			
	28 d	18.61	18.69	0.35			
DMI							
kg/box/week	0-7 d	3.16	3.58	0.66	0.544	<.0001	0.805
	7-14 d	7.69	8.52	0.66			
	14-21 d	11.10	10.65	0.66			
	21-28 d	12.41	12.77	0.66			
	0-28 d	8.59	8.88	0.33			
GAIN							
Kg/box/week	0-7 d	0.85	0.74	0.24	0.577	<.0001	0.076
	7-14 d	2.41	2.90	0.24			
	14-21	3.92[a]	3.14[b]	0.24			
	21-28 d	4.07	4.08	0.24			
	0-28 d	2.81	2.71	0.12			
FCR							
	0-7 d	2.44	2.61	0.18	0.986	<.0001	0.595
	7-14 d	1.77	1.52	0.18			
	14-21 d	1.55	1.72	0.18			
	21-28 d	1.64	1.56	0.18			
	0-28 d	1.85	1.85	0.09			
CARCASS WEIGHT							
kg		14.73	14.72	0.69	0.998		
DRESSING %							
		79.41	78.82	0.66	0.377		

[A,B] $P < 0.01$; [a,b] $P < 0.05$

Table 4 Faecal score of C (control) and T (treated) animals

Item	Time	Treatment		SEM	*P*-value		
		C	T		Treatment	Time	Treatment*time
FAECAL SCORE							
	7 d	3.55[A]	3.22[B]	0.084	0.069	<.0001	0.097
	14 d	3.17	3.06	0.084			
	18 d	3.28	3.39	0.084			
	21 d	3.00	3.00	0.084			
	26 d	3.11	2.94	0.084			

[A,B] $P < 0.01$; [a,b] $P < 0.05$

Table 5: Effect of administration of nucleotides in the diet of post weaning piglets on blood cells counts.

Item	Time	Treatment		SEM	P-value		
		C	T		Treatment	Time	Treatment*time
WBC							
(10^3/µl)	0 d	1093.78	1355.00	178.21	0.8687	0.0005	0.6138
	13 d	1724.78	1815.56	178.21			
	18 d	1308.00	1225.44	178.21			
	26 d	1961.44	1776.67	178.21			
RBC							
(10^6/µl)	0 d	632.56	623.67	16.13	0.2047	0.0396	0.9845
	13 d	639.44	626.67	16.13			
	18 d	614.56	595.00	16.13			
	26 d	600.67	581.56	16.13			
HGB							
(g/dl)	0 d	111.56	108.22	2.73	0.1621	<.0001	0.9964
	13 d	103.22	101.11	2.73			
	18 d	97.00	93.89	2.73			
	26 d	91.78	89.00	2.73			
HTC							
(%)	0 d	369.44	360.56	8.54	0.0902	<.0001	0.9677
	13 d	337.56	326.78	8.54			
	18 d	319.11	303.22	8.54			
	26 d	306.00	298.00	8.54			
MCV							
(%)	0 d	587.78	579.22	16.56	0.6399	0.0002	0.9865
	13 d	529.11	523.44	16.56			
	18 d	521.33	511.67	16.56			
	26 d	512.33	513.89	16.56			
MCH							
(%)	0 d	177.33	173.78	5.01	0.799	0.0005	0.9795
	13 d	161.89	162.00	5.01			
	18 d	158.33	158.33	5.01			
	26 d	153.78	153.56	5.01			
MCHC							
(g/L)	0 d	302.00	300.44	3.50	0.5311	0.0554	0.6447
	13 d	306.00	309.44	3.50			
	18 d	303.89	309.78	3.50			
	26 d	300.22	298.78	3.50			

(To be continued)

PLT							
(10^3/μl)	0 d	352.44	331.78	57.64	0.5373	0.0706	0.8147
	13 d	228.67	255.00	57.64			
	18 d	315.56	403.33	57.64			
	26 d	233.22	242.56	57.64			
RDWSD							
(%)	0 d	461.56	441.78	24.03	0.1461	0.1179	0.852
	13 d	453.75	403.56	24.03			
	18 d	426.11	399.56	24.03			
	26 d	398.11	390.00	24.03			
RDWCV							
(%)	0 d	232.89	228.11	12.08	0.2651	0.7896	0.9071
	13 d	250.89	230.22	12.08			
	18 d	240.22	232.33	12.08			
	26 d	233.00	226.89	12.08			
NEUT							
(%)	0 d	822.22	840.11	19.23	0.6053	<.0001	0.4172
	13 d	819.33	829.78	19.23			
	18 d	832.89	802.11	19.23			
	26 d	697.11	728.22	19.23			
LYMPH							
(%)	0 d	123.67	113.56	16.43	0.8544	<.0001	0.3626
	13 d	137.78	131.22	16.43			
	18 d	116.22	148.56	16.43			
	26 d	230.00	205.67	16.43			
MONO							
(%)	0 d	14.89	11.33	2.18	0.4285	<.0001	0.0137
	13 d	13.00	13.67	2.18			
	18 d	17.78^{b}	23.89^{a}	2.18			
	26 d	30.00^{A}	21.78^{B}	2.18			
EO							
(%)	0 d	31.11	27.78	6.66	0.6475	0.1662	0.7801
	13 d	22.56	19.33	6.66			
	18 d	23.89	16.00	6.66			
	26 d	29.78	35.44	6.66			
BASO							
(%)	0 d	8.11	7.22	1.04	0.0500	0.0007	0.1866
	13 d	7.33	6.00	1.04			
	18 d	9.22	9.44	1.04			
	26 d	13.11^{A}	8.89^{B}	1.04			

WBC: white blood cells; RBC: red blood cells; HGB: haemoglobin; HCT haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, RDW: red cells distribution width; PLT: platelet; MPV: mean platelet volume; NEUT: neutrophil; LYMPH: lymphocytes; MONO: monocytes; EO: eosinophils; BASO: basophils. ^{A,B} $P < 0.01$; ^{a,b} $P < 0.05$

Table 6: Effect of administration of dietary nucleotides on haematological parameters

Item	Time	Treatment		SEM	P-value		
		C	T		Treatment	Time	Treatment*time
HB							
mg/mL	0 d	0.594	0.538	0.0321	0.0035	0.0053	0.6890
	13 d	0.569	0.524	0.0321			
	18 d	0.5611[a]	0.4533[b]	0.0321			
	26 d	0.5044[a]	0.4022[b]	0.0321			
IgA							
mg/mL	0 d	0.449	0.460	0.0201	0.3820	<.0001	0.8355
	13 d	0.381	0.411	0.0201			
	18 d	0.321	0.338	0.0201			
	26 d	0.258	0.251	0.0201			
IgG							
mg/mL	0 d	4.961	5.183	0.1258	0.0641	0.1144	0.8923
	13 d	4.838	5.019	0.1258			
	18 d	4.868	4.930	0.1258			
	26 d	4.638	4.880	0.1258			
Fe							
ng/dL	0 d	292.720	300.960	6.0567	0.1399	<.0001	0.9520
	13 d	267.270	269.600	6.0567			
	18 d	229.290	237.260	6.0567			
	26 d	201.220	209.300	6.0567			
B12							
ng/dL	0 d	236.440	246.890	8.0470	0.0251	0.0387	0.9394
	13 d	230.220	246.560	8.0470			
	18 d	224.780	235.440	8.0470			
	26 d	210.110	228.890	8.0470			

[A,B] $P < 0.01$; [a,b] $P < 0.05$

Table 7: Effect of administration of dietary nucleotides on interleukins gene expression

Item	Time	Treatment		SEM	P-value
		C	T		Treatment
IL1a					
*(RAU)	28 d	0.8412	0.7735	0.1038	0.5166
IL1b					
*(RAU)	28 d	0.8948	0.6657	0.1267	0.0759
IL6					
*(RAU)	28 d	1.2499^{A}	1.0314^{B}	0.0789	0.0075
IL 10					
*(RAU)	28 d	1.0033^{A}	0.7931^{B}	0.0752	0.0070
TNF					
*(RAU)	28 d	1.1637^{A}	0.8953^{B}	0.0754	0.0007
TLR2					
*(RAU)	28 d	1.1780^{A}	0.9180^{B}	0.0810	0.0022
TLR4					
*(RAU)	28 d	1.1399^{A}	0.8768^{B}	0.0748	0.0009
PPARG					
*(RAU)	28 d	10.2388	3.1022	4.9185	0.1524

*RAU: Relative Arbitrary Unit ^{A,B} $P < 0.01$; ^{a,b} $P < 0.05$

CHAPTER 4

4 In vitro antimicrobial activity of a polyphenol-rich olive extract and application on growth performances and meat quality in broiler chickens

4.1 Abstract

The aim of the present study was to investigate the antimicrobial potential of a polyphenol-rich olive extract in vitro and its effects on growth performance, caecum microbial population, slaughtering performance and meat quality. Antimicrobial in vitro trials were performed on test cultures (E. coli ATCC 25922, L. monocytogenes ATCC 7644, S. Enteritidis ATCC 12592 and S. aureus ATCC 6833). Growth was assessed every 12 h for 48 h as optical density of both cultures added with different concentrations of the product (0.1 %, 0.5 %, 1 %, and 5 %) incubated at 20°C; an evident inhibition was observed with the increasing of the product concentration and a total inhibition was observed with the highest concentration.

The in vivo trial considered a total of 720 female ROSS308 broiler chicks, one day-old. Chicks were allocated into 4 dietary treatments: basal diet (BD), CTR; BD supplemented with vitamin E (T1, 200 I.U./kg) or with Polyphenols olive extract (T2, 1g/kg and T3, 5 g/kg respectively). Each group consisted of 9 pens (replicates) with 20 birds per pen. Growth performances were determined on days 0, 10, 20 and 35. On day 35, two birds from each replicate were chosen on pen weight basis and slaughtered. From one bird, cecum content was collected for microbial analyses and measured dressing and breast muscle percentages; from the other bird breast was collected for the measurements of pH, TBARS, and colour at day 0 and after 9 days, mimicking the commercial preservation after slaughter.

Polyphenols olive extract supplementation tended to reduce FCR during the last phase ($P=0.09$) and overall period ($P=0.07$). No effect was observed in dressing and breast muscle percentage. No difference was observed in cecum microbial population and gut health indexes. TBARS showed a statistical tendency ($P=0.075$), T1 resulted with lower oxidation if compared to all other groups ($P<0.05$); pH value and colour of breast, skin and thigh, expressed with L*, a*

and b*, did not show any difference. In conclusion, polyphenols clearly showed antimicrobial effect when in vitro tested. However, these results were not confirmed from dietary treatment. More investigations are needed to explain the potential reduction of FCR at the end of in vivo trial.

4.2 Introduction

An important field of livestock research is the improvement of the quality and safety of meat. It is well recognized that pathogens, such as *Campylobacter* spp. and *Salmonella* spp. can be transmitted along the food chain and can be the source of human illnesses (Gaggia et al., 2010). The increasing antimicrobial resistance of pathogens isolated from humans and animals combined with the ban of the use of antibiotics as feed additives occurred and led to investigations of alternative options for more efficient antimicrobials in animal production (Viveros et al., 2011). In the past, antibiotics have been included in animal feed at sub-therapeutic levels, acting as growth promoters (Antibiotic Growth Promoters, AGPs) (Dibner and Richards, 2005). The removal of antibiotic growth promoters from feed is expected to result in reducing feed efficiency and increasing incidence of intestinal disorders as a result of proliferation of gut pathogens (Hughes et al., 2005). As an alternative, several researchers have reported the antimicrobial effects of various plant extracts against certain pathogens (Tepe et al., 2004; Papadopoulou et al., 2005; Viveros et al., 2011). In recent years, naturally occurring antimicrobial and antioxidant compounds derived from plant sources have been preferably employed in meat productions because of their potential health benefits and safety compared with synthetic preservatives (de Ciriano et al., 2009; Sebranek al., 2005). The potential health risks shown to be associated with synthetic antioxidants have prompted strict regulations for their use in foods and consequently, interest in the development

and use of natural antimicrobial agents and antioxidants derived from plant sources as safe alternatives has increased markedly (Hayes et al., 2010). Olives (*Olea europaea L.*) and olive oil contains polyphenols such as oleuropein, hydroxy-tyrosol and tyrosol (Briante et al., 2002), rutin (Boitia et al., 2001), quercetin (Obied et al., 2007) as well as caffeic (Papadopoulos & Boskou, 1991), vanillic and o- and p-coumaric acids (Brenes et al., 1999) possess excellent antioxidant properties (Dejong & Lanari, 2009). Olive extract is a phenolic compound and is well known to have antioxidative, antimicrobial, antiviral and anti-inflammatory properties (Bouaziz et al., 2008; Khayyal et al., 2002; Micol et al., 2005; Visioli & Galli, 1994; Hayes et al., 2010). Hydroxytyrosol [(3,4-dihydroxyphenyl)ethanol] is one of the major natural phenolic compounds present in olive fruits, virgin olive oil, table olives, and waste streams generated during olive processing (Amiot et al., 1986; Mulinacci et al., 2001). This compound has shown antimicrobial, hypoglycemic, hypolipidemic, and hypocholesterol properties of particular interest with regard to food and human health (Ruiz-Barba et al., 1993; Aruoma et al., 1998; Romero et al., 2002). It has been largely demonstrated that phenolic compounds have antimicrobial properties (Pereira et al., 2006; Proestos et al., 2005; Rauha et al., 2000; Zhu at al., 2004; Puupponen-Pimiä et al., 2001). It is also assured that many more benefits can be obtained from a full extract that the individual and isolated component; however, the properties of the individual single bioactive component can be changed in the presence of other compounds in the totality of the extract (Borchers et al., 2004). According to Liu (2003) additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their strong bioactive properties and the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods. This explains why no single antimicrobial can replace the combination of natural phytochemicals to achieve the health benefits. In

previous studies, olive leaf aqueous extracts were screened for their antimicrobial activity against gram positive and gram negative bacteria such as *B. cereus*, *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*. Aside from concerns with food quality degradation, these microorganisms may be causal agents of intestinal infections in humans (Pereira et al., 2007). The flavonoid polyphenols in olive are natural antioxidants that have a host of health beneficial effects (Visioli & Galli 1994). Hydroxytyrosol and tyrosol are some of the many phenol compounds in olives that contribute to bitter taste, astringency, and resistance to oxidation (McDonald et al., 2001; Visioli et al., 1998; Singh et al., 2008). There is a growing interest in the use of natural antioxidants as bioactive components in food, and such foods have been termed "functional foods" (Hertog et al., 1993). Due to their ability to scavenge reactive oxygen species (ROS), antioxidants are capable of inhibiting the process of low-density lipoprotein (LDL) cholesterol oxidation subsequently decreasing the risk of cardiovascular diseases (Diaz et al., 1997). Although oxidation of LDL can be prevented by the addition of synthetic antioxidants, greater attention is now focused on natural antioxidants because of their better safety compared to that of synthetic compounds (Amro et al., 2002; Singh et al., 2008). Lipid oxidation plays an important role in the development of undesirable flavours commonly known as "warmed-over-flavour" (WOF) and in the formation of toxic carcinogenic compounds in cooked meat products. For many years, meat processors used synthetic antioxidants like butyl hydroxyanisol (BHA) or butyl hydroxytoluene (BHT) to prevent, or reduce, flavour deterioration. However, concerns about their safety and consumer's preference for more natural foods has resulted in a high demand for "natural" additives, that can extend the shelf life of both processed and unprocessed meat products (Dejong & Lanari, 2009). Several publications concluded that the remarkable olive oil resistance to oxidation was closely linked to its total polyphenol content (Franconi et al., 2006; Hrncirik & Fritsche, 2005; Matos et al., 2007; Dejong &

Lanari, 2009). Microbial growth, lipid oxidation and colour are key factors for the determination of shelf-life and consequently for consumer acceptance of fresh meat (Zhao et al., 1994; Hayes et al., 2010). Lipid oxidation is one of the main factors limiting the quality and acceptability of lipid-containing foods as it affects the sensory quality, due to off-flavour and off-odour development, and the production of potentially toxic compounds (Morrisey et al., 1994). In addition, colour changes are also an important factor influencing the quality and acceptability of meat and meat products. Colour tends to be used as an indicator of perceived quality and freshness of meat. It is regarded as one of the first limiting factors in determining the shelf-life (Smith et al., 2000) and is also an important characteristic perceived by consumer during the exposition of meat. Carpenter et al. (2001) and Hayes et al (2010) confirmed that there is a close link between colour preference and the decision to purchase. The use of natural preservatives to increase the shelf-life of meat products is a promising technology since many plant-derived substances have antioxidant and antimicrobial properties (Hayes et al., 2010). Aim of the present study was to investigate the antimicrobial potential of a polyphenol-rich olive extract in vitro and evaluate its effects on growth performance, caecum microbial population, slaughtering performance and meat quality of broilers chickens.

4.3 Material and methods

Two experiments, in vitro and in vivo on poultry, were carried with a product containing polyphenols-rich olive extract (Ethifenol, Ethivet s.r.l). The product consisted in a water-soluble olive natural extract of *Olea europaea* in powder form. Polyphenols were expressed as GAE – Gallic Acid Equivalent > 4 %; Hydroxithirosol (HPLC - PDA) > 2,5 %) and were extracted with eco

technologies using water as the only extracting solvent. Polyphenols content was 250 mg/g of product.

4.3.1 In vitro trial

The in vitro was carried out considered test cultures (*E. coli* ATCC 25922, *L. monocytogenes* ATCC 7644, *S. Enteritidis* ATCC 12592 and *S. aureus* ATCC 6833) kept at -30°C in microbank containing 20% glycerol. Working cultures were prepared by transferring a single colony into Tryptone Soy Broth, TSB (Oxoid Ltd.) and incubated 37°C for 24 h. Then 100 µl were transferred to 10 ml TSB tubes before the experiment and incubated for 24 h at 37°C. The product containing polyphenols olive extract, was dissolved at a concentration of 1, 5, 10 and 50 % in distilled water and sterilized by filtration through 0.20 µm Millipore filters. Blank tubes were also prepared inoculating the microorganisms in broth. An aliquot of 1 ml of each of the solutions was then added to 9 mL of TSB tubes reaching final concentrations of the product of 0.1, 0.5, 1 and 5 % respectively. The cultures were collected in exponential growth phase, defined as a change of absorbance of 0.05 at 540 nM. The bacterial concentration was assessed before the inoculation of the tubes by microscope examination (phase contrast, 1000x). When necessary precultures were diluted in physiological water (0.85% NaCl) before inoculation in order to reach the starting concentration of 3 Log ufc/ml; the inoculum volume was defined at 50 µl. The optical density (OD) of each inoculated tube was recorded before the incubation (540 wavelength, Jenway 6320) and every 12 h for 48 h in order to show any difference in growth ability and relative rate. The eventual bacterial growth was evidenced as an increase in optical density of the broths. For each product concentration level the test was performed in duplicate for each microorganism for a total number of 200 measurements.

4.3.2 In vivo experiment

The in vivo experimental protocol was reviewed and approved by the Animal Care and Use Committee of the University of Milan. The experiment was performed at the facility for Animal Production Research and Teaching Centre of the Polo Veterinario, Università degli Studi di Milano (Lodi, Italy).

Seven-hundred and twenty female ROSS 308 chickens for fattening (45.02 ± 0.74 g L.W.) were used from birth to slaughtering 35 days. Chicks obtained from a local hatchery at 1 day of age and allocated to 36 pens (replicates) with 20 birds in each pen, measuring 1.25 × 2.00 m. All chickens were vaccinated against Marek's disease, Newcastle disease and infectious bronchitis via coarse spraying at hatching. The birds were housed in an environmentally controlled room in separate floor pens with white wood shavings as bedding, under a photoperiod of 24 h of light from day 0 to day 7 and 23 h light: 1 h dark from day 7 to day 35. Room temperature was maintained at 35°C from day 1 to day 3 and was then decreased by 2.5°C/week, to a final temperature of 24°C at day 35. Feed and water were provided ad libitum consumption. The animals were checked twice daily and any dead animal was removed, weighed and recorded.

The 36 pens were randomly assigned to 4 treatments, consisting of a non-supplemented basal diet (CTR), basal diet supplemented with 200 I.U./kg vitamin E (T1), basal diet supplemented with 1 g/kg Phenofeed (T2), basal diet supplemented with 5 g/kg Phenofeed (T3). Diets (Table 1) consisted in a 3-phase feeding program and were formulated to meet or exceed the nutrient requirements defined by the genetic producers company (Aviagen, Ross 308, nutrition specification, 2014). Feed were provided in crumble form during the starter and grower periods, while during the finisher period was as pellet. The

product was mixed with other ingredients before the pelleting feed process. Added fat consisted of only vegetable oils (soybean oil).

All birds were individually weighted on day 0, 10, 20 and 35 of age. The average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) values were calculated for each pen. On day 35, all chickens were slaughtered. Following a 12-h overnight fast, chickens were stunned in water bath (125 Hz AC, 80 mA/birds, 5 s) before killing via exsanguination. The dressing percentage was calculated by dividing the eviscerated weight by the live weight. Breast muscle was removed and weighted, and the breast muscle yield was calculated as the percentage of eviscerated weight.

Al slaughtering, 2 birds per replicate were chosen on the basis of average pen weight exhibiting the BW nearest to the average for the pen were chosen and marked. Intact intestinal tract was removed from one bird per replicate (36 samples, 9 per treatment) as explained in the previous section, and samples placed in sterile labelled plastic bags and kept refrigerated during the transport to the laboratory. Microbiological analyses were carried out on the same day. From the second bird (9 per treatment), physical and chemical analysis were carried out on breast thigh meat and skin.

4.3.3 Microbiological analysis

From each sample, the cecum was isolated, and, after an external disinfection, the content was aseptically collected and used for the microbiological analyses. One gram of each sample was diluted in a tube with 9 mL of saline solution (NaCl 0.85%, tryptone 0.1%) and homogenized by a Vortex for 40 s (MRCLab, London, UK). Serial 10-fold dilutions were spread by sterile spatula onto the following media: MRS agar incubated in anaerobic jars (Anaerojar, Oxoid, Basingstoke, UK) with Anaerogen kit (Oxoid) at 37°C for 48 h (Oxoid) for the

enumeration of Lactobacilli, on APT agar (Biolife, Milano, IT) incubated at 30°C for 48 h in anaerobic conditions for the enumeration of Lactic Acid Bacteria, on TBX agar (Oxoid) for the enumeration of *Escherichia coli* (ISO 16649-2), on VRB and VRBG agar (Oxoid) for the enumeration of Coliforms (ISO 4832) and *Enterobacteriaceae* (ISO 21528-2) respectively, on Sabouraud agar (Scharlab, Barcelona, ES) for the research of Yeasts and Moulds (ISO 21527-2), on Slanetz-Bartley agar incubated at 37°C for 48 h in order to count *Enterococci,* and on mCCDA (Oxoid) in order to count *Campylobacter* spp, following the ISO 10272-2 method. The second one gram share of caecal content was diluted 1:10 with Buffered peptone water (Oxoid) and incubated at 37°C for the detection of *Salmonella* spp. according with (ISO 6579) method. Finally, another 1 g sample was transferred a tube with 9 ml of Bolton broth, for the detection of thermophilic *Campylobacter* spp., following the ISO 10272-1 method. As suggested by the method, the inoculation mCCDA (Oxoid) was combined with another method based on a different principle, to improve the sensitivity of the analysis. In this case, the method described by Steele and McDermott (1984), with some modifications, was applied. An aliquot of 0.3 ml of the enrichment broth was put onto 47 mm diameter, 0.45 µm pore size cellulose membrane filters (Sigma Aldrich Italy, Milan, I) laid on the surface of non-selective blood agar plates (Columbia Agar base added with 5% of defibrinated sheep blood, Oxoid). The membranes were left for 45 min and then removed, taking care to avoid the spilling of the broth; the filtered inoculum was spread on the surface by a sterile 10 µl loop. The plates were then incubated at 42°C for 48 h. Typical colonies were isolated by subculturing on Columbia-blood agar and submitted to further confirmation steps.

From the obtained counts, *Lactobacilli*/*E. coli, Lactobacilli*/*Enterobacteriaceae, Lactobacilli*/*Coliforms* and LAB/*E. coli*, LAB/*Enterobacteriaceae* and LAB/*Coliforms*

ratios were calculated as the log difference between the two parameters (Abu Tarboush, 1996).

4.3.4 Meat, skin and fat colour

The surface colour of the meat, skin and fat (breast thigh meat and skin) was assessed 24 h after slaughtering on six randomly chosen spots of each sample surface using a Minolta CR-200 Chromameter (Minolta, Osaka, J). L* (lightness), a* ("red" index) and b* ("yellow" index) parameters were determined. Chroma was calculated as a2+b2, the hue angle (h) was calculated as h = arctan (b*⁄a*), where h = 0 for red hue and h = 90 for yellowish hue. Total colour differences (ΔE*) between treated and control samples were calculated as: $\sqrt{(L1^*-L2^*)^2 + (a1^*-a2^*)^2 + (b1^*-b2^*)^2}$. A ΔE* more than 2.3 means a variation hardly perceptible to the human eye, while ΔE* more than 3.0 a variation well perceptible to the human eye.

4.3.5 Physical and chemical analyses

Muscle pH was measured by a pH meter: three independent measurements were performed on each of the 36 subjects considering breast muscle 24 hour after slaughter and after 9 days of storage: means were calculated for each time.
All meat samples were used for measurement values of TBARS by the extraction method as described by Salih et al. (1987). For extraction, ground meat (5 g) was first mixed with 25 mL of a 20% aqueous solution of trichloroacetic acid (TCA) and 20 mL distilled water. Then, the mixture was macerated with a glass rod and allowed to stand for 1 h at room temperature at 25°C. Next, the mixture was centrifuged at 2000 rpm for 10 min, and the filtrate was diluted with distilled

water to 50 mL. Afterward, 5 mL of the new filtrate was taken to mix with 5 mL 0.02 M aqueous solution of 2-thiobarbituric acid (TBA) in a stoppered test tube, kept at 95 °C for 20 min in a water-bath, and subsequently cooled for 5 min in cold water at 0 °C. Absorbance was measured at 532 nm by the spectrophotometer against a distilled water blank. In order to achieve accurate measurement, each filtrate was measured two times, and their average value was taken as the final reference value. The TBARS value was expressed as mg/100 g chicken meat according to the following formula: TBARS value (mg/100 g) = A532*7.8.

4.4 Statistical analysis

Growth performance were analysed by a multivariate ANOVA for repeated measurements of SAS version 9.2 (SAS Institute Inc., Cary, NC, USA.). Statistical analysis included in the model the effect of treatment, time and relative interaction. Microbiological analysis, slaughtering performance and meat quality were analysed by a General Linear Model (GLM). Pen was considered the experimental unit for growth performance, while the broiler represented the experimental unit for microbiological parameters and meat quality. The least square treatments means and standard errors are presented in tabular form, and the significance was declared at $P \leq 0.05$

4.5 Results and discussion

Microbial activity is one of the primary mode of spoilage of many foods and is often responsible for the loss of quality and safety. Concern over pathogenic and spoilage microorganisms in foods is increasing due to the increase in outbreaks

of food borne diseases (Tauxe, 1997). Currently there is a growing interest in the use of natural antibacterial compounds, like plant extracts of herbs and spices for the preservation of foods, as these possess a characteristic flavour and sometimes show antioxidant activity as well as antimicrobial activity (Smid & Gorris, 1999; Jayaprakasha et al., 2003).

According to literature, synergistic effects of polyphenols, responsible for their potent bioactive properties and the health benefit, are attributed to the complex mixture of phytochemicals present in whole foods. In addition, to support this sentence, individual component can change its properties in the presence of other compounds present in the extracts (Liu, 2003; Borchers et al., 2004; Pereira et al., 2007).

In *in vitro* study the potential ability of a polyphenol-rich olive extract against different pathogenic bacteria was evaluated. The results obtained from *in vitro* trial are reported in table 2. All the microorganisms showed a rapid growth in the blank samples in 48 h at 20°C, reaching in almost all the cases the highest absorbance recorded. With the increase of the concentration of the product in the broths, an evident inhibition was recorded with a decreasing optical density in all the times of measurements. The highest effect was recorded when the product was added at the highest concentration (5%) while no increasing optical density was recorded in any of the microorganisms inoculated after 48h. *Salmonella* spp. and *E. coli* showed to reach a high optical (around 1.7/1.8) density before the other two bacteria considered (36 h): this could be due to their ability to growth faster at these conditions. Moreover, these two microorganisms, although showed a slight decrease with the increasing of the concentration of the product, were strongly inhibited only in the broths inoculated with 5%.

The Gram positive bacteria tested, *L. monocytogenes* and *S. aureus,* resulted to be more sensitive to the presence of the product especially after 36 and 48 h of inoculation: the mechanism of action of polyphenols against these

microorganisms is not completely known. The chemical composition of olive extract is likely to have a role in the antimicrobial action exerted thanks to the high content in polyphenols, as already described by Pereira et al. (2007). Markin et al. (2003) in particular showed a minimal bactericidal concentration for *E. coli* of 0.3% (w/v) and for *S. aureus* of 0.6% (w/v) of ground powdered leaves at a 20% (w/v) concentration.

Anyway, additional studies should be considered in order to understand the action against several other microorganisms of interest and eventually different storage conditions.

After *in vitro* stage, where promising results were obtained, we extended the experiment with a second *in vivo* step with the aim to evaluate growth performance, cecum microbial population and the properties exerted by the product on meat oxidative stability (TBARS), pH and color.

In vitro results showed that antimicrobial activity, although slight, was already observed at the lower dosages. Taking into account these results and considering the economical sustainability in intensive farming, the two applied dosages for the *in vivo* trial fell on 0.1% and 0.5%. In the experimental design an additional dietary treatment with inclusion of vitamin E was also included with the aim to compare its antioxidant potential with those exerted by polyphenols associated to meat characteristics and lipid oxidation.

The effects of polyphenol extract from olive on growth performances are shown in table 3. No detectable effect of dietary treatment and its interaction with time on Body Weight (BW), Average Daily Feed Intake (ADFI), Average Daily Gain (ADG) was observed.

Feed conversion ratio (FCR) outlined a positive trend for treatment x time interaction (P= 0.09) with T1 group showing higher values than CTR when both accounting for each experimental phase and overall the experimental period (P= 0.07).

This tendency was supported by some significant differences in the comparison between treatments at each time: in the second phase (11-20 d) FCR of CTR animals were significantly higher than T1 (P= 0.03); in the third phase FCR of animals from T1 was significant higher than those obtained from T2 (P=0.010) and tended to increase against T3 (P= 0.06). In overall period (0-35 d) FCR from T2 animals resulted significantly lower than CTR (P= 0.01) and the tendency was lower than T1 (P=0.08). Mortality ratio was reported like a percentage of death animals divided by total animals per treatment. Mortality was very low or absent in polyphenols treated animals (T2= 0 %, T3= 0.56 %) with respect to CTR (2.2 %) and Vitamin E supplemented (2.2).

No differences were observed in carcass yield at slaughtering. Partially in agreement with our results, King et al. (2014) reported that Dried Olive Extract did not affect the growing performance of broiler chicks.

As reported by some authors, polyphenols are able to improve low-density lipoprotein (LDL) metabolism (Zhang et al., 1997; Yang et al., 2003), limiting the peroxidation of LDL (Pearson et al., 1998; Yokozawa et al., 2002) and fat (Sano et al., 1995) and inhibiting liver estrone glucuronidation (Zhu et al., 1998; Cao et al., 2005). Additionally, hydroxytyrosol, as the main polyphenol present in the extract of olive, exerted its more effective antioxidant activity in emulsion (Medina et al., 2009). In the present trial, the last dietary phase showed a decrease of FCR in T2: this could be probably associated to emulsification. This hypothesis can be explained with complete development of gastrointestinal tract: endogenous emulsifier produced from animal organism helps hydroxytyrosol to explain better its antioxidant potential on over mentioned fat and LDL metabolism.

Antioxidant and anti-inflammatory properties and improvements in endothelial dysfunction and the lipid profile have been reported for dietary polyphenols (Zern & Fernandez, 2005). Olive oil, rich in oleic acid (a monounsaturated fatty

acid), is the main fat of the Mediterranean diet in humans (Psaltopoulou et al., 2004). To date, most of the protective effect of olive oil within the Mediterranean diet has been attributed to its high monounsaturated fatty acid content (FDA, 2004). However, if the effect of olive oil can be attributed solely to its monounsaturated fatty acid content, any type of olive oil, rapeseed or canola oil, or monounsaturated fatty acid– enriched fat would provide similar health benefits. In this study feed composition were formulated including three principal raw materials: corn (more than 55% in 3 phases), soybean meal and soybean oil, with around 27% of total fat of corn and 24% of soybean oil being oleic acid. Whether the beneficial effects of olive oil on the cardiovascular system are exclusively due to oleic acid remains to be elucidated but in experimental studies, phenolic compounds in olive oil showed strong antioxidant properties (Owen et al., 2000; Visioli et al., 2000). Oxidized low-density lipoprotein (LDL) is currently thought to be more damaging to the arterial wall than native LDL cholesterol (Navamb et al., 1996).

Some researchers asserted that the effect of daily doses of olive oil can achieve benefits against cardiovascular risk factors (Covas et al., 2006), accounting for its phenolic content, lipid profile and decreasing effect on lipid oxidative damage and LDL cholesterol levels. The major diseases that can cause death in broiler chickens are sudden death syndrome; no other causes were found in death animals in present study. These illnesses are popularly accompanied by excessive accumulation of abdominal fat (Giordani et al., 1994). In fact, some reports guessed that the cause of death was related to the faster growth rate (Leeson and Summers, 1997) and metabolic disturbances (Gonzales et al., 1998; Gonzalez et al., 2000) ended with heart attack as cause of death (Cao et al., 2005).

Cecum microbial population was not influenced by Polyphenols dietary treatment; these results are not in agreement with the data reported in literature

(Terada et al., 1993; Cao et al., 2005), as these authors reported an antimicrobial effect on gut microbial population.

Salmonella spp. and *Campylobacter* spp. were not detected in any of the samples analysed: this could be due to the particular rearing conditions at which the animals were subjected. In fact, the experimental facility could not be compared to an intensive farm, and may have equalized the potential dietary treatment effect on intestinal population of chicks for fattening. Considering the other parameters, no influences due to polyphenols from olive extract and vitamin E administration were observed for all the other microbial groups considered. Thus, all the ratios calculated (*Lactobacilli*/*E. coli, Lactobacilli*/*Enterobacteriaceae, Lactobacilli*/*Coliforms* and LAB/*E. coli*, LAB/*Enterobacteriaceae* and LAB/*Coliforms* ratios) for the evaluation of gut health status did not show any differences between the treatments. Moreover, the optimal rearing conditions and the absence of stresses in the experimental facility prevented the possibility of having animal with weak intestinal condition.

The high *E. coli* concentrations in the caecum (7.6/7.7 Log CFU/g) were expected, as this microorganism is a usual commensal bacterium of intestine in many animals, including food-producing species. These microorganisms should be carefully considered as different potentially pathogenic strains since they could be carried asymptomatically by chickens and excreted with feces.

In this study dietary Polyphenols olive extract did not delay lipid oxidation in breast chicken meats; only vitamin E showed a tendency to reduce oxidation at 9 days of storage. Meat quality, evaluated with TBARS values, pH and colour at day 0 and 9 are presented in table 5.

The mean TBARS values measured after 9 days in control, T2 and T3 were not statistically different while the TBARS values of T1 were significantly lower ($P<0.05$) than those from the other groups.

Administration of polyphenols and vitamin E showed a tendency (P=0.07) on the interaction between treatment and time; especially at day 9, T1 resulted significantly lower compared with CTR group and T2 and T3 administered with polyphenols extract.

Brenes et al. (2008) report that polyphenols from Grape pomace concentrate supplementation showed the same antioxidant potential as vitamin E. Contrarily to this conclusion and in agreement with review of Surai (2014), in our study the antioxidant effect evidenced by vitamin E, was not exerted by Polyphenols. It is well known that vitamin E possesses membrane-stabilizing effects (Surai, 2002; Surai and Fisinin, 2010) responsible for decreasing lipid peroxidation in the meat during storage; results from the present study showed that dietary olive extract polyphenols could not delay lipid oxidation in breast chicken meats, while vitamin E showed only a tendency to reduce oxidation at 9 days of storage, according to other mentioned authors. Dejong and Lanari (2009) showed that polyphenol extract from the wastewater of olive oil's pomace inoculated on pre-cooked meat inhibited lipid oxidation in pre-cooked ground beef and pork. In detail, in the TBARS values of cooked beef patties stored from zero to six days, hydroxy-tyrosol were the most potent antioxidants: no significant differences resulted from data at different times.

In this study, the values of pH were not influenced from dietary treatment in both f measures on day 0 and 9 (table 5).

Colour parameters were not affected by the addition of olive extract polyphenols and vitamin E, but some differences in a single parameter considered in the total evaluation of the colour were detected; L parameter of breast muscle showed a tendency effect on interaction between treatment and time (P=0.064) and b parameter of skin showed a treatment effect in the model (P=0.003).

No other differences were detected in colour parameters on thigh; fat colour was detected only at day 9 because of its consistency.

4.6 Conclusions

In this study, a polyphenol-rich olive extract was tested for antimicrobial activity *in vitro* and the effects on growth performance, caecum microbial population, slaughtering performance and meat quality were evaluated. Evident promising inhibition towards pathogenic bacteria was observed with the increase of the product concentrations. Considering growth performances, just a tendency in Feed Conversion Ratio was evidenced. Meat quality was not significantly affected by the administration of the extract, considering oxidative stability and color.

It would be interesting to evaluate the potential of the administration of this type of polyphenols in animals subjected to the stress of intensive livestock production, where more significant results due to potential antimicrobial and antioxidant activity could be obtained.

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4.8 Tables

Table 1: Ingredients and calculated analysis of the basal diet for chickens for fattening (as-fed basis)

INGREDIENT	Starter	Grower	Finishing
Corn	55.05	57.40	61.67
Soybean Meal 48	37.30	34.10	29.20
Soybean Oil	3.00	4.30	5.30
Dicalcium Phosphate	2.50	2.50	2.10
Calcium Carbonate	0.70	0.45	0.50
Mineral+Vitamin Premix	0.50	0.50	0.50
Salt	0.40	0.40	0.40
DL-Methionine	0.32	0.18	0.16
L-Lysine HCL	0.23	0.17	0.17
Vitamin E	±	±	±
Poliphenols	±	±	±
CHEMICAL ANALYSIS, % as fed			
ME, kcal/kg	3003.36	3100.26	3200.48
Crude Protein	22.63	21.16	19.14
Lysine (%) SID	1.28	1.16	1.04
Met+Cys (%) SID	0.91	0.74	0.68
Methionine (%) SID	0.62	0.46	0.42
Lysine (%) total	1.40	1.27	1.13
Met+Cys (%) total	1.00	0.83	0.76
Methionine (%) total	0.64	0.49	0.44

1Provided the following per kilogram of diet: vitamin A, 10000 IU; vitamin D_3, 2000 IU; vitamin E, 20 mg; $MnSO_4 \cdot 1H_2O$, 60 mg; $ZnSO_4 \cdot 1H2O$, 94.25 mg; $FeSO_4 \cdot 1H_2O$,50 mg; $CuSO_4 \cdot 5H_2O$, 5 mg; KI, 1 mg; Na_2SeO3, 150 μg.

Table 2: Potential ability of polyphenol-rich olive extract against different potential pathogenic bacteria (values expressed like means of two optical absorbances at 540 nM)

Microorganisms	Time	Treatment*				
		0.00%	0.10%	0.50%	1.00%	5.00%
		CTRL	T1	T2	T3	T4
L. monocytogenes						
	0 h	0.098	0.008	-0.115	0.047	-0.101
	12 h	0.098	0.040	-0.101	0.052	-0.129
	24 h	0.138	0.085	-0.108	0.044	-0.131
	36 h	1.381	1.190	0.450	0.129	-0.083
	48 h	1.551	1.437	1.021	0.642	-0.093
Salmonella spp.						
	0 h	0.072	0.029	-0.074	0.097	-0.161
	12 h	0.083	0.073	-0.065	0.054	-0.161
	24 h	0.345	0.297	0.003	0.130	-0.194
	36 h	1.782	1.452	1.455	1.356	-0.122
	48 h	1.732	1.666	1.401	1.359	-0.125
Escherichia coli						
	0 h	0.061	0.001	-0.034	0.039	-0.107
	12 h	0.042	0.017	-0.043	-0.002	-0.096
	24 h	0.183	0.068	0.042	0.055	-0.148
	36 h	1.836	0.764	1.559	1.446	-0.077
	48 h	1.785	1.227	1.542	1.480	-0.089
Staphylococcus aureus						
	0 h	0.132	-0.057	-0.052	0.047	-0.044
	12 h	0.114	-0.044	-0.166	0.024	-0.086
	24 h	0.114	0.013	-0.026	0.064	-0.111
	36 h	0.446	0.703	0.674	0.194	-0.034
	48 h	1.164	1.246	0.940	0.677	-0.046

*=CTR: microbial growing without any included % of Polyphenol-rich olive extract; T1: microbial growing with included 0.1% of Polyphenol-rich olive extract; T2: microbial growing with included 0.5% of Polyphenol-rich olive extract; T3: microbial growing with included 1.0% of Polyphenol-rich olive extract; T4= microbial growing with included 5.0% of Polyphenol-rich olive extract.

Table 3: Effect of polyphenols and vitamin E supplementation on growth performance of broiler chicks.

Items	Time	Treatment				SEM	*P*-value		
		CTR	T1	T2	T3		Treatment	Time	Treatment*time
BODY WEIGHT (g)									
	0 d	45.1	45.0	44.9	45.1	14.46	0.190	<.0001	0.514
	10 d	283.5	270.5	276.8	278.4				
	20 d	798.1	784.0	787.8	807.4				
	35 d	1963.1	1941.7	1998.2	1999.5				
ADFI (g/day)									
	0-10 d	29.64	28.94	29.33	29.40	1.85	0.773	<.0001	0.756
	11-20 d	72.84	68.18	70.42	72.07				
	21-35 d	133.09	134.91	134.67	135.13				
	0-35 d	87.44	85.16	86.21	86.96	1.49	0.459		
ADG (g/day)									
	0-10 d	23.68	22.55	23.18	23.32	1.11	0.244	<.0001	0.443
	11-20 d	51.78	51.35	51.10	52.90				
	21-35 d	77.66	76.53^{B}	80.69^{A}	79.48				
	0-35 d	54.80	53.92	55.81	55.84	1.10	0.268		

(To be continued)

FCR (feed/gain)									
	0-10 d	1.251	1.285	1.266	1.261	0.02	0.645	<.0001	0.090
	11-20 d	1.407[a]	1.329[b]	1.380	1.364				
	21-35 d	1.716	1.768[a]	1.671[b]	1.701				
	0-35 d	1.596[A]	1.581	1.545[B]	1.557	0.02	0.070		
MORTALITY % [1]									
		2.22	2.22	0.00	0.56				
CARCASS YIELD: [2]									
DRESSING %		72.18	71.93	71.72	71.87	0.47	0.809		
BREAST MUSCLE %									
		26.73	27.87	26.78	27.54	0.75	0.360		

CTR = basal diet without supplementation; T1 = CTR+VIT E (200 I.U. /kg from day 0 to 35), T2 = CTR + polyphenols (1 g/kg Phenofeed from day 0 to 35), T3 = CTR + polyphenols (5 g/kg Phenofeed from day 0 to 35), ADG = average daily gain; ADFI = average daily feed intake; FCR =feed conversion ratio; [1]Mortality percentage was calculated by dividing the number of died animals per treatment by the total animals per treatment; [2] Dressing percentage was calculated by dividing the eviscerated weight by the live weight; breast muscle yield was calculated as the percentage over the eviscerated weight. [A,B] $P < 0.01$; [a,b] $P < 0.05$

Table 4: Effect of polyphenols and vitamin E supplementation on cecum microbiological analysis of broiler chicks.

Item	Treatment					
	CTR	T1	T2	T3	SEM	P-value
Lactic Acid Bacteria (log CFU/g)	8.36	8.17	8.39	8.20	0.36	0.894
Lactobacilli (log CFU/g)	8.89	8.70	8.90	8.56	0.23	0.426
Enterococci (log CFU/g)	7.40	7.24	7.18	7.53	0.29	0.601
Enterobacteriaceae (log CFU/g)	7.89	7.84	8.01	8.04	0.34	0.921
Coliforms (log CFU/g)	7.91	7.86	7.96	8.01	0.32	0.971
Escherichia coli (log CFU/g)	7.61	7.78	7.76	7.77	0.36	0.956
Yeasts (log CFU/g)	7.43	7.37	7.05	7.23	0.33	0.700
Clostridia (log CFU/g)	2.93	3.62	4.22	3.04	0.72	0.556
Salmonella spp. (log CFU/g)	absent	absent	absent	absent		
Campylobacter spp. (log CFU/g)	absent	absent	absent	absent		
Lactobacilli/*E. coli* ratio	1.28	0.92	1.14	0.79	0.33	0.465
Lactobacilli/*Enterobacteriaceae* ratio	1.00	0.86	0.89	0.52	0.33	0.519
LAB/*E. coli* ratio	0.75	0.39	0.63	0.42	0.25	0.394
LAB/*Enterobacteriaceae* ratio	0.47	0.34	0.38	0.13	0.18	0.309
Lactobacilli/*Coliforms* ratio	0.98	0.84	0.94	0.55	0.32	0.542
LAB/*Coliforms* ratio	0.45	0.31	0.43	0.19	0.17	0.403

CTR = basal diet without supplementation; T1 = CTR+VIT E (200 I.U. /kg from day 0 to 35), T2 = CTR + polyphenols (1 g/kg Phenofeed from day 0 to 35), T3 = CTR + polyphenols (5 g/kg Phenofeed from day 0 to 35)

Table 5: Effect of dietary supplementation with vitamin E and polyphenol olive extract on meet oxidation, pH and meat colour

Items		Time	Treatment				SEM	P-value		
			CTR	T1	T2	T3		Treatment	Time	Treatment*time
TBARS		0d	0.15	0.15	0.14	0.15	0.03	0.160	<.0001	0.075
		9d	0.30	0.20	0.35	0.32				
pH		0d	6.27	6.26	6.25	6.17	0.03	0.455	0.768	0.349
		9d	6.23	6.27	6.23	6.23				
COLOURS	L	0d	53.97	55.22	56.53	55.92	0.97	0.508	<.0001	0.064
Breast		9d	58.05	60.07	57.01	57.68				
	a	0d	-0.42	-0.43	-0.57	-0.87	0.35	0.548	<.0001	0.468
		9d	-1.74	-2.10	-1.18	-1.84				
	b	0d	10.21	10.34	10.77	9.62	0.76	0.379	<.0001	0.209
		9d	13.49	13.84	11.58	11.94				
Skin	L	0d	71.11	71.17	70.65	71.33	0.76	0.620	0.0001	0.959
		9d	68.75	68.60	68.06	69.46				
	a	0d	-2.04	-1.41	-0.77	-1.74	0.39	0.214	0.198	0.630
		9d	-1.15	-1.22	-0.87	-1.26				
	b	0d	20.84	19.20	17.91	18.23	0.82	0.003	<.0001	0.544
		9d	22.46	23.01	20.26	20.08				
Thigh	L	0d	52.02	52.28	53.72	52.80	0.58	0.178	<.0001	0.252
		9d	55.44	57.09	56.22	56.04				
	a	0d	0.55	0.88	0.33	0.70	0.32	0.567	0.062	0.595
		9d	0.58	0.04	-0.06	0.20				
	b	0d	8.44	8.19	8.91	8.40	0.50	0.397	<.0001	0.124
		9d	11.83	11.44	10.35	10.18				
Fat	L	9d	74.13	74.88	75.01	72.78	0.96	0.310		
	a	9d	-0.26	-0.03	0.12	0.56	0.49	0.674		
	b	9d	22.78	22.65	22.21	21.64	0.72	0.649		

CTR = basal diet without supplementation; T1 = CTR+VIT E (200 I.U. /kg from day 0 to 35), T2 = CTR + polyphenols (1 g/kg Phenofeed from day 0 to 35), T3 = CTR + polyphenols (5 g/kg Phenofeed from day 0 to 35)

CHAPTER 5

5 The effects of a novel synthetic emulsifier product on growth performance of chickens for fattening and weaned piglets

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5.1 Abstract

Two experiments were conducted to evaluate the effects of a novel synthetic emulsifier product (AVI-MUL TOP) on the growth performance of chickens for fattening and weaned piglets. The emulsifier product consists of 50% vegetal bi-distillated oleic acid emulsified with 50% glyceryl polyethyleneglycol ricinoleate. In Exp. 1, four hundred and eighty 1-d-old female Cobb500 chickens for fattening were assigned to two treatments: 1) a control diet (CTR); and 2) the control diet + the emulsifier (AMT, 1 g/kg from day 0 to day 10, 0.75 g/kg from day 10 to day 20 and 0.5 g/kg from day 20 to day 34). AMT supplementation increased body weight on days 20 and 34 ($P < 0.01$). Dietary AMT increased the average daily gain (ADG) and average daily feed intake (ADFI) from day 10 to day 20, from day 20 to day 34 and from day 0 to 34 ($P < 0.01$). A reduced feed conversion ratio was observed in the AMT group from day 10 to day 20 ($P < 0.01$). In Exp. 2, ninety-six Stambo HBI × Dalland piglets were weaned at 24 days and assigned to 2 treatments (the basal diet without the product (CTR) or with 2 g/kg emulsifier from day 0 to day 14 and 1.5 g/kg from day 14 to day 42 (AMT)). There was an increase in the ADFI associated with AMT supplementation from day 14 to day 42 ($P = 0.04$). These results indicated that supplementation with the synthetic emulsifier may significantly improve the growth performance of chickens for fattening and numerically improve that of weaned piglets.

Keywords: Chickens for fattening, Emulsifier, Growth performance, Weaned piglets.

5.2 Implications

This study determined whether a novel synthetic emulsifier product, consisting of vegetal bi-distillated oleic acid and glyceryl polyethyleneglycol ricinoleate, could have beneficial effects on the growth performance of chickens for fattening and weaned piglets. The current observations may be of significant value to commercial feed manufacturers and farmers as they may give rise to important savings in the industry.

5.3 Introduction

Low hepatic bile acid synthesis may result in poor lipid digestion in weaned piglets (Lewis et al., 2000), and inefficient digestion and absorption of fat also occurs in young chickens, possibly due to a low level of natural endogenous lipase production (Al-Marzooqi and Leeson, 1999). These observations have generated considerable interest in and research on the use of emulsifiers to improve the utilization of fats in young chicks (Al-Marzooqi and Leeson, 1999) and post-weaning piglets (Jones et al., 1992). Lecithin, an emulsifier, has been reported to depress free fatty acid absorption, probably by increasing the size of bile salt micelles, which diffuse more slowly through the luminal water interface, retarding the delivery of free fatty acids to the absorptive cell surface (Saunders and Sillery, 1976). Compared with lecithin, glyceryl polyethyleneglycol ricinoleate is more hydrophilic and dissolves free fatty acids, which are largely insoluble in bile salt micelles alone (Dierick and Decuypere, 2004). Roy et al. (2010) reported that

supplementation of exogenous emulsifiers in diets containing moderate quantities of added vegetable fats may substantially improve broiler performance. In addition, emulsification of fat (as in sow milk) may improve the growth performance of weaned pigs fed supplemental fat (Xing et al., 2004).

To the best of our knowledge, the use of emulsifiers in association with vegetable oils in animal feed has not yet been thoroughly investigated, even though the interest in using exogenous emulsifiers has increased in the last several decades. Experiments were therefore conducted with the aim of assessing the effect of a novel synthetic emulsifier product (AVI-MUL TOP), consisting of vegetal bi-distillated oleic acid and glyceryl polyethyleneglycol ricinoleate, on the growth performance of chickens for fattening and weaned piglets.

5.4 Materials and methods

The experimental protocol was reviewed and approved by the Animal Care and Use Committee of the University of Milan. Both experiments were performed at the facility for Animal Production Research and Teaching Centre of the Polo Veterinario, Università degli Studi di Milano (Lodi, Italy).

The emulsifier product (AVI-MUL TOP, SEVECOM S.P.A., Milan, Italy), consisting of 50% vegetal bi-distillated oleic acid emulsified with 50% ethoxylated castor oil E484, which belongs to the glyceryl polyethyleneglycol ricinoleate family (Community Register of Feed Additives - EU Reg. No. 1831/2003), was mixed with other ingredients before the pelleting process. The animal fat consisted of

50% poultry fat and 50% lard and vegetable oils (soybean oil) were used in experiment 1, whereas only vegetable oils (soybean oil and coconut oil) were used in experiment 2. The experimental diets were produced by Veronesi Verona S.P.A., Verona, Italy

5.4.1 Experiment 1

Four hundred and eighty female Cobb500 chickens for fattening (44.46 ± 0.96 g) were obtained from a local hatchery at 1 d of age and allocated to 48 pens with 10 birds in each pen, measuring 1.25 m ×1.00 m. All of the chickens were vaccinated against Marek's disease, Newcastle disease and infectious bronchitis via coarse spraying at hatching. The 48 pens were randomly assigned to 2 treatments, consisting of a non-supplemented basal diet (CTR) and the basal diet supplemented with the emulsifier (AMT, 1 g/kg inclusion rate from day 0 to day 10, 0.75 g/kg from day 10 to day 20 and 0.5 g/kg from day 20 to day 34 of the trial. The diets (Table 1) were formulated to meet the nutrient requirements defined by the NRC (1994). The diet during the starter and grower periods was provided in crumble form, and the diet during the finisher period was provided as pellets. The birds were housed in an environmentally controlled room in separate floor pens with white wood shavings as bedding, under a photoperiod of 24 h of light from day 0 to day 7 and 23 h light:1 h dark from day 7 to day 34. Room temperature was maintained at 35°C from day 1 to day 3 and was then decreased by 2.5°C per week, to a final temperature of 24°C at day 34. Feed and water were provided for *ad*

libitum consumption. The animals were checked twice daily and any dead animals were removed, weighed, and recorded. All birds were individually weighed on days 0, 10, 20 and 34 of age. The average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) values were calculated for each pen. At days 20 and 34, the gait score of each bird was characterized according to a 3-point gait-scoring system: 0 = no impairment of walking ability; 1 = obvious impairment, but still ambulatory; 2 = severe impairment and inability to walk without great difficulty. At 34 days of age, one bird exhibiting the average BW for the pen was chosen and marked to measure the carcass yield. Following a 12 h overnight fast, all of the chickens were sent to a commercial slaughterhouse and stunned in water bath (125 Hz AC, 80 mA/birds, 5 s) before killing via exsanguination. The dressing percentage was calculated by dividing the eviscerated weight by the live weight. Breast muscle was removed and weighed, and the breast muscle yield was calculated as the percentage of eviscerated weight.

5.4.2 Experiment 2

Ninety-six crossbred weaned barrow piglets (Stambo HBI × Dalland, 24 days old, 8.04 ± 1.32 BW) were selected for a 42-day experiment from a commercial swine herd. All of the piglets were vaccinated for *Mycoplasma hyopneumoniae* at two days of age. The piglets were randomly allotted to two dietary treatments according to their initial BW (12 replicates of 4 piglets each per treatment). The experimental unit was defined as one pen. The two dietary treatments consisted of two different diets: 1)

control (CTR), basal diet; and 2) AMT, basal diet + emulsifier (AMT, 2 g/kg from day 0 to day 14 and 1.5 g/kg from day 14 to day 42 of the trial). All of the diets were provided in pellet form and were formulated to meet the recommended requirements of the NRC (2012) for a feeding programme (Table 2). The piglets were housed in one environmentally regulated room with a slatted plastic floor (4 piglets/pen, 1.20 m × 1.00 m). Each pen was equipped with a one-sided self-feeder and a nipple waterer to allow the pigs *ad libitum* access to feed and water throughout the experimental period. The temperature of the pig barn was set between 26 and 28° C, with a 12 h light:dark cycle. All of the piglets were individually weighed on days 0, 14, 28 and 42 of the trial. The ADG, ADFI and FCR values were calculated for each pen.

5.5 Statistical analysis

The data from both experiments were analysed in accordance with the GLM Procedure of SAS v. 9.2 (SAS Institute Inc., Cary, NC). The pen was used as the experimental unit for the growth performance of the chickens and piglets, whereas the individual chicken was considered to be the experimental unit for the carcass yield of chickens. The gait score data were analysed using the MIXED procedure for repeated measurements, and the pen represented the experimental unit. Treatment differences were assessed via the least squares means with the Tukey adjustment. Treatment effects were considered significant at $P \leq 0.05$, whereas a trend for a treatment effect was noted for $P \leq 0.10$.

5.6 Results

The effects of the AMT on the body weight (BW), average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) of chickens for fattening are shown in Table 3. There was no detectable effect of the diet on BW from day 0 to day 10 ($P > 0.05$). AMT supplementation increased BW compared with the CTR group on days 20 and 34 ($P < 0.01$). An increased ADG was observed in the AMT chickens in comparison with the CTR birds from day 10 to day 20, from day 20 to day 34 and from day 0 to day 34 ($P < 0.01$). Compared with the CTR group, dietary AMT supplementation increased the ADFI from day 10 to day 20, from day 20 to day 34 and from day 0 to day 34 ($P < 0.01$). Additionally, AMT supplementation tended to increase the ADFI from day 0 to day 10 compared with the CTR diet ($P = 0.09$). A reduced FCR was observed in the AMT group compared with the CTR group from day 10 to day 20, whereas the AMT chickens exhibited a higher FCR than the CTR birds from day 0 to day 10 and from day 20 to day 34 ($P < 0.01$). AMT supplementation significantly increased the percentages of dressing and breast muscle ($P < 0.01$; $P < 0.01$). The gait scores of the AMT chickens were lower than those of the CTR birds on days 20 and 34 ($P < 0.01$; Table 4). A significant interaction between the diet and age was observed ($P < 0.01$), which was due to the decreased values recorded from day 20 to day 34 in the CTR group ($P < 0.01$), whereas there was no change in the AMT group.

The effects of AMT supplementation on the growth performance of weaned piglets are shown in Table 5. The AMT piglets presented a higher ADFI compared with the CTR group from day 28 to day 42 (P = 0.04). In addition, AMT supplementation tended to increase the ADFI from day 0 to day 42 (P = 0.098) and numerically increased the ADG from day 28 to day 42 (P = 0.11). The addition of AMT to diet also promoted greater mean BW on day 42 (30.40 kg), although in this case, the difference compared with the CTR group (28.98 kg) was not significant (P = 0.29).

5.7 Discussion

This experiment was performed with the aim of evaluating the effect of a synthetic emulsifier, consisting of vegetal bi-distillated oleic acid emulsified with glyceryl polyethyleneglycol ricinoleate, on the growth performance of chickens for fattening and weaned piglets. The results showed that the addition of emulsifier to the feed significantly increased the ADG and ADFI of chickens for fattening and tended to increase the ADFI of weaned piglets during the entire experimental period, which is in agreement with the findings of previous studies (Xing et al., 2004; Roy et al., 2010; Price et al., 2013). The increased growth and feed intake may be due to certain effects of the dietary emulsifier on pellet quality and fat digestibility (Jones et al., 1992; Roy et al., 2010).

It is well known that emulsifiers can reduce the surface tension of water and increase the penetration and improve the distribution of water in press meal (van

der Heijden and de Haan, 2010). In the current study, the AMT product was mixed with feed compounds before pelleting process, which may increase humidity, reduce pellet press energy consumption and improve pellet quality by modulating the moisture content during the pelleting process and, consequently, improve feed intake and performance of animals. Lecithin, as an emulsifier, has been reported to enhance the apparent digestibility of unsaturated fatty acids in lard (Soares and Lopez-Bote, 2002). Dierick and Decuypere (2004) reported that the addition of an emulsifier improved the digestibility of major nutrients, which may reduce the viscosity of the digestive contents and increase the transit of the digesta as well as feed intake (Lázaro et al., 2004). In this study, the incorporation of vegetal bi-distillated oleic acid and glyceryl polyethyleneglycol ricinoleate may have also improved the growth performance of animals via the emulsification of supplemental fatty acids (Xing et al., 2004). However, there was no significant influence of AMT supplementation on the ADFI during the first phase in the chickens and piglets, possibly due to the insufficient digestion and absorption of fat in young animals (Dierick and Decuypere, 2004).

In experiment 1, a high incidence of tibial dyschondroplasia, an abnormality of the growth cartilage that occurs in chickens, was observed in the CTR chickens on days 20 and 34, which may have been due to the rapid growth rate (Kestin et al., 1999) and the broiler strain used (Dinev et al., 2012). Leg problems are of serious consequence for welfare as lame birds have difficulty reaching diet and water (Mc Geown et al., 1999), and seriously lame birds may lose weight (Kestin et al., 1999).

However, the growth of the CTR chickens in our study was normal compared with the reference value (weight on day 34, 1792 g vs. 1829 g). Interestingly, our results indicated that there was no incidence of poor gait in the AMT group during the entire experimental trial, which may due to the possible improvements in the pellet quality and nutrients digestibility of birds by AMT supplementation. However, the present data are insufficient to demonstrate that the lack of leg weakness in the AMT chickens was due to emulsifier supplementation, more research should be conducted to identify the effect of emulsifier on nutrients absorption and determine whether it would influence the incidence of leg weakness. Additionally, increased feed intake was observed in the weaned piglets during the last period in experiment 2, which may suggest that the synthetic emulsifier can positively affect growth performance for both chickens and piglets by increasing feed intake. However, the effect of the emulsifier on the growth of piglets was weaker than on chickens, possibly due to the high copper intake of the piglets. The piglets' diets contained 140 mg/kg copper, which is close to the maximum authorised level for weaned piglets (175 mg/kg) according to the EU (European Commission, 2003) and could affect the gut flora (Højberg et al., 2005) and the metabolism of bile acids (Deol et al., 1992) and eventually interfere with the effect of the emulsifier. It has been reported that the dietary fat level may influence the apparent fat digestibility (Lauridsen et al., 2007). In this study, the fat intake of piglets was lower than chickens, which may also influence the effect of the emulsifier. The digestive system of swine is monogastric that is different from the avian digestive system

found in poultry. Jones et al. (1992) concluded that addition of emulsifiers increased digestibility of nutrients but had minimal effect on growth performance in weanling pigs. In addition, the CTR chickens had better FCR than the AMT birds during the finishing phase, possibly due to the improved feed efficiency during compensatory growth when the tibias of the CTR chickens were recovering from day 20 to 34 (Zubaira and Leeson, 1996). Scheele (1997) noted that the growth of the pectoral muscles primarily occurs during the late stages of developmental growth in fast-growing birds. In the present study, an increased slaughter yield was also observed in the treated group, suggesting that the relatively rapid growth of the AMT-fed chickens in the grower and finisher phases may have contributed to the comparable increases in carcass and breast muscle yields. The increased growth performance recorded in the present work was probably supported by digestive and physiological mechanisms/processes (Dierick and Decuypere, 2004; Roy et al., 2010); however, these processes were not considered in our experiments. Future studies are in progress to specifically assess the possible improvement of digestibility and fat utilization.

In conclusion, the current observations indicated that the supplementation of a novel synthetic emulsifier consisting of vegetal bi-distillated oleic acid and glyceryl polyethyleneglycol ricinoleate has the potential to significantly improve the growth performance of chickens for fattening, although only numerical improvements were noted in the growth performance of weaned piglets under supplementation with the synthetic emulsifier product.

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5.10 Tables

Table 1: Ingredient and calculated analysis of the basal diet for chickens for fattening (as-fed basis)

	Starter (0-10 d)		Grower (10-20 d)		Finisher (20-34 d)	
	CTR	AMT	CTR	AMT	CTR	AMT
Ingredients, %						
Maize	45.17	45.17	37.31	37.31	30.28	30.28
Soybean meal, 49% CP	32.50	32.50	25.00	25.00	25.50	25.50
Wheat	15.00	15.00	15.00	15.00	20.00	20.00
Sorghum	0	0	10.00	10.00	15.00	15.00
Soybean full fat	0	0	5.00	5.00	0	0
Soybean oil	3.00	3.00	0	0	0	0
Poultry fat and lard	0	0	4.00	4.00	6.00	6.00
Sodium chloride	0.40	0.40	0.35	0.35	0.25	0.25
Calcium carbonate	1.20	1.20	1.00	1.00	0.95	0.95
Dicalcium phosphate 18%	1.50	1.50	1.2	1.2	1.00	1.00
DL-Methionine	0.34	0.34	0.30	0.30	0.25	0.25
L-Threonine	0.11	0.11	0.09	0.09	0.04	0.04
L-Lysine HCl	0.28	0.28	0.25	0.25	0.23	0.23
Premix[1]	0.50	0.50	0.50	0.50	0.50	0.50
EM[2]	0	0.10	0	0.075	0	0.05
Calculated composition						
ME, MJ/kg	12.77	12.77	13.19	13.19	13.65	13.65
Crude protein, %	21.80	21.80	20.20	20.20	19.00	19.00
Crude fat, %	5.20	5.20	6.70	6.70	8.20	8.20
Calcium, %	0.92	0.92	0.80	0.80	0.70	0.70
Phosphorous, %	0.65	0.65	0.60	0.60	0.54	0.54
Analyzed composition						
Crude protein, %	21.50	21.87	20.70	20.10	19.42	19.34
Crude fat, %	5.16	5.86	6.49	6.58	7.69	7.47

[1] Provided the following per kilogram of diet: vitamin A, 11250 IU; vitamin D_3, 5000 IU; vitamin E, 60 mg; $MnSO_4 \cdot 1H_2O$, 308 mg; $ZnSO_4 \cdot 1H_2O$, 246 mg; $FeSO_4 \cdot 1H_2O$, 136 mg; $CuSO_4 \cdot 5H_2O$, 39 mg; KI, 2.4 mg; Na_2SeO_3, 657 μg; 6-Phytase EC 3.1.3.26, 750 FTU; Endo-1, 4-beta-xylanase EC 3.2.1.8, 2250 U.

[2] AMT group: 1 g/kg during d 0-10; 0.75 g/kg during 10-20; 0.5 g/kg during d 20-34.

Table 2: Ingredient and calculated analysis of the basal diet for weaned piglets

(as-fed basis)	Prestarter (0-14 d)		Starter (14-42 d)	
	CTR	AMT	CTR	AMT
Ingredients, %				
Maize	14.00	14.00	15.00	15.00
Barley	16.28	16.28	16.39	16.39
Wheat	15.00	15.00	12.00	12.00
Rolled Barley	10.00	10.00	6.00	6.00
Soybean meal, 49% CP	7.00	7.00	10.00	10.00
Whey	8.00	8.00	6.00	6.00
Wheat bran	4.00	4.00	6.00	6.00
Corn flakes	8.00	8.00	6.00	6.00
Corn gluten meal	3.00	3.00	3.00	3.00
Soy protein concentrate	4.00	4.00	3.00	3.00
Rice flour	0	0	4.00	4.00
Soybean oil	1.50	1.50	2.50	2.50
Coconut oil	1.50	1.50	1.50	1.50
Beet pulp	4.00	4.00	4.00	4.00
Sugar cane molasses	0	0	1.00	1.00
Sodium chloride	0.25	0.25	0.30	0.30
Calcium carbonate	0.60	0.60	0.65	0.65
Dicalcium phosphate 18%	0.65	0.65	0.50	0.50
Citric acid	0.50	0.50	0.50	0.50
DL-Methionine	0.24	0.24	0.20	0.20
L-Tryptophane	0.06	0.06	0.04	0.04
L-Threonine	0.20	0.20	0.22	0.22
L-Lysine HCl	0.72	0.72	0.70	0.70
Premix[1]	0.50	0.50	0.50	0.50
EM[2]	0	0.20	-	0.15
Calculated composition				
ME, MJ/kg	13.40	13.40	13.65	13.65
Crude protein, %	16.60	16.60	17.20	17.20
Crude fat, %	4.50	4.50	5.50	5.50
Calcium, %	0.55	0.55	0.55	0.55
Phosphorus, %	0.50	0.50	0.48	0.48
Copper, mg/kg	140	140	140	140
Analyzed composition				
Crude protein, %	17.50	17.26	18.60	18.05
Crude fat, %	4.30	4.03	5.53	5.56

[1] Provided the following per kilogram of diet: vitamin A, 20000 IU; vitamin D_3, 2000 IU; vitamin E, 100 mg; $MnSO_4 \cdot 1H_2O$, 154 mg; $ZnSO_4 \cdot 1H_2O$, 356 mg; $FeSO_4 \cdot 1H_2O$, 425 mg; $CuSO_4 \cdot 5H_2O$, 628 mg; KI, 3.1 mg; Na_2SeO_3, 657 μg; 6-Phytase EC 3.1.3.26, 1500 FTU; Endo-1, 4-beta-xylanase EC 3.2.1.8, 1220 U; Endo-1, 3(4)-beta-glucanase EC 3.2.1.6, 125 U; Citric acid, 5000mg.

[2] AMT group: 2 g/kg during d 0-14; 1.5 g/kg during d 14-42.

Table 3: Effect of emulsifier (EM) supplementation on growth performance of chickens for fattening

Item	CTR	AMT	SEM	*P*-value
No. of pens	24	24		
BW (g)				
d 0	44.3	44.6	0.2	0.37
d 10	306	303	1	0.20
d 20	728	855	6	<0.01
d 34	1792	2101	17	<0.01
ADG (g/d)				
d 0 to 10	26.2	25.8	0.2	0.16
d 10 to 20	42.2	55.2	0.6	<0.01
d 20 to 34	76.0	89.0	1.0	<0.01
d 0 to 34	51.4	60.5	0.5	<0.01
ADFI (g/d)				
d 0 to 10	34.3	34.9	0.2	0.09
d 10 to 20	67.8	79.3	0.6	<0.01
d 20 to 34	119.7	144.2	1.2	<0.01
d 0 to 34	79.3	93.0	0.6	<0.01
FCR (feed/gain)				
d 0 to 10	1.31	1.35	0.01	<0.01
d 10 to 20	1.61	1.44	0.01	<0.01
d 20 to 34	1.58	1.62	0.01	<0.01
d 0 to 34	1.54	1.54	0.01	0.52
Mortality (%)	0.83	0.83	0.58	-
Carcass yield at d 35 (%)[1]				
Dressing	68.44	71.18	0.29	<0.01
Breast muscle	28.84	31.92	0.48	<0.01

CTR = basal diet without supplementation; AMT = CTR + emulsifier (1 g/kg during d 0-10; 0.75 g/kg during d 10-20; 0.5 g/kg during d 20-34).

[1] The dressing percentage was calculated by dividing the eviscerated weight by the live weight, and breast muscle yield was calculated as the percentage of eviscerated weight.

Table 4: Effect of emulsifier (EM) supplementation on gait score[1] of chickens for fattening

				P-value		
	CTR	AMT	Pooled s.e.m.	Diet	Age	Interaction
d 20	0.588[A]	0.004[C]	0.027	<0.01	<0.01	<0.01
d 34	0.139[B]	0.004[C]				

CTR = basal diet without supplementation; AMT = CTR + emulsifier (1 g/kg during d 0-10; 0.75 g/kg during d 10-20; 0.5 g/kg during d 20-34).

[A-C] Values within the same column or row with different superscripts differ significantly at $P < 0.01$.

[1] Gait scores were recorded using a 3-point scoring system: 0 = none; 1 = obvious impairment; 2 = severe impairment.

Table 5: Effect of emulsifier (EM) supplementation on growth performance of weaned piglets

	CTR	AMT	SEM	*P*-value
No. of pens	12	12		
BW (kg)				
d 0	8.03	8.05	0.39	0.98
d 14	11.66	11.96	0.53	0.70
d 28	19.17	19.87	0.76	0.52
d 42	28.98	30.40	0.91	0.29
ADG (g/d)				
d 0 to 14	259	279	14	0.34
d 14 to 28	536	567	20	0.31
d 28 to 42	701	752	21	0.11
d 0 to 42	499	532	15	0.14
ADFI (g/d)				
d 0 to 14	358	388	21	0.32
d 14 to 28	770	829	34	0.23
d 28 to 42	1131	1264	43	0.04
d 0 to 42	753	827	30	0.098
FCR (feed/gain)				
d 0 to 14	1.38	1.39	0.03	0.88
d 14 to 28	1.44	1.46	0.02	0.26
d 28 to 42	1.63	1.68	0.04	0.40
d 0 to 42	1.51	1.55	0.02	0.23
Mortality (%)	0	0	-	-

CTR = basal diet without supplementation; AMT = CTR + emulsifier (2 g/kg during d 0-14; 1.5 g/kg during d 14-42).

CHAPTER 6

6 Influence of a novel synthetic emulsifier product on growth performance, plasma lipid profile and related genes expression of broiler chicks

6.1 Abstract

The aim of present study was to investigate the influence of a novel synthetic emulsifier product (AVI-MUL TOP) on growth performance, plasma lipid profile and hepatic expression of related genes in female and male broiler chicks. A total of 1200 ROSS308 broiler chicks with day-old were allocated into 4 treatments with a 2 × 2 factorial design comparing sex (female or male) and different dietary treatments (basal diet supplemented without (CTR) or with the emulsifier (AMT, 1 g/kg from d 0 to 12, 0.75 g/kg from d 12 to 22 and 0.5 g/kg from d 22 to 44, respectively)). Each group consisted of 15 pens, 20 birds per pen. Growth performance was determined at days 0, 12, 22 and 44 (37 for females). At day 44 (37 for females), one bird of each pen was chosen and slaughtered to collect blood and livers samples and determine the dressing and breast muscle percentages. Males had higher BW than female chicks on days 0 ($P = 0.014$), 12 ($P < 0.01$) and 22 ($P < 0.01$). Compared to females, male chicks had higher ADG and ADFI from day 0 to 12 and from day 12 to 22 ($P < 0.01$), and lower FCR from day 12 to 22 ($P < 0.01$). AMT supplementation increased the BW compared to the CTR group on days 12 ($P = 0.02$), and AMT chicks had higher ADG compared to the CTR birds from day 0 to 12 ($P = 0.02$). AMT male chicks had lower FCR compared to the CTR males from day 22 to 44 and day 0 to 44 ($P = 0.047$ and 0.02). However, there was no diet effect on growth performance of female chicks during the trial. AMT supplementation increased dressing percentage compared to the CTR group ($P = 0.02$). In addition, dietary AMT increased cholesterol, HDL and LDL contents compared to the CTR group ($P < 0.01$; $P = 0.02$; $P < 0.01$). In conclusion, supplementation of AMT may have beneficial effect on growth performance and plasma lipid profile of broiler chicks.

Key words: chicks, emulsifier, growth performance, hepatic gene expression, plasma lipid profile

6.2 Introduction

Low hepatic bile acid synthesis may result in inefficient digestion and absorption of fat occurs in young chickens, possibly due to a low level of natural endogenous lipase production (Al-Marzooqi and Leeson, 1999). These observations have generated considerable interest in and research on the use of emulsifiers to improve the utilization of fats in young chicks (Al-Marzooqi and Leeson, 1999). Lecithin, an emulsifier, has been reported to depress free fatty acid absorption, probably by increasing the size of bile salt micelles, which diffuse more slowly through the luminal water interface, retarding the delivery of free fatty acids to the absorptive cell surface (Saunders and Sillery, 1976). Compared with lecithin, glyceryl polyethyleneglycol ricinoleate is more hydrophilic and dissolves free fatty acids, which are largely insoluble in bile salt micelles alone (Dierick and Decuypere, 2004). Our previous study observed that supplementation with the synthetic emulsifier improve the growth performance of broiler chicks. In addition, Roy et al. (2010) reported that supplementation of exogenous emulsifiers in diets containing moderate quantities of added vegetable fats may substantially improve broiler performance. Altered concentrations of adipose ApoA-I and ApoB are strongly associated with lipid transport and fat deposition (Jiang et al., 2014). The liver is the major site of apoA-I synthesis and secretion in the adult chicken (Smith et al., 1978), and dietary and physiological factors affect concentrations of ApoA-I and plasma HDL in mammals (Azrolan et al., 1995; Jin et al., 1996).

However, the effect of emulsifiers in association with vegetable oils on fat utilization has not yet been thoroughly investigated, even though the interest in using exogenous emulsifiers has increased in the last several decades. Thus, this study was conducted to assess the effect of a novel synthetic emulsifier product (AVI-MUL TOP), consisting of vegetal bi-distillated oleic acid and glyceryl polyethyleneglycol ricinoleate, on the growth performance, plasma lipid profile and hepatic expression of related genes in broiler chicks.

6.3 Materials and methods

The experimental protocol was reviewed and approved by the Animal Care and Use Committee of the University of Milan. Both experiments were performed at the facility for Animal Production Research and Teaching Centre of the Polo Veterinario, Università degli Studi di Milano (Lodi, Italy).

The emulsifier product (AVI-MUL TOP, SEVECOM S.P.A., Milan, Italy), consisting of 50% vegetal bi-distillated oleic acid emulsified with 50% ethoxylated castor oil E484, which belongs to the glyceryl polyethyleneglycol ricinoleate family (Community Register of Feed Additives - EU Reg. No. 1831/2003), was mixed with other ingredients before the pelleting process. The animal fat consisted of 50% poultry fat and 50% lard and vegetable oils (soybean oil) were used.

6.3.1 Animals and housing

A total of 1200 birds ROSS308 (half females and half males) were obtained from a local hatchery at 1 d of age and were weighed and randomly assigned into 4 experimental dietary groups consisting of 15 replicates. Each pen (replicate) contained 20 birds, measuring 2.5 m ×1.00 m. All of the chickens were vaccinated against Marek's disease, Newcastle disease and infectious bronchitis via coarse spraying at hatching. Chicks were raised in 4 different groups with a 2 × 2 factorial design comparing sex (female or male) and different dietary treatments (basal diet supplemented without (CTR) or with AVI-MUL TOP/GP10 (AMT)). Pens were equally distributed among treatments. Broilers had the same kind of feed (chain feeders) and water (nipple with cup) distribution. Pens were bedded with shaving of white wood, and chickens had the same light cycle and temperature. The photoperiod was 24 h of light from day 0 to day 7 and 23L:1D from day 7 to day 34. The room temperature was maintained at 35°C from day 1 to day 3, and then decreased by 2.5°C per week to a final temperature of 21°C at day 44. Feed and

water were provided for ad libitum consumption. Mortality was removed, weighed, and recorded twice daily. At the end of the trial, the females were slaughtered at 37 d of age, while the males at 44 d. The basal diet was a typical corn–soybean diet formulated to meet nutrient requirements of broiler chicks (National Research Council, 1994), for starter from 0 to 12 days, grower from 12 to 22 days and finisher from 22 to 44 days of age. The diets were prepared in the form of crumbled (starter and grower) and pellet (finisher). Compositions of basal diets and nutrients level are presented in Table 1.

6.3.2 Data collection and sampling

The body weight and feed intake were recorded at 12, 22 and 44 (37 for females) days of age for each replicate to determine the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR). Mortality was removed, weighed, and recorded twice daily. At 44 (37 for females) days of age, one bird from each pen, close to the pen average weight, was selected and slaughtered by cutting jugular vein. Before killing by exsanguination, the birds were stunned in a water bath (125 Hz AC, 80 mA/birds, 5 s). Blood samples were collected into heparinized test tubes to analyse the plasma lipid profile differences among dietary treatment, and were immediately centrifuged at 3000 × g for 10 min at 4°C to separate plasma, and stored at -20°C until analysis. Portions (50–100 mg) of liver were sampled and snap-frozen in liquid nitrogen and stored at -80°C until analysis for total lipid, DNA and RNA isolation. The dressing percentage was calculated by dividing the eviscerated weight by the live weight. Breast muscle was removed and weighed, and the breast muscle yield was calculated as the percentage of eviscerated weight. The breast muscles were stored individually in plastic bags at 4°C for analysis of meat quality.

6.3.3 Plasma lipid profile

The concentrations of all the plasma parameters were measured with an automated spectrophotometer (ILAB 300 plus, Instrumentation Laboratory S.p.a., Milan, Italy) with commercial assay kits according to the manufacturer instructions. For the plasma triglyceride, cholesterol, high-density lipoprotein cholesterol (HDL), glucose, total protein and urea, the kits from Instrumentation Laboratory S.p.a., Milan, Italy were used. For non-esterified fatty acids (NEFA), the kits from Randox Laboratories Ltd., Crumlin, Co. Antrium, UK were used. Low-density lipoprotein cholesterol (LDL) was obtained by subtraction of the measured concentration of HDL from the measured concentration of total cholesterol (cholesterol-HDL).

6.3.4 Determination of lipid/DNA ratio in liver samples

Lipids were extracted by the Folch's method (Folch et al., 1957) with slightly modification. Briefly, liver samples (±1g) were thawed and homogenized in an excess of chloroform:methanol (2:1, v/v) solution for 2min. The homogenate was filtered and placed in separator funnels and mixed with saline solution containing KCl 088%. After separation in two phases, the lipid chloroform fraction (bottom layer) was evaporated using rotary evaporator and weighted.

Genomic DNA was isolated from liver samples using a commercial kit (Sigma Aldrich) based on silica membrane column. Briefly, frozen liver tissue was quickly minced, weighted and digested with the lysis solution and proteinase K provided by the kit. Samples were incubated in the lysis solution up to 4h until the tissue was completely digested (no remaining particles). The lysate was accurately loaded in the column in order to carefully isolate the entire genomic DNA present in the sample. The DNA extracted was finally eluted and maintained in 200µl of TE buffer. The concentration and quality of the total DNA was determined by UV spectrophotometry at 260nm and by *agarose gel electrophoresis* followed by Image

Lab™ software analysis. The Liver total DNA content and the total lipid concentration have been used to calculate total lipid/DNA ratio.

6.3.5 Hepatic expression of genes involved in lipid metabolism

Total RNA was extracted from liver tissue using a commercial kit (Promega) according to manufacturer' protocols. The RNA integrity was assessed by gel electrophoresis in order to detect 18s and 28s rRNA bands. An aliquot of total RNA was reverse transcripted using the iScript cDNA synthesis kit. The primer sequences used are listed in Table 2. Primers pairs were first tested for their specificity in qualitative PCR, using the pooled cDNA as template (Jiang et al., 2013). The cycling profile for the assay consisted of 95°C per 10', which was followed by 40 cycles of amplification (95°C for 15s, 63° for 60s). The quantitative analysis of mRNA was carried out by SYBR green methodology using a real time PCR system (Strategene Mx3000p). The comparative CT method was used (Livak and Schmittgen, 2001), determining fold changes in genes expression, calculated as $2^{-\Delta\Delta CT}$. Relative quantity values were normalized to the internal control gene, 18s rRNA since its expression did not fluctuate in liver tissues in response to the experimental treatments.

6.3.6 Meat quality

At 24 h after killing, the breast muscle pH was tested at a depth of 2.5 cm below the surface. This was done using a combined glass-penetrating electrode (Ingold, Mettler Toledo, Greifensee, Switzerland). Color measurements were assessed on the carcass surface over the breast muscles and on a freshly exposed cut surface of muscle. A Minolta CR-300 chromameter (Minolta, Osaka, Japan) was set to the L* (lightness), a* (redness), and b* (yellowness) CIE scale.

The Water Holding Capacity was determined on breast muscle using the method of Jauregui et al. (1981), with some modifications. Briefly, 1.5 ± 0.3 g of lean muscle

were inserted into a pre-weighed (W1) funnel made of 4 layers of grade 1 filter paper (Whatman International, Maidstone, UK). The funnel with the sample was weighed (W2), put into a centrifuge tube and centrifugated at 15000 rpm for 15 minutes at 4°C. Then, the muscle sample was removed from the funnel, and was weighed again (W3). The WHC was calculated as percentage of water weight lost from the sample, with the formula:

(P3-P1)/(P2-P1)*100

where: P3-P1 = water weight (absorbed by the paper), and P2-P1 = initial meat weight.

Each breast was weighted sealed in a plastic cooking bag and cooked by immersion in an 85°C water bath until the internal endpoint temperature was 80°C. Internal temperatures were assessed with cooking thermometers introduced into the thickest part of each of the breast muscle in each cooking batch. After cooking, the pieces were chilled by immersion into the bags in ice water bath for 30 min. Each piece was then removed from its bag and weighted (wtc) and cooking loss was calculated. Muscles were tempered at 20°C for 30 min to equilibrate temperatures and then six probes were obtained from the center of each muscle in line with the fibers. Finally, the tenderness of the cooked breast samples was determined as Warner Bratzler Shear Force (WBSF) by an Instron universal testing machine (Model 5542, Instron Engineering Corp., Canton, MA, U.S.A.); the analysis was performed on 6 shares (1.27 cm in diameter) from each sample. The shares were cut parallel to the longitudinal orientation of muscle fibres; the peak shear force was measured (Warner-Bratzler blade speed 200 mm/min), and mean values were recorded (expressed in Newton).

6.3.7 Cecum microbial count

The cecum content of each sacrificed chick was collected during necropsy. Each sample was placed in a small sterile container and immediately sent to the

laboratory for microbiological analysis. Three samples with the same treatment were pooled. The samples were placed in sterile plastic bags and kept refrigerated during the transport to the laboratory; the analyses were performed on the same day. From each sample, the caecum was isolated, and, after an external disinfection, the content was aseptically collected and used for the microbiological analyses. Pools of three subjects were randomly obtained (10 pools of caecum of males and 10 of females). From one to three grams of each sample were diluted 1:10 with saline solution (NaCl 0.85%, tryptone 0.1%) and homogenized by a Vortex for 40 s (MRC Lab, London, UK). Serial 10-fold dilutions were spread by sterile spatula onto MRS agar medium incubated in anaerobic jars (Anaerojar, Oxoid, Basingstoke, UK) with Anaerogen kit (Oxoid) at 37°C for 48 h (Oxoid) for the enumeration of Lactobacilli, and on TBX agar (Oxoid) incubated aerobically at 44°C for 24 h for the enumeration of *Escherichia coli* (ISO 16649-2). Other one to three grams of caecal content were diluted 1:10 with Buffered peptone water (Oxoid) and incubated at 37°C for the detection of *Salmonella* spp. according with ISO 6579 method. From the obtained counts, *Lactobacilli*/*E. coli* ratio was calculated as the log difference between the two parameters (Abu Tarboush, 1996).

6.4 Statistical analysis

The data were analyzed as a completely randomized design with a 2 × 2 factorial treatment arrangement by ANOVA using the MIXED procedure of SAS v. 9.2 (SAS Inst. Inc., Cary, NC). The statistical model included effects of sex (female or male), diet (CTR or AMT) and their interaction. The pen represented the experimental unit for growth performance, while individual chicks were the experimental units for the carcass yield, plasma lipid profile and hepatic gene expression. Treatment comparisons were done using a Tukey's honestly significant difference test for multiple testing. Probability values < 0.05 were considered to be significant.

6.5 Results and discussion

6.5.1 Growth performance and carcass yield

The effects of AMT on body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), feed conversion ratio (FCR) and mortality of female and male chicks are shown in Table 3. No significant interaction between diet and sex was observed. Males had higher BW than female chicks on days 0 ($P = 0.014$), 12 ($P < 0.01$) and 22 ($P < 0.01$). Compared to females, male chicks had higher ADG and ADFI from day 0 to 12 and from day 12 to 22 ($P < 0.01$), and lower FCR from day 12 to 22 ($P < 0.01$). However, mortality of females had a tendency to be lower than males ($P = 0.07$). AMT supplementation increased the BW compared to the CTR group on days 12 ($P = 0.02$), and AMT chicks had higher ADG compared to the CTR birds from day 0 to 12 ($P = 0.02$). In the male chicks, dietary AMT increased BW on days 12 ($P = 0.04$) and ADG from day 0 to 12 ($P = 0.02$) compared to the CTR chicks. AMT male chicks had lower FCR compared to the CTR males from day 22 to 44 and day 0 to 44 ($P = 0.047$ and 0.02). Our results that emulsifier supplementation increased growth performance of male chicks is in agreement with the findings of previous studies (Xing et al., 2004; Roy et al., 2010; Price et al., 2013). In addition, our previous work observed that the addition of emulsifier to the feed significantly increased the ADG and ADFI of broiler chicks (Bontempo et al., 2015). It is well known that emulsifiers can reduce the surface tension of water and increase the penetration and improve the distribution of water in press meal (van der Heijden and de Haan, 2010). In the current study, the AMT product was mixed with feed compounds before pelleting process, which may increase humidity, reduce pellet press energy consumption and improve pellet quality by modulating the moisture content during the pelleting process and, consequently, improve feed intake and performance of animals. Dierick and Decuypere (2004) reported that the addition of an emulsifier improved the digestibility of major nutrients, which may

reduce the viscosity of the digestive contents and increase the transit of the digesta as well as feed intake (Lázaro et al., 2004). In this study, the incorporation of vegetal bi-distillated oleic acid and glyceryl polyethyleneglycol ricinoleate may have also improved the growth performance of animals via the emulsification of supplemental fatty acids (Xing et al., 2004). However, there was no diet effect on growth performance of female chicks during the trial, which may be due to the low consumption of diet compared to the male chicks.

The effects of AMT on carcass characteristics of female (37 days of age) and male (44 days of age) chicks are shown in Table 3. No significant interaction between diet and sex was observed ($P > 0.05$). Males had higher dressing percentage than females ($P < 0.01$), while breast muscle percentage of males had tendency to be lower than females ($P = 0.07$). AMT supplementation increased dressing percentage compared to the CTR group ($P = 0.02$). Scheele (1997) noted that the growth of the pectoral muscles primarily occurs during the late stages of developmental growth in fast-growing birds. In the present study, an increased slaughter yield was also observed in the treated group, suggesting that the relatively rapid growth of the AMT-fed chicks in the finisher phase may contribute to the comparable increases in carcass yield.

6.5.2 Plasma lipid profile

The effects of AMT on plasma lipid profile of female (37 days of age) and male (44 days of age) chicks at slaughtering are shown in Table 4. No significant interaction between diet and sex was observed ($P > 0.05$). Males had higher plasma cholesterol, LDL and glucose contents than females ($P = 0.02$; $P = 0.04$; $P < 0.01$), and HDL content in plasma of male chicks had a tendency to be higher than female chicks ($P = 0.06$). However, low levels of plasma total protein and triglyceride were observed in males compared to females ($P = 0.013$; $P < 0.01$). AMT supplementation increased cholesterol, HDL and LDL contents compared to the CTR group ($P <$

0.01; $P = 0.02$; $P < 0.01$), whereas dietary AMT tended to reduced plasma urea content in comparison with the CTR group ($P = 0.098$).

6.5.3 Lipid and DNA contents and hepatic expression of relevant genes in liver samples

The effect of AMT on total lipid and DNA contents and hepatic expression of selected genes in the livers of female and male chicks are shown in Table 5. Males had lower lipid content and ApoA-I expression than the females ($P < 0.01$ and $P = 0.03$), and tended to reduce the lipid/DNA ratio ($P = 0.07$) and down-regulate the ApoB expression ($P = 0.09$). In the present study, the reduction of total lipid content and lipid/DNA ratio may suggest reduced hepatocellular lipid accumulation in the liver of male chicks. Male chicks had lower ApoA-I and ApoB expression and higher plasma cholesterol, HDL and LDL concentration than females, which is consistent with the previous finding by Kamanna and Kashyap (2008) that dietary nicotinic acid decreased HDL-ApoA-I catabolism whereas increased HDL half-life and concentrations of Lp(A-I) HDL subfractions, thereby augmenting cholesterol efflux and reverse cholesterol transport. However, no diet effect and significant interaction between gender and diet were observed in the hepatic parameters.

6.5.4 Meat quality and cecum microbial counts

The effect of AMT on meat quality and cecal microbial count of female and male chicks are shown in Table 6. Male chicks had higher a* (redness) value ($P = 0.04$), cooking loss percentage ($P < 0.01$) and shear force ($P = 0.07$) than the females. Dietary AMT increased b* (yellowness) significantly ($P < 0.01$) and reduced cooking loss ($P = 0.03$). There was a significant interaction between gender and diet in cooking loss ($P = 0.049$), which may be due to the different effect of AMT in females and males. Meat color is one of the first characteristics noted by customers, especially in boneless products, and is also an indictor of meat quality. In our study,

the source of variability of diet influenced the colorimetric indexes of muscles. The changes in b* (yellowness) in the breast muscle in the group fed the AMT diet may be explained by higher lipid content and there may be higher lipid-soluble pigments such as xanthophylls. However, no gender and diet effects and no significant interaction between gender and diet on *E. coli* and *Lactobacilli* counts were observed, which indicates that the supplementation of AMT and gender do not affect the gastrointestinal environment.

6.6 Conclusions

The current observations indicated that the supplementation of a novel synthetic emulsifier consisting of vegetal bi-distillated oleic acid and glyceryl polyethyleneglycol ricinoleate has the potential to improve the growth performance and plasma lipid profile of broiler chicks. In addition, male chicks showed better growth performance and lipid utilization than female chicks.

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6.8 Tables

Table 1: Ingredient and calculated analysis of the basal diet for broiler chicks (as-fed basis)

	Starter 1 (0-12 d)	Grower 2 (12-22 d)	Finisher 3 (22-44 d)
Ingredients, %			
Maize	45.17	37.31	30.28
Soybean meal, 49% CP	32.50	25.00	25.50
Wheat	15.00	15.00	20.00
Sorghum	0	10.00	15.00
Soybean full fat	0	5.00	0
Soybean oil	3.00	0	0
Animal fat	0	4.00	6.00
Sodium chloride	0.40	0.35	0.25
Calcium carbonate	1.20	1.00	0.95
Dicalcium phosphate 18%	1.50	1.2	1.00
DL-Methionine	0.34	0.30	0.25
L-Threonine	0.11	0.09	0.04
L-Lysine HCl	0.28	0.25	0.23
Premix[1]	0.50	0.50	0.50
EM[2]	-/0.10	-/0.075	-/0.05
Calculated nutrient content			
Metabolizable energy, MJ/kg	12.77	13.19	13.65
Crude protein, %	21.8	20.2	19.0
Crude fat, %	5.2	6.7	8.2
Calcium, %	0.92	0.80	0.70
Phosphorous, %	0.65	0.60	0.54

[1] Provided the following per kilogram of diet: vitamin A, 11250 IU; vitamin D_3, 5000 IU; vitamin E, 60 mg; $MnSO_4 \cdot 1H_2O$, 308 mg; $ZnSO_4 \cdot 1H_2O$, 246 mg; $FeSO_4 \cdot 1H_2O$, 136 mg; $CuSO_4 \cdot 5H_2O$, 39 mg; KI, 2.4 mg; Na_2SeO_3, 657 μg; 6-Phytase EC 3.1.3.26, 750 FTU; Endo-1, 4-beta-xylanase EC 3.2.1.8, 2250 U.

[2] AMT group: 1 g/kg during d 0-12; 0.75 g/kg during 12-22; 0.5 g/kg during d 22-44.

Table 2: Oligonucleotide primer sequences

Gene	Primer	Sequence	Accession number	Product size, pb
Apolipoprotein A-I (Apo A-I)	Forward	5'GTGACCCTCGCTGTGCTCTT3'	NM205525	217
	Reverse	5'CACTCAGCGTGTCCAGGTTGT3'		
Apolipoprotein B (Apo B)	Forward	5'GACTTGGTTACACGCCTCA3'	M18421	196
	Reverse	5'TAACTTGCCTGTTATGCTC3'		
18s rRNA	Forward	5'GCGGCTTTGGTGACTCTA3'	AF173612	194
	Reverse	5'CTGCCTTCCTTGGATGTG3'		

Table 3: Effect of Avi-Mul Top (AMT) on growth performance and carcass yield of female (37 days of age) and male (44 days of age) broiler chicks

	Interaction effect					Main effect						*P*-value		
	Female		Male			Gender			Diet					
Item	CTR	AMT	CTR	AMT	SEM	Female	Male	SEM	CTR	AMT	SEM	Gender	Diet	G × D
No. Pen	15	15	15	15										
BW, g														
d 0	40.54	40.39	40.89	40.97	0.18	40.46	40.93	0.13	40.72	40.68	0.13	0.014	0.84	0.55
d 12	368	370	378	388	2	369^{b}	383^{a}	2	373^{b}	379^{a}	2	<0.01	0.02	0.13
d 22	984	986	1070	1092	8	985^{b}	1081^{a}	5	1027	1039	5	<0.01	0.12	0.16
d 37	2333	2338	-	-	25	-	-	-	2333	2338	25	-	0.88	-
d 44	-	-	3411	3475	28	-	-	-	3411	3475	28	-	0.12	-
ADG, g/d														
d 0 to 12	27.30	27.47	28.09	28.88	0.21	27.39^{b}	28.49^{a}	0.15	27.70^{b}	28.18^{a}	0.15	<0.01	0.02	0.14
d 12 to 22	61.62	61.54	69.15	70.49	0.63	61.58^{b}	69.82^{a}	0.44	65.38	66.02	0.44	<0.01	0.32	0.27
d 22 to 37	89.93	90.20	-	-	1.38	-	-	-	89.93	90.20	1.38	-	0.89	-
d 0 to 37	61.97	62.11	-	-	0.68	-	-	-	61.97	62.11	0.68	-	0.88	-
d 22 to 44	-	-	106.4	108.3	1.2	-	-	-	106.4	108.3	1.2	-	0.27	-
d 0 to 44	-	-	76.60	78.06	0.64	-	-	-	76.60	78.06	0.64	-	0.12	-

(To be continued)

ADFI, g/d														
d 0 to 12	31.68	31.61	32.28	32.72	0.31	31.65[b]	32.50[a]	0.22	31.98	32.17	0.22	<0.01	0.55	0.42
d 12 to 22	83.53	84.43	92.29	94.45	0.79	83.98[b]	93.37[a]	0.56	87.91	89.44	0.56	<0.01	0.06	0.43
d 22 to 37	149.1	148.6	-	-	2.1	-	-	-	149.1	148.6	2.1	-	0.88	-
d 0 to 37	93.29	93.33	-	-	1.05	-	-	-	93.29	93.33	1.05	-	0.98	-
d 22 to 44	-	-	180.9	180.6	1.7	-	-	-	180.9	180.6	1.7	-	0.91	-
d 0 to 44	-	-	120.2	120.7	0.9	-	-	-	120.2	120.7	0.9	-	0.74	-
FCR														
d 0 to 12	1.161	1.151	1.149	1.133	0.010	1.156	1.141	0.007	1.155	1.142	0.007	0.14	0.19	0.74
d 12 to 22	1.356	1.373	1.335	1.340	0.007	1.364[b]	1.337[a]	0.005	1.346	1.356	0.005	<0.01	0.14	0.44
d 22 to 37	1.661	1.651	-	-	0.025	-	-	-	1.661	1.651	0.025	-	0.79	-
d 0 to 37	1.506	1.503	-	-	0.014	-	-	-	1.506	1.503	0.014	-	0.88	-
d 22 to 44	-	-	1.700[a]	1.669[b]	0.011	-	-	-	1.700[a]	1.669[b]	0.011	-	0.047	-
d 0 to 44	-	-	1.570[a]	1.547[b]	0.007	-	-	-	1.570[a]	1.547[b]	0.007	-	0.02	-
Mortality, %	1.33	0.33	2.00	2.33	0.72	0.83	2.17	0.51	1.67	1.33	0.51	0.07	0.64	0.36
Carcass yield, %														
Dressing	73.62	73.96	75.14	76.09	0.26	73.79[b]	75.62[a]	0.19	74.46[b]	74.95[a]	0.19	<0.01	0.02	0.24
Breast muscle	32.97	33.58	31.96	32.67	0.52	33.27	32.32	0.37	32.43	33.16	0.37	0.07	0.21	0.92

[a,b] Means listed in the same row with different superscripts are significantly different ($P < 0.05$).

Female = female broiler chicks; Male = male broiler chicks; CTR = basal diet without supplementation; AMT = CTR + Avi-Mul Top (1 g/kg from d 0 to 12; 0.75 g/kg from d 12 to 22; 0.5 g/kg from d 22 to 37 for females and from d 22 to 44 for males); Gender = gender effect; Diet = diet effect; G × D = interaction between gender and diet effects.

Table 4: Effect of Avi-Mul Top (AMT) on plasma lipid profile of female (37 days of age) and male (44 days of age) broiler chicks

	Interaction effect					Main effect						*P*-value		
	Female		Male			Gender			Diet					
Item	CTR	AMT	CTR	AMT	SEM	Female	Male	SEM	CTR	AMT	SEM	Gender	Diet	G × D
No. Chick	15	15	15	15										
Cholesterol, mg/Dl	115	125	123	134	3	120^{b}	128^{a}	2	119^{b}	129^{a}	2	0.02	<0.01	0.90
HDL, mg/dL	72.51	75.49	74.37	81.91	2.16	74.00	78.14	1.53	73.44^{b}	78.70^{a}	1.53	0.06	0.02	0.30
LDL, mg/dL	42.68	49.72	48.46	51.81	1.90	46.20^{b}	50.13^{a}	1.35	45.57^{b}	50.77^{a}	1.35	0.04	<0.01	0.34
NEFA, mmol/L	0.74	0.90	0.82	0.64	0.13	0.82	0.73	0.09	0.78	0.77	0.09	0.46	0.95	0.20
Glucose, mg/dL	231	230	256	256	4	230^{b}	256^{a}	3	244	243	3	<0.01	0.87	0.86
Total protein, g/dL	3.08	3.16	2.90	2.96	0.07	3.12^{a}	2.93^{b}	0.05	2.99	3.06	0.05	0.013	0.37	0.88
Triglyceride, mg/dL	55.85	68.90	37.57	36.26	4.34	62.37^{a}	36.91^{b}	3.07	46.71	52.58	3.07	<0.01	0.18	0.10
Urea, mg/dL	5.25	4.38	5.32	4.97	0.36	4.82	5.14	0.26	5.28	4.67	0.26	0.37	0.098	0.47

^{a,b} Means listed in the same row with different superscripts are significantly different ($P < 0.05$).

Female = female broiler chicks; Male = male broiler chicks; CTR = basal diet without supplementation; AMT = CTR + Avi-Mul Top (1 g/kg from d 0 to 12; 0.75 g/kg from d 12 to 22; 0.5 g/kg from d 22 to 37 for females and from d 22 to 44 for males); Gender = gender effect; Diet = diet effect; G × D = interaction between gender and diet effects.

Table 5: Effect of Avi-Mul Top (AMT) on total lipid and DNA content (%) and hepatic expression of selected genes in the liver of female (37 days of age) and male (44 days of age) broiler chicks

	Interaction effects					Main effects						*P*-value		
	Female		Male			Gender			Diet					
	CTR	AMT	CTR	AMT	SEM	Female	Male	SEM	CTR	AMT	SEM	Gender	Diet	G × D
n. chicks	10	10	10	10										
Lipid, %	9.49	9.67	6.54	7.39	0.79	9.58[a]	6.97[b]	0.56	8.01	8.53	0.56	<0.01	0.52	0.67
DNA, %	0.16	0.18	0.20	0.17	0.02	0.17	0.19	0.01	0.18	0.18	0.01	0.43	0.80	0.18
L:D ratio	89.39	57.67	37.24	44.79	17.41	73.53	41.01	12.31	63.31	51.23	12.31	0.07	0.49	0.27
n. chicks	5	5	5	5										
ApoA-I	1.00	0.96	0.46	0.38	0.24	0.98[a]	0.42[b]	0.17	0.73	0.67	0.17	0.03	0.81	0.94
ApoB	1.00	3.37	0.80	0.72	0.79	2.19	0.76	0.56	0.90	2.04	0.56	0.09	0.17	0.14

[a,b] Means listed in the same row with different superscripts are significantly different ($P < 0.05$).

ApoA-I = Apolipoprotein A-I; ApoB = Apolipoprotein B; Female = female broiler chicks; Male = male broiler chicks; CTR = basal diet without supplementation; AMT = CTR + Avi-Mul Top (1 g/kg from d 0 to 12; 0.75 g/kg from d 12 to 22; 0.5 g/kg from d 22 to 37 for females and from d 22 to 44 for males); Gender = gender effect; Diet = diet effect; G × D = interaction between gender and diet effects.

Table 6: Effect of Avi-Mul Top (AMT) on meat quality and cecal microbial count (log_{10} cfu/g) of female (37 days of age) and male (44 days of age) broiler chicks

	Interaction effect					Main effect						*P*-value		
	Female		Male			Gender			Diet					
Item	CTR	AMT	CTR	AMT	SEM	Female	Male	SEM	CTR	AMT	SEM	Gender	Diet	G × D
No. Chick	15	15	15	15										
pH_{24}	6.08	6.10	6.18	6.12	0.04	6.09	6.15	0.03	6.13	6.11	0.03	0.17	0.64	0.32
Colour														
L^* (lightness)	52.28	54.70	54.77	54.11	0.86	53.49	54.44	0.60	53.52	54.41	0.60	0.27	0.30	0.08
a^* (redness)	-1.72	-0.30	-0.12	-0.14	0.42	-1.01^b	-0.13^a	0.30	-0.92	-0.22	0.30	0.04	0.098	0.09
b^* (yellowness)	6.60	8.05	6.64	8.58	0.44	7.33	7.61	0.31	6.62^b	8.31^a	0.31	0.53	<0.01	0.58
WHC, %	34.89	33.21	36.07	35.27	1.01	34.05	35.67	0.72	35.48	34.24	0.72	0.12	0.22	0.66
Cooking loss, %	25.05^a	19.86^b	26.64^a	26.45^a	1.24	22.45^b	26.55^a	0.88	25.85^a	23.15^b	0.88	<0.01	0.03	0.049
Shear force, N	14.75	16.75	17.25	18.89	1.25	15.75	18.07	1.88	16.00	17.82	1.88	0.07	0.15	0.89
No. Chick	5	5	5	5										
E. coli	7.62	7.65	7.34	7.60	0.16	7.64	7.47	0.12	7.48	7.63	0.12	0.33	0.39	0.49
Lactobacilli	8.21	7.96	8.10	7.98	0.24	8.09	8.04	0.17	8.16	7.97	0.17	0.86	0.45	0.78

[a,b] Means listed in the same row with different superscripts are significantly different ($P < 0.05$).

WHC = water-holding capacity; Female = female broiler chicks; Male = male broiler chicks; CTR = basal diet without supplementation; AMT = CTR + Avi-Mul Top (1 g/kg from d 0 to 12; 0.75 g/kg from d 12 to 22; 0.5 g/kg from d 22 to 37 for females and from d 22 to 44 for males); Gender = gender effect; Diet = diet effect; G × D = interaction between gender and diet effects.

CHAPTER 7

7 General discussion

Reinvigorating animal agricultural research is essential to sustainably address the global challenge of food security. The global demand for food from animal agriculture is anticipated to nearly double by 2050. Increased demand is due, in part, to a predicted increase in world population from 7.2 billion to between 9 billion and 10 billion people in 2050. The increase in population puts additional pressure on the availability of land, water, and energy needed for animal and crop production (National Research Council, 2015). The growth in white meat (pork and poultry) production in developing countries from 1989 to 1999 has been remarkable - more than double the growth of red meat (cattle, sheep and goats). There are, however, major regional differences. Growth in poultry production has been spectacular in East Asia (11.7 percent p.a.) and South Asia (7.2 percent p.a.) and reflects the rapid intensification of the poultry industry in these regions. Latin America saw annual growth rates of 9 percent. Yet in sub-Saharan Africa the annual growth rate was only 2.6 percent. Red meat accounted for almost 37 percent of total meat production in the developing countries in the late 1980s, but declined to 31 percent in 1997/99 and this proportion is expected to decline further (http://www.fao.org/docrep/005/y4252e/y4252e07.htm#TopOfPage).

Worldwide, the Food and Agriculture Organization (FAO) estimates that there will be a 73 percent increase in meat and egg consumption and a 58 percent increase in dairy consumption over 2011 levels by the year 2050 (McLeod, 2011; National Research Council, 2015). Increased production can be achieved by a combination of expansion in animal numbers and increased productivity. Higher productivity is a compound of higher offtake rates (shorter production cycles by, for example, faster fattening), and higher carcass weight or milk or egg yields. The projections show that the increase in livestock numbers will remain significant, but less so than in the past. Higher carcass weights will play a more important role in beef production, while higher offtake rates (shorter production

cycles) will be more important in pig and poultry meat production. The changes in global demand for animal products and the increasing pressures on resources have important implications for the principal production systems found in developing countries (http://www.fao.org/docrep/005/y4252e/y4252e07.htm#TopOfPage).

Advances in animal agriculture have been a result of R&D and new technologies, particularly in areas such as food safety, genetics, reproductive efficiencies, nutrition, animal welfare, disease control, biotechnology, and the environment. Animal science research has improved animal productivity and thus decreased the costs of animal products to consumers, increased food safety and food security, decreased environmental impacts of livestock and poultry production, and addressed public concerns about animal welfare (National Research Council, 2015). Considerable challenges to the health and well-being of animals and humans are presented by animal disease pathogens. Understanding and developing effective measures to control animal infectious diseases are sometimes problematic mainly due to the lack of direct and continuous monitoring of the animal status as would otherwise occur with humans (National Research Council, 2015). A further challenge is the overuse of medically important antibiotics leading to an increased risk of infectious disease in humans and in animals, particularly with increasing globalization leading to more rapid spread of disease (National Research Council, 2015).

The World Health Organization (WHO) has recognized that the worldwide upswing in resistance to antibiotics is based on a combination of factors that includes "overuse in many parts of the world by both human and animals, particularly for minor infections, and misuse due to lack of access to appropriate treatment" (WHO, 2001; National Research Council, 2015). Feed additives have been used in livestock production for a long time and they are considered a viable alternative to replacing partial/total AGP (Summons, 1968). Today it is a

huge industry and according to one market report, the sector will be worth over $18 billion globally by 2018 (Watt Global Media, 2013; Lewis et al., 2015).

European livestock production is also a significant user of feed additives, particularly the pig and poultry industries (Watt Global Media, 2013; Lewis et al., 2015).

Additives used in piglet nutrition are nucleotides, which are intracellular compounds involved in numerous metabolic processes that are essential for cell growth (Mateo et al., 2004). Nucleotides are precursors of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), which are key sources of energy, especially in the form of ATP (de Santanal et al., 2015).

Diarrhoea in neonatal and early-weaned piglets due to enterotoxigenic Escherichia coli (ETEC) is an important problem in the pig farming industry. Entero-toxigenic Escherichia coli F4 (K88) is a major cause of diarrhoea and death in neonatal and weaned pigs (Francis et al., 1998) and K88-caused is the most prevalent form of diarrhoea in the pig farming industry (Fairbrother et al., 2005). In our study, where a chronic infection with E. coli K88 was successfully created, growth performance in piglets was not influenced in contrast with Superchi et al. (2011). Serum level of vitamin B12 was significantly higher in Treated group of piglets indicating that normal absorption occurred in ileum due to nucleotide supplementation, might increase VH: LPD in the ileum. The latter may be caused by improved intestinal health (Moore et al., 2009). Anyway the most interesting result obtained from this trial can be associated to the improvement of immune response of treated animals than control: Our finding of lower Hp serum content in nucleotide-fed animals when challenged than respective control groups are in agreements with others authors (Eurell et al., 1992; Petersen et al., 2003; Pineiro et al., 2007) who evidenced as the use of dietary nucleotides has positive effects on serum acute phase proteins content, especially haptoglobin. Similarly with the observation of many studies (Weble et

al., 1997; Llamas Moya et al., 2006), in our experiment, challenge with *E. coli* induced symptoms of acute inflammation response by reducing production of proinflammatory cytokines (IL-6, TNF-α, TLR2 and TLR4), anti-inflammatory cytokine (IL-10) after inoculation, while there was no difference of inflammatory cytokines concentrations. The benefits of nucleotide supplementation may be related to improvements in histological features and organ morphometry (Andrade et al., 2011). Furthermore, dietary nucleotides benefit gastrointestinal morphology and function, the immune system and the intestinal microbiota of monogastric species (Sauer et al., 2011; de Santanal et al., 2015).

Many plants have multifunctional beneficial properties due to their specific bioactive components. Most biologically active plant constituents are secondary metabolites, such as terpenoids (mono- and sesquiterpenes, steroids, etc.), phenolics (tannins), glycosides, and alkaloids (present as alcohols, aldehydes, ketones, esters, ethers, lactones, etc.) (Huyghebaert et al., 2011). These herbal extracts improve feed palatability, stimulate the secretion of endogenous enzymes, enhance nutrient absorption, modulate the intestinal microbiota and improve performance (Windisch et al., 2008). According to Lambert et al. (2001), the antimicrobial activity of extracts lies in their ability to disrupt bacterial cells by altering membrane permeability (de Santal et al., 2015).

According to literature, synergistic effects of polyphenols, responsible for their potent bioactive properties and the health benefit, are attributed to the complex mixture of phytochemicals present in whole foods. In addition, to support this sentence, individual components can change their properties in the presence of other compounds present in the extracts (Liu, 2003; Borchers et al., 2004; Pereira et al., 2007). In the first step of our study, we deepened the potential ability of a polyphenol-rich olive extract against different potential pathogenic bacteria. The results obtained on all the microorganisms, showed a rapid growth in the blank samples in 48 h at 20°C, reaching in almost all the cases the highest

absorbance recorded. With the increase of the concentration of the product in the broths, an evident inhibition was recorded with a decreasing optical density in all the times of measurements. The gram positive bacteria tested, *L. monocytogenes* and *S. aureus,* resulted to be more sensitive to the presence of the product especially after 36 and 48h of inoculation: the mechanism of action of this product against these microorganisms is not completely known. After the *in vitro* stage, where promising results were obtained, we extended the experiment with a second *in vivo* step with the aim to evaluate growing performance, cecum microbial population and the properties exerted by the product on meat oxidative stability (TBARS), pH and color. Taking into account these results and considering the economical sustainability in intensive farming, the two applied dosages for the *in vivo* trial were 0.1% and 0.5%. In the experimental design, an additional dietary treatment with inclusion of vitamin E was also included with the aim to compare its antioxidant potential with those exerted by polyphenols associated to meat characteristics and lipid oxidation.

The effects of polyphenol extract from olive on growth performances showed no detectable effect of dietary treatment and its interaction with time on Body Weight (BW), Average Daily Feed Intake (ADFI), Average Daily Gain (ADG). Feed conversion ratio (FCR) outlined a positive trend for treatment x time interaction (P=0.09) with T1 group showing higher values than CTR when both accounting for each experimental phase and overall the experimental period (P=0.07). Mortality ratio presented in this study showed a very low or absent value in animals treated with polyphenols compared to CTR and T1 animals (vitamin E supplemented): CTR and T1 showed the same percentage (2.2%) followed by T3 with 0.56 % and T2 with no dead animals. This datum may be associated to daily doses of olive oil, as a function of phenolic content that can obtain benefits against cardiovascular risk factors, probable main cause of death in this broiler chicks.

No differences were observed in carcass yield at slaughter under effect of administration of polyphenols and vitamin E. Partially in agreement with our results, King et al. (2014) reported that Dried Olive Extract did not affected the growing performance of broiler chicks. As reported by some authors, polyphenols are able to improve low-density lipoprotein (LDL) metabolism (Zhang et al., 1997; Yang et al., 2003), limiting the peroxidation of LDL (Pearson et al., 1998; Yokozawa et al., 2002) and fat (Sano et al., 1995) and inhibiting liver estrone glucuronidation (Zhu et al., 1998; Cao et al., 2005). Additionally, hydroxytyrosol, as the main polyphenol present in the extract of olive, exerted its more effective antioxidant activity in emulsion (Medina et al., 2009). In the present trial, the last dietary phase showed a decrease of FCR in T2: this could be probably associated to emulsification. This hypothesis can be explained with complete develoment of gastrointestinal tract: endogenous emulsifier produced by animal organism help hydroxytyrosol to explain better its antioxidant potential on overmentioned fat and LDL metabolism. Whether the beneficial effects of olive oil on the cardiovascular system are exclusively due to oleic acid remains to be elucidated but in experimental studies, phenolic compounds in olive oil showed strong antioxidant properties (Owen et al., 2000; Visioli et al., 2000). Oxidized low-density lipoprotein (LDL) is currently thought to be more damaging to the arterial wall than native LDL cholesterol (Navamb et al., 1996). Some researchers asserted that the effect of daily doses of olive oil can achieve benefits against cardiovascular risk factors (Covas et al., 2006), accounting for its phenolic content, lipid profile and decreasing effect on lipid oxidative damage and LDL cholesterol levels. The major diseases that can cause death in broiler chickens are sudden death syndrome; no other causes were found in death animals in present study. This desease is normally accompanied by excessive accumulation of abdominal fat (Giordani et al., 1994). In fact, some reports speculated that the cause of death was related to the faster growth rate (Leeson

and Summers, 1997), and metabolic disturbances ended with heart attack as cause of death (Gonzales et al., 1998; Gonzalez et al., 2000; Cao et al., 2005).

About cecal microbial population and relative calculated ratios, no influences due to the administration of polyphenols olive extract and vitamin E were observed in all microbial categories considered. In this case, no animal with weak intestinal condition were observed because of the optimal rearing conditions and the absence of stresses in the experimental facility. As regard of meat quality, evaluated with TBARS analysis, pH and colour, polyphenols olive extract did not delay lipid oxidation in breast chicken meats; only vitamin E showed a tendency to reduce oxidation at 9 days of storage ($P=0.075$) on the interaction between treatment and time. In agreement with several studies reviewed by Surai (2014), the antioxidant effect evidenced by vitamin E, was not exerted by polyphenols. In this study, the values of pH and colour were not influenced by dietary treatment in both measures on day 0 and 9. Fat colour were only measured at day 9 because of its consistency. It would be interesting to evaluate the potential of the administration of this type of polyphenols in animals subjected to the stress of intensive livestock production, where more significant results due to potential antimicrobial and antioxidant activity could be obtained.

Another potential approach is the use of technological additives to improve livestock diets. Feed intake and feed type can be adjusted with reduction of dietary crude protein, reducing also ammonia emissions from livestock excreta (Hayes et al., 2004). Taking this a step further, research has also been undertaken to explore the potential of diet supplementation to deliver environmental benefits (FAO, 2011). Certain substances added to livestock feeds (i.e. feed additives) can be used to improve animal digestive processes such that nutrients are used more efficiently leading to a reduction in waste production. (Lewis et al., 2015)

It is well known that emulsifiers can reduce the surface tension of water and increase the penetration and improve the distribution of water in press meal (van der Heijden and de Haan, 2010). In the current study, the emulsifier product was mixed with feed compounds before pelleting process, which may increase humidity, reduce pellet press energy consumption and improve pellet quality by modulating the moisture content during the pelleting process and, consequently, improve feed intake and performance of animals. Lecithin, as an emulsifier, has been reported to enhance the apparent digestibility of unsaturated fatty acids of lard (Soares and Lopez-Bote, 2002). Dierick and Decuypere (2004) reported that the addition of an emulsifier improved the digestibility of major nutrients, which may reduce the viscosity of the digestive contents and increase the transit of the digesta as well as feed intake (Lázaro et al., 2004). In a study, the incorporation of vegetal bi-distillated oleic acid and glyceryl polyethyleneglycol ricinoleate may have also improved the growth performance of animals via the emulsification of supplemental fatty acids (Xing et al., 2004). The first phase of our investigation on emulsifier was conducted on weaned piglets and broiler for fattening. Effects of a synthetic emulsifier, consisting of vegetal bi-distillated oleic acid emulsified with glyceryl polyethyleneglycol ricinoleate, showed that the addition of this novel product to the feed, significantly increased ADG and ADFI of chickens for fattening and tended to increase ADFI of weaned piglets during the entire experimental period, in agreement with the findings of previous studies (Xing et al., 2004; Roy et al., 2010; Price et al., 2013). The increased growth and feed intake may be due to certain effects of the dietary emulsifier on pellet quality and fat digestibility (Jones et al., 1992; Roy et al., 2010; Bontempo et al., 2015).

A second specific study was conducted to investigate the influence of a novel synthetic emulsifier product (AVI-MUL TOP) on growth performance, plasma lipid profile and hepatic expression of genes related to lipid metabolism in female and male broiler chicks.

The increased growth performance of male chicks supplemented with the emulsifier is in agreement with the findings of previous studies (Xing et al., 2004; Roy et al., 2010; Price et al., 2013). In addition, in our previous work we observed that the addition of emulsifier to the feed significantly increased the ADG and ADFI of broiler chicks (Bontempo et al., 2015). However, there was no diet effect on growth performance of female chicks during the trial, which may be due to the low consumption of feed compared to the male chicks. Scheele, (1997) noted that the growth of the pectoral muscles primarily occurs during the late stages of developmental growth in fast-growing birds. In the present study, an increased slaughter yield was also observed in the treated group, suggesting that the relatively rapid growth of the AMT-fed chicks in the finisher phase may contribute to the comparable increases in carcass yield. In the present study, the reduction of total lipid content and lipid/DNA ratio may suggest reduced hepatocellular lipid accumulation in the liver of male chicks. Male chicks had lower ApoA-I and ApoB expression and higher plasma cholesterol, HDL and LDL concentration than females, which is consistent with the previous finding by Kamanna and Kashyap, (2008). They observed that dietary nicotinic acid decreased HDL-ApoA-I catabolism whereas increased HDL half-life and concentrations of Lp(A-I) HDL subfractions, thereby augmenting cholesterol efflux and reversing cholesterol transport. However, no diet effect and significant interaction between gender and diet were observed in the hepatic parameters. Indeed, no gender and diet effects and no significant interaction between gender and diet on E. coli and Lactobacillus counts were observed, which indicates that the supplementation of AMT and the gender do not affect the gastrointestinal environment. Additionally no effects on meat quality of chicken breast fed with AMT were detected. The current observations indicated that the supplementation of a novel synthetic emulsifier consisting of vegetal bi-distillated oleic acid and glyceryl polyethyleneglycol ricinoleate has the

potential to improve the growth performance and plasma lipid profile of broiler chicks. In addition, male chicks showed better growth performance and lipid utilization than female chicks.

Poultry production is a global industry providing a major source of meat. It faces competition for feed ingredients by other animal industries such as pork and aquaculture. Health and nutrition play a key role in livestock production and research in these fields are essential to identify alternative feed ingredients, increasing digestibility of existing ingredients, and to find novel ways to use new ingredients and feed additives.

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CHAPTER 8

8 Summary

The global demand for food from animal agriculture is anticipated to nearly double by 2050. The growth in white meat (pork and poultry) production in developing countries between 1989 and 1999 has been remarkable - more than double the growth of red meat (cattle, sheep and goats). Increased production can be achieved by a combination of expansion in animal numbers and increased productivity. Higher productivity is a compound of higher offtake rates (shorter production cycles by, for example, faster fattening), and higher carcass weight or milk or egg yields. The projections show that the increase in livestock numbers will remain significant, but less so than in the past. Higher carcass weights will play a more important role in beef production, while higher offtake rates (shorter production cycles) will be more important in pig and poultry meat production.

In order to increase health and feed efficiency in monogastric animals, four trials were carried out to evaluate the role of some additives used to increase health in post-weaning piglets and in poultry meat production.

The aim of first study was to evaluate the effects of nucleotides administration to post-weaning piglets on ileal proinflammatory interleukin gene expression. Thirty-six weanling piglets (28 d of age, 7.85 ± 0.25 kg L.W.) were used in 28 days study. Piglets were allotted to 2 homogeneous groups (C, T) and fed the basal diet supplemented with oral nucleotides (UMP 88.05%, GMP 5.51%, AMP 3.82%, CMP 1.94% and IMP 0.68%; 0.8 g/piglet/day in 2.1 ml water solution) or saline. On day 14 both experimental groups were challenged with 1x109 CFU/g E. coli 0149:F4(K88). Growth performance and faecal score were evaluated weekly, while blood samples for immunological serum parameters, Fe and Vitamin B12 serum content were collected on days 0, 13, 18 and 26. Proinflammatory IL1a, IL1b, IL6, IL10, and TNF, TLR2 and TLR4 gene expression in ileal Peyer patches were evaluated at slaughtering after individual tissue sample collection by RT-PCR and β-actin as housekeeping gene. Growth

performances were not affected by dietary treatment, while faecal score was ameliorated in T piglets after one week on trial ($P< 0.05$). Haptoglobin serum content was decreased in treated pigs ($P<0.01$). Dietary treatment did not affect serum Fe content, while vitamin B12 level was higher in nucleotide-fed animals ($P< 0.05$). At slaughtering IL6, IL10, TNF, TLR2 and TLR4 gene expression were decreased in nucleotide-fed pigs ($P< 0.01$). By these findings, dietary nucleotides supplementation in post-weaning piglets can positively affect gut health status, ameliorating inflammatory response and digestibility of nutrients in microbial stress conditions.

Second study was carried out with the aim to investigate the antimicrobial potential of a polyphenol-rich olive extract *in vitro* and its effects on growth performance, caecum microbial population, slaughtering performance and meat quality. *In vitro* antimicrobial trials were performed on test cultures (*E. coli* ATCC 25922, *L. monocytogenes* ATCC 7644, *S. Enteritidis* ATCC 12592 and *S. aureus* ATCC 6833); the growth was assessed every 12 h for 48 h as optical density of broth cultures added with different concentrations of the product (0.1/0.5/1/5%) incubated at 20°C; an evident inhibition was observed with the increasing of the product concentration and a total inhibition was observed with the highest one. Considering these results, an in vivo trial was performed. A total of 720 female ROSS308 broiler chicks with day-old were allocated into 4 treatments with different dietary treatments (basal diet supplemented without (CTR) or with vitamin E (T1, 200 I.U. /kg for each dietary phase) or with Polyphenols olive extract (T2, 1g/kg and T3, 5 g/kg respectively in each dietary phase). Each group consisted of 9 pens with 20 birds per pen. Growth performances were determined at days 0, 10, 20 and 35. At day 35, two birds of each pen were chosen on average pen weight basis and slaughtered: from one was collected the cecum for microbial analyses and for the determination of the dressing and breast muscle percentages and the other one was collected for the

measurements of pH, TBARS content and color at day 0 and after 9 days, mimicking the commercial preservation after slaughter.

Supplementation with polyphenols olive extract showed only a tendency in Feed Conversion Ratio, in particular in the three phase of administration and in overall performance period (0-35 d) of animals (respectively P=0.09 and P=0.07) and T2 result lower compared to control group. No other evidence was observed in growth performances measured included carcass measures on dressing and breast muscle percentage. In addition, no differences were observed in cecum microbial population and gut health indexes. TBARS showed a statistical tendency (P=0.075), T1 resulted with lower oxidation if compared to all other groups (P<0.05); pH value and color of breast, skin and thigh, expressed with L*, a* and b*, did not explain any statistically difference. In conclusion in addition to promising in vitro data obtained, it would be interesting to evaluate the potential of the administration of this type of polyphenols in animals subjected to the stress of intensive livestock production, where more interesting results due to potential expressed with antimicrobial and antioxidant activity could be obtained.

Third and fourth trials were carried out to evaluate different effects of a novel synthetic emulsifier.

First step, the third trial, consist in two experiments conducted to evaluate the effects of a novel synthetic emulsifier product (AVI-MUL TOP) on the growth performance of chickens for fattening and weaned piglets. The emulsifier product consists of 50% vegetal bi-distillated oleic acid emulsified with 50% glyceryl polyethyleneglycol ricinoleate. In Exp. 1, four hundred and eighty 1-d-old female Cobb500 chickens for fattening were assigned to two treatments: 1) a control diet (CTR); and 2) the control diet + the emulsifier (AMT, 1 g/kg from day 0 to day 10, 0.75 g/kg from day 10 to day 20 and 0.5 g/kg from day 20 to day 34). AMT supplementation increased body weight on days 20 and 34 (P <

0.01). Dietary AMT increased the average daily gain (ADG) and average daily feed intake (ADFI) from day 10 to day 20, from day 20 to day 34 and from day 0 to 34 ($P < 0.01$). A reduced feed conversion ratio was observed in the AMT group from day 10 to day 20 ($P < 0.01$). In Exp. 2, ninety-six Stambo HBI × Dalland piglets were weaned at 24 days and assigned to 2 treatments (the basal diet without the product (CTR) or with 2 g/kg emulsifier from day 0 to day 14 and 1.5 g/kg from day 14 to day 42 (AMT)). There was an increase in the ADFI associated with AMT supplementation from day 14 to day 42 ($P = 0.04$). These results indicated that supplementation with the synthetic emulsifier may significantly improve the growth performance of chickens for fattening and numerically improve that of weaned piglets.

Second step consist in to investigate the influence of a novel synthetic emulsifier product (AVI-MUL TOP) on growth performance, plasma lipid profile and hepatic expression of related genes in female and male broiler chicks. A total of 1200 ROSS308 broiler chicks with day-old were allocated into 4 treatments with a 2 × 2 factorial design comparing sex (female or male) and different dietary treatments (basal diet supplemented without (CTR) or with the emulsifier (AMT, 1 g/kg from d 0 to 12, 0.75 g/kg from d 12 to 22 and 0.5 g/kg from d 22 to 44, respectively)). Each group consisted of 15 pens, 20 birds per pen. Growth performance was determined at days 0, 12, 22 and 44 (37 for females). At day 44 (37 for females), one bird of each pen was chosen and slaughtered to collect blood and livers samples and determine the dressing and breast muscle percentages. Males had higher BW than female chicks on days 0 ($P = 0.014$), 12 ($P < 0.01$) and 22 ($P < 0.01$). Compared to females, male chicks had higher ADG and ADFI from day 0 to 12 and from day 12 to 22 ($P < 0.01$), and lower FCR from day 12 to 22 ($P < 0.01$). AMT supplementation increased the BW compared to the CTR group on days 12 ($P = 0.02$), and AMT chicks had higher ADG compared to the CTR birds from day 0 to 12 ($P = 0.02$). AMT male

chicks had lower FCR compared to the CTR males from day 22 to 44 and day 0 to 44 ($P = 0.047$ and 0.02). However, there was no diet effect on growth performance of female chicks during the trial. AMT supplementation increased dressing percentage compared to the CTR group ($P = 0.02$). In addition, dietary AMT increased cholesterol, HDL and LDL contents compared to the CTR group ($P < 0.01$; $P = 0.02$; $P < 0.01$). In conclusion, supplementation of AMT may have beneficial effect on growth performance and plasma lipid profile of broiler chicks.

CHAPTER 9

9 Acknowledgements

Thanks God, he's still alive!