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PhD IN VETERINARY SCIENCE

XXX CYCLE

Thesis

Use of alternative protein sources in poultry and fish nutrition

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Summary

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The global increase of the world population started during the ‘60s and the most credited FAO estimates predicted that by the mid-twentieth century the human population will still grow to reach about 9 billion.
Likewise, in order to satisfy the nutritional needs of both humans and livestock and pet animals, the demand of raw materials and in particular protein sources will increase. It is estimated that in the near future the production of meat will increase of 50%, and the demand of fish, milk and eggs of 75%.
The most important protein sources used in animal nutrition are the soybean meal and the fish meal; however, the production of these two raw materials are linked to several environmental problems.
Recently, the researchers are studying the use of innovative protein sources alternative to the soybean and fish meal presenting the best possible characteristics such as: environmental sustainability, low production costs, high nutritional value and no antinutritional factor content. The insects and the processed animal proteins (PAPs) could be suitable protein sources in poultry and fish nutrition.
The present thesis includes 6 experimental trials conducted along the three years of my PhD in Italy and in Greece in order to evaluate the possible utilization of innovative and alternative protein sources in poultry and in fish species.
Regarding poultry, the studies has been conducted in Italy. The first step was to investigate the in vitro crude protein digestibility of different insect meals from *Tenebrio molitor* and *Hermetia illucens* and their correlation with chemical composition traits. The results showed that chitin is the main constituent of insect body able to affect the crude protein digestibility of *Tenebrio molitor* and *Hermetia illucens* larvae meals. Based on these results two in vivo trials were carried out on the use of the insect meals in the poultry diets.
The first in vivo study was carried out on 80 broilers (from 30 to 62 days of age) equally divided into two groups fed, respectively, a corn-soybean meal based diet and a diet in which *Tenebrio molitor* larvae meal totally replaced the soybean meal. The diets were isoproteic and isoenergetic. No differences were observed on growth parameters, physical and chemical properties of the meat between the groups. The presence of the chitin in the insect meal increased the length and the weight of the full intestine, the weight of the spleen, the production of the caecal volatile fatty acids; and it affected negatively the apparent ileal digestibility of nutrients and in particular of the proteins. In addition, broilers fed the insects had a lower albumin to globulin

ratio in the blood samples than the group fed soybean meal and this suggests a better disease resistance and immune response of birds linked to the prebiotic activity of the chitin.
The second in vivo study was carried out on 108 laying hens (from 24 to 45 weeks of age). Following a scheme similar to that of the previous trial, the hens were equally dived into two groups fed two isoproteic and isoenergetic diets. The control group fed a corn-soybean meal based diet and the other group fed a diet in which an *Hermetia illucens* larvae meal was included as total replacement of the soybean meal. The use of the insect meal did not show negative effects on the health status of the animals and on the feed conversion ratio. However, the complete replacement of soybean meal showed negative effects on the feed intake and, as a consequence, on the laying performance. The chitin content of the the Hermetia illucens diet negatively affected the ileal apparent digestibility of the nutrients and, in particular, of the proteins. The eggs quality was positively affected by the insect meal based diet, in particular the yolk resulted richer in PUFA, n-6 and n-3 and showed lower cholesterol content than the hens fed on the soybean meal based diet.
The three studies on fish species were carried out in Greece. The first step was to evaluate the suitability of some processed animal proteins carrying out a preliminary in vivo digestibility trial; a control diet (with only fish meal) was compared to six isoproteic and isoenergetic diets including, respectively, poultry feather meal (FeM) alone (4 diets with 15, 25, 50 and 75% of fish meal substitution) and a mix of processed animal proteins meals (2 diets including feather meal, offal meal and blood meal (BOF) added to the diet in equal amounts to replace 50 and 75 % of the fish meal). The trial evidenced a decrease of nutrients digestibility for feather meal containing diets starting from the 15% fish meal substitution rate diet. The diets containing a mix of processed animal proteins, instead, showed better results, with the 50% fish meal substitution rate diet presenting values comparable to the control diet. A worsening of digestibility was registered, instead for the 75% substitution rate diet. After this preliminary trial two growth trials were carried out in order to evaluate the growth performance of the European sea bass fed on the only feather meal based diets (360 European sea bass juveniles) and the mix of ingredients previously described (225 juveniles European sea bass). The diets with the only inclusion of feather meal put in evidence that the only suitable diet was the 15% FM. The experimental diet with the mix of the ingredient showed promising results,

because the inclusion of 50% BOF showed growth performance results comparable to the control diet.

I Introduction

The English economist T.R. Malthus (1766-1834) was the first to describe the demographic increase in his book “An Essay on the Principle of Population”. He prophesied that the most important consequence of the human increase was the consumption of raw materials. The unavailability of food and feed would have led to the exploitation of new cultivable land with their consequent impoverishment, and in the meantime, the human population would continue to grow. Demographic increase would stop only when food and feed shortages would have led to wars, famines and epidemics. Furthermore, Malthus was convinced that the human population would have reached 9 billion individuals at the end of the Nineteenth Century. The English economist's assumptions were wrong, as he had not taken into account that wars and epidemics contained the human population increase.

During the 1960s, after about 20 years since the end of World War II, improvement in hygiene conditions, medical and scientific progress, economic boom, increased longevity, and decreasing neonatal mortality led to a new increase in human population.

The global increase of world population during the 1960s was about 75millions (+ 1.1% of human population). In the 2012 the world population reached 7billions, according to the most credited FAO estimates (2013) and the same FAO predicted that by the mid-twentieth century the human population will still grow by two billions, to reach about 9 billions.

Likewise, in order to satisfy all human needs, but also for livestock and pet animals will increase the demand of raw materials and in particular protein sources. It is estimated that in the near future it will increase the production of meat of 50%, and it is expected that the demand of fish, milk and eggs will increase of 75%. (FAO, 2013).

The most important protein sources used in animal nutrition are the soybean meal (SBM) and the fish meal (FM) the production of these two raw materials is linked to several environmental problems (Mungkung et al 2013, Sanchez Muros et al.,2014).

The increase in soy production caused: the deforestation of one million hectares of Brazilian rainforest (Carvalho 1999, Osava 1999), threatening animal and plant biodiversity, a massive utilization of water (Steinfeld et al., 2006), fertilizer and pesticides (Carvaho, 1999), responsible for the impoverishment of the land (Osava et al., 1999).

The quantity and the quality of fishmeal is not constant, because this protein source is obtained from:

- The catch
- Fish stocks harvested for this purpose, such as herring, anchovy, horse mackerel (they are small, bony and rich in lipids)
- Trimming and offal left over from the fish processing for human consumption (FIN, 2008).

The fishing contributed to the pollution and deterioration of the marine environment, for this reason the quantity of fish caught decreased and increased the fish meal price. The increasing price of fish meal has a negative impact on aquaculture, in particular on carnivorous fish because need a high amount of protein.
In recent years, researchers are studying the use of alternative protein sources to soybean and fish meal that have characteristics such as:

- The environmental sustainability
- low production costs
- high nutritional value
- no antinutritional factor.

The aim of this PhD thesis was to investigate the use of insect larvae meals and protein animal processed (PAP) as a possible protein sources alterative to soybean and fish meal in animal nutrition (in particular broilers chicken, laying hens and sea bass).

This thesis is composed by six experimental trials:

- *In vitro* crude protein digestibility of *Hermetia illucens* and *Tenebrio molitor* larvae meal.
- *Tenebrio molitor* larvae meal as a possible alternative protein to soybean meal in broiler chickens
- *Hermetia illucens* larvae meal as a possible alternative protein to soybean meal in laying hens
- *In vivo* digestibility of feather meal (FeM), blood meal (BM) and offal meal (OM) in sea bass
- Growth trial carried out on juveniles sea bass (Dicentrarchus labrax) fed on feather meal at different level of inclusion (15- 25-50% of protein inclusion)

- A mix of processed animal protein (Feather meal, offal meal and blood meal) as a suitable alternative protein in the juveniles sea bass in growth.

The trials regarding the use of insect larvae meal in broilers and lying hens were performed in two private farms located in southern Italy while analysis of diets, blood and animal products were performed at the Department of Veterinary Medicine and Animal Production of University of Napoli Federico II.
The three trials on the use of processed animal protein (PAP) in sea bass nutrition were designed and performed at the laboratories and facilities of Hellenic Center for Marine Research (HCMR), located in Heraklion (Crete, Greece).

The insects are a suitable protein source.
The use of the insect as food and feed can be promoted for health, environmental, economic and social factors.
Insects are healthy, nutritious alternatives to mainstream staples such as chicken, pork, beef and even fish (from ocean catch). Many of them are rich in crude proteins and fat, and presents a high amount in micro and macronutrients such as calcium, iron and zinc. In many regions of the world they are considered a delicacy and are important to prevent the malnutrition.
Insects promoted as food emit considerably fewer greenhouse gases (GHGs) than most livestock (methane, for instance, is produced by only a few insect groups, such as termites and mealworms). Insect rearing is not necessarily a land-based activity and does not require land clearing to expand production. Feed is the major requirement for land (FAO, 2013).
The ammonia emissions associated with insect rearing are also far lower than those linked to conventional livestock, such as pigs (FAO, 2013).
Because they are cold-blooded, insects are very efficient in converting feed into protein (crickets, for example, need 12 times less feed than cattle, four times less feed than sheep, and half as much feed as pigs and broiler chickens to produce the same amount of protein). Insects can be fed on organic waste streams (FAO, 2013).

The PAP as a suitable protein sources for animal nutrition.
The PAP can be suitable because present a high nutritional value and for environmental and economic reasons.

Processed animal proteins come from animal by-products, mostly consisting of non-edible parts of animals reared for human consumption (such as fat trims, meat viscera, blood, bones, feathers, hides and skins), and are included in the category 3 of the animal by products. Between 32 and 48% of the weight of food-producing animals is removed during slaughter and further meat processing (Alm, 2012a).
Generally, the processed animal proteins are very rich in crude proteins (and in essential aminoacids), micronurients and macronutrients (such as calcium and phosphorus), have a variable fat content (the viscera are richer in fat than the blood), and generally are palatable (except same of them such as the blood meal, that is considered unpalatable for the hemoglobin content).
According to European's authorities, around 18 million t of animal fat and meat industry by-products arise annually in the European Union (EU) from slaughterhouses, dairies and plants producing food for human consumption. Another 8 to 12 million t emerge every year as former foodstuffs. Recycling of slaughter by-products and other animal products, sometimes considered as waste materials, into animal feed can bring major benefits to the economics of livestock production and the environment in the EU (Jedrejeck et al.,2016).

Standing these preliminary considerations, the insects and the PAP seem to have an interesting environmental and economic sustainability associated to a high nutritional value. These properties induced in the recent years the European Union to modify the legislation to allow at least part of the use of these as ingredients for feed (and in the case of insects also as food).
The European legislation on novel food and feed will be described in the subsequent chapters.

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The major protein sources used to product feed are fish meal and soybean meal.

Fish meals

Fish meals have been used as feedstuffs since 19th century in Northern Europe and they are now used worldwide. Global production of fish meal has been stable for the past two decades at around 5 to 6 million tons, Peru and Chile being the main producers.

The fresh raw fish and the fish trimmings are cooked, pressed, dried and ground in order to produce the fish meal (IFFOO, 2006); the final product presents a brownish colour.

The quality and type of fish meal depends not only on the fish species and on fisheries by- products used but also on the processing technology adopted.

The most important sources to produce the fish meal are:

- Small, bony and fat fish (as anchovy, horse mackerel, menhaden, capelin, sand eel, blue whiting herring) that are captured specifically to this purpose;
- by-catches of capture fishery activity;
- by-products deriving from fish processing considered unpalatable or fast spoiling (as trimmings and viscera) (FIN, 2008).

According to IFOMA (2001), fish meal is an excellent source of highly digestible protein, long chain omega-3 fatty acids (EPA and DHA) and essential vitamins (as B and D) and minerals (calcium, potassium, phosphorous).

The raw material is composed of:

- solids (include fat- free dry matter)
- oil
- water.

The raw materials at first are cooking at temperatures between 85-90° C, after that the cooked fish is pressed through a screw press in order to remove the water, at the end of the pressing procedure is obtained a “press-cake”. The water obtained from the pressing procedure are decanted and the surnatant is centrifuged in order to obtain the “stick- water”; after this product is concentrated through evaporation.

The final step to produce the fish meal is drying the press cake and the stick water previously mixed together; the final product presents a moisture of the 10%. At each of these processing steps, there can be variations, leading to obtain fish meals of variable qualities (FAO, 1986).
A fish meal of good quality usually presents a crude protein content of about 66%, lipids content between 8-11%, and ash less than 12%. Other fish meal processing by-products include fish protein concentrates with high protein levels (more than 70%) (Kaushik, 2010).

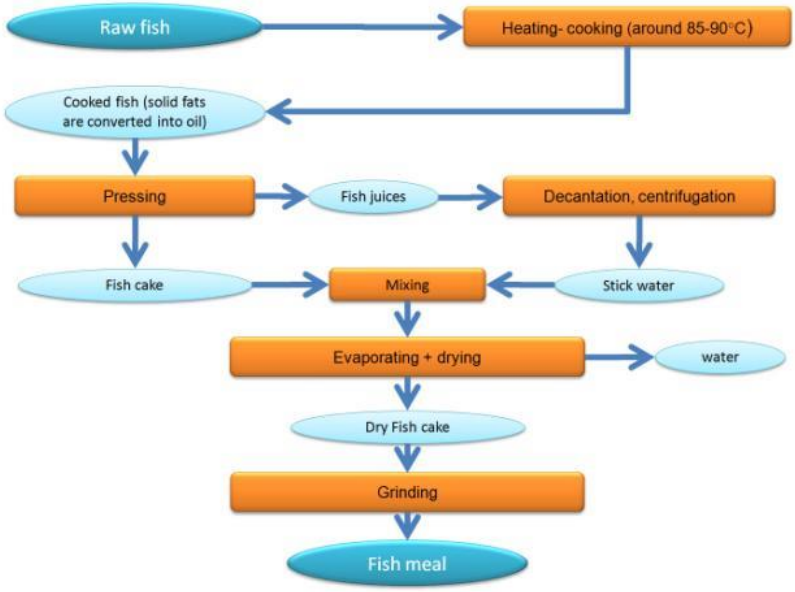

Figure 2.1: Processing Fish meal (Credits: Valérie Heuzé, Gilles Tran / AFZ).

Figure 2.2: Fish meal (Credits: Sadasivam Kaushik / INRA - https://www.feedipedia.org/node/208

A major portion (more than 60%) of fish meal produced globally is used for aquaculture (farming of finfish and shrimp). The intensification of aquaculture in Asia, and particularly in China, is increasing the demand for fish meal even though the supply cannot grow accordingly. Natural phenomena such as the El Niño-Southern Oscillation affect the fisheries along Central American Pacific coasts, and the impoverishment of natural stocks due to overfishing, had led to seasonal scarcities and increased prices. Due to these factors, the fish meal market is volatile and prices often shoot up. The search for suitable and cost-effective alternative protein sources to be used in industrial aquafeeds will be the most critical factor in the development of intensive aquaculture in Asia (Kaushik, 2010; Steinfeld et al., 2006), where aquaculture is expending at very high rate, or in fish species requiring high protein content diets such as carnivorous fish mainly reared in developed country.

The best quality fish meal is obtained from raw fish. However, in order to prevent protein and oil breakdown, raw fish is often processed by draining, chilling (chilled water systems, mixing of ice with fish) or chemical preservation (with sodium nitrite or formaldehyde).

Given that the indispensable amino acid profile of fish meal reflects that of the ideal protein pattern for fish or shrimp, fish meal is a major protein source in aquaculture. Protein digestibility of good quality fish meal is very high with equally high amino acid availability (Anderson et al., 1995). Fish meal is also a source of essential fatty acids, minerals and trace elements.
Currently available data show that out of the 6 million tons of fish meal available globally, more than 65% is used in feeds for fish and crustacean farming. The levels of incorporation of fish meal can range from 40 to 60% in feeds for marine fish to less than 5% in feeds for carp, catfish or tilapia (Tacon et al., 2008). Most cyprinids (carp) reared in semi-intensive ponds are fed with feeds practically devoid of fish meal. In recent years, much progress has been made towards the substitution of fish meal by mixtures of different plant protein sources even in intensively-reared salmonids or marine finfish, thus leading to significant economy as well as addressing sustainability issues (Kaushik, 1990; Kaushik et al., 2004; Kaushik et al., 2008).
Like fish feed, feeds for marine or freshwater shrimp contain high levels of fish meal (up to 40%). However, plant ingredients are being increasingly incorporated as an alternative to fish meals, or other marine-derived protein sources such as shrimp meal or squid meal, in order to ensure the sustainable development of shrimp farming (Amaya et al., 2008).
Depending on the protein content we could identify three types of fish meal available on the market:

- fish meal high protein
- fish meal average protein (68-70%)
- fish meal low protein.

Table 2.1: Chemical composition of the fish meals

	High protein	**average protein**	**Low protein**
Dry matter (% as fed)	92.1	92.2	92.5
Crude protein (% DM)	75.4	70.6	48.4
ether extract (% DM)	11.0	9.9	10.3
Ash	13.6	18.4	35.2
Gross energy (Mj/Kg DM)	21.9	20.4	19.0

Minerals			
Calcium (g/Kg DM)	26.5	43.4	79.3
Phosphorus(g/Kg DM)	22.3	27.9	39.8
Potassium(g/Kg DM)	11.9	8.7	11.1
Sodium(g/Kg DM)	10.9	11.3	28.4
magnesium(g/Kg DM)	3.1	2.3	---
Manganese (mg/Kg DM)	10	16	---
Zinc(mg/Kg DM)	99	96	---
Iron(mg/Kg DM)	---	7	---
Copper(mg/Kg DM)	---	367	---
Alanine (%protein)	6.1	6.3	6.2
Arginine(%protein)	5.8	6.2	5.2
Aspartic acid(%protein)	8.7	9.1	9.6
Cysteine(%protein)	0.8	0.8	1.2
Glutemic acid(%protein)	12.6	12.6	12.6
Glycine(%protein)	5.9	6.4	7.0
Histidine (%protein)	2.2	2.4	2.4
Isoleucine(%protein)	4.3	4.2	4.1
Leucine(%protein)	7.0	7.2	7.5
Lysine(%protein)	7.5	7.5	7.0
Methionine(%protein)	2.8	2.7	2.6
Phenilalanine(%protein)	3.8	3.9	4.0
Proline(%protein)	3.8	4.2	4.8
Serine(%protein)	4.0	3.9	2.5
Threonine(%protein)	4.1	4.1	4.0
Tryptophan(%protein)	1.1	1.0	3.2
Tyrosine(%protein)	2.9	3.1	5.2
Valine(%protein)	4.9	4.9	

Contaminants and toxic substances

Since proteins and lipids from fish are highly degradable, adequate processing has to be performed in order to prevent protein breakdown into biogenic amines (especially histamines) or fatty acids breakdown into oxidized compounds. Bacterial development, although low, should be avoided given the low levels of moisture and the absence of carbohydrates.

Cooking fish meal above 80°C normally destroys bacteria but the whole chain-process is susceptible to re-infection (FAO, 1986).
Fish meal is also susceptible to chemical contamination with harmful substances (chlorinated hydrocarbons, dieldrin, lindane, PCBs, dioxins, heavy metals) (Erne et al., 1979), due to the accumulation of those anthropogenic substances in the marine food chain and finally in the fatty tissues of fish used for the manufacture of fish meal. The levels of such contaminants (PCBs, dioxins) in fish meal depend on the fish source: fish meals from Central America have lower levels than those from the Northern hemisphere (New et al., 2002).
Due to the ever increasing demand for fish meal and fish oil to be used in feeds for farmed fish and crustaceans, there has been concern that the over-reliance on capture fishery-derived fish products for aquaculture would contribute to the over-exploitation of certain types of fisheries, with concomitant effects on the stocks of other wild fish (Naylor et al., 2000). Based on current developments in fish feed formulations, it is now recognized that aquaculture contributes to global fisheries supply and does not deplete the marine fishery resources (Naylor et al., 2009). Besides, the fish meal industry has committed itself and set forth several stringent measures to ensure that the feed-grade fisheries respect sustainability criteria.

Soybean meal
Soybean meal is the most important protein source used to feed farm animals. It represents two-thirds of the total world output of protein feedstuffs, including all other major oil meals and fish meal (Oil World, 2015). Its feeding value is unsurpassed by any other plant protein source and it is the standard to which other protein sources are compared (Cromwell, 1999). While it has been an accepted part of livestock and poultry diets in the USA since the mid-1930s (Lewis et al., 2001), soybean feed production took off in the mid-1970s and then accelerated in the early 1990s due to a growing demand from developing countries. The expansion of aquaculture and prohibitions on the feed use of slaughterhouse by-products have also fuelled the demand for this high-quality source of protein (Steinfeld et al., 2006).
Soybean meal is the by-product of the extraction of soybean oil. Several processes exist, resulting in different products. Soybean meal is usually classified for marketing by its crude protein content. There are two main categories of soybean meal, the “high-protein” soybean meal with 47-49%

protein and 3% crude fibre, obtained from dehulled seeds, and the "conventional" soybean meal, with 43-44% protein, that contain the hulls. In solvent-extracted soybean meals, the oil content is typically lower than 2% while it exceeds 3% in mechanically-extracted meals (Cromwell, 2012). Soybean meal is available worldwide. In 2014, soybean meal production reached 190 million tons and accounted for 62.5% of oil meals (FAO, 2016). Main producers were China (54 MT), USA (37 MT), Argentina (29 MT), Brazil (27 MT), and EU-28 (10 MT). Main exporters were Argentina and Brazil (Oil World, 2015). The EU-28 was the most important importer of soybean meal (22 MT) followed by South-East Asian countries like Indonesia, Malaysia, Thailand and the Philippines (Oil World, 2015). In the EU-28, soybean meal represented 61% of the proteins used to feed livestock, 16% of compound feeds, and an amount of 24 MT (Booth, 2015).

Processes

There are 3 ways to extract soybean for oil and soybean meal. The most commonly used worldwide is solvent extraction. In the USA, virtually all soybeans (99%) are solvent extracted. This method effectively extracts the oil from the beans and only 1,5% residual oil can be found in the soybean meal. The second method consists in mechanical extraction of the soybean flakes with a screw press to extract oil, without using any solvent. This method produces less oil and a high fat soybean meal. The third method combines extruding and expelling of soybean flakes, and neither uses solvent for oil extraction (Johnson et al., 2004).

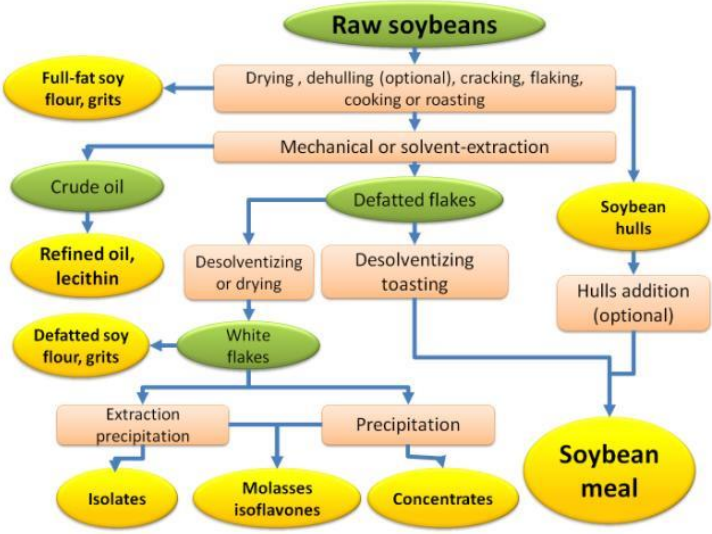

Figure 2.3: Soybean meal: Processing method (Credits: Gilles Tran, Valérie Heuzé, AFZ; www.feedipedia.org/node/674)

Environmental impact

The high phytate content of soybean meal requires supplementation with inorganic sources of phosphorus in monogastric animals. Dietary P in excess of animal requirements is excreted into the environment and becomes an environmental pollutant (Dilger et al., 2006).

In general and in comparison to other plant protein sorces, the high digestibility of the amino acids of soybean meal in diets for monogastrics and the high content of lysine allow the formulation of diets that contain less total protein and less excess nitrogen in the feed, thereby reducing nitrogen excretion into the biosphere (Pettigrew et al., 2008).

On the other hand, soybean meals are usually extracted with hexane, a solvent that is extremely flammable and non-biorenewable, poses health risks and is regulated as a hazardous air pollutant (O'Quinn et al., 1997).

There are three type of soybean meal used as ingredient in animal feed:

- low protein (non dehulled)
- high protein (dehulled)
- high oil (expeller).

The average nutritional values are reported in the table 2.

Table 2.2: Nutritional values of soybean meal

	Non dehulled	**Dehulled**	**High oil**
Dry matter (% as feed)	87.9	88.1	90.7
Crude protein (% DM)	51.8	53.5	49.3
Crude fibre(% DM)	6.7	4.9	4.9
NDF(% DM)	13.7	11.0	11.1
ADF(% DM)	8.3	5.9	5.9
Lignin(% DM)	0.8	0.5	0.5
Ether extract(% DM)	2.0	1.8	7.7
Ash(% DM)	7.1	7.2	6.8

Total sugars(% DM)	9.4	10.6	9.3
Gross Energy (MJ/Kg DM)	19.7	19.7	20.8
Minerals (g/Kg DM)			
Calcium(g/Kg DM)	3.9	3.6	4.6
Phosphorus(g/Kg DM)	6.9	7.6	7.2
Potassium(g/Kg DM)	23.7	25.1	21.0
Sodium(g/Kg DM)	0.1	0.1	0.2
Magnesium(g/Kg DM)	3.1	3.4	3.2
Manganese (mg/Kg DM)	45	40	
Zinc (mg/Kg DM)	54	57	
Copper (mg/Kg DM)	18	18	
Iron (mg/Kg DM)	346	169	129
Amminoacids (% protein)			
Alanine	4.4	4.3	4.3
Arginine	7.4	7.3	7.5
Aspartic acid	11.3	11.4	11.6
Cysteine	1.5	1.6	1.6
Glutamic acid	17.7	17.9	17.9
Glycine	4.2	4.2	4.2
Histidine	2.6	2.7	2.7
Isoleucine	4.6	4.6	4.6
Leucine	7.5	7.7	7.7
Lysine	6.1	6.3	6.3
Methionine	1.4	1.4	1.4
Phenilalanine	5.0	5.1	5.1
Proline	4.9	5.0	4.8
Serine	5.0	4.6	4.4
Threonine	3.9	3.8	3.7
Tryptophan	1.3	1.4	1.4
Tyrosine	3.5	3.5	3.5

Valine	4.8	4.8	4.5

Nutritional attributes

A highly palatable feedstuff, soybean meal is characterised by a high protein content (from 43 to 53%) and a low crude fibre content (less than 3% for the dehulled soybean meals). It has a very good amino acid balance and contains high amounts of lysine, tryptophane, threonine and isoleucine, which are often lacking in cereal grains. However, the concentration of cystine and methionine are suboptimal for monogastric animals, and methionine supplementation is necessary (McDonald et al., 2002). Amino acid digestibility is also very high (more than 90% for lysine in pigs and poultry) (Sauvant et al., 2004).

Soybean meal contains oligosaccharides such as raffinose and stachyose that cannot be digested by monogastric animals, due to the lack of a specific endogenous alpha-galactosidase. Raffinose and stachyose can cause flatulence and diarrhoea that may increase the digesta passage rate, and decrease digestion and absorption of dietary nutrients. In poultry, these oligosaccharides have been shown to decrease nitrogen-corrected true metabolizable energy, fibre digestion, and transit time (Parsons et al., 2000; Coon et al., 1990; Rackis, 1975 and Reddy, 1984 cited by Zuo et al., 1996). Low-oligosaccharide soybean meals are now available.

About 60-70% of phosphorus in soybean meal is bound to phytic acid, which is nutritionally unavailable to monogastric animals and reduces the availability of P and other minerals (Wilcox et al., 2000). Supplementation with inorganic phosphorus is required, and the addition of phytase may alleviate the problem. Low-phytate soybeans are under development but their productivity is still low (Waldroup et al., 2008).

Soybean meal is a poor source of B vitamins and lack of B vitamin supplementation in soybean meal-based diets may cause reproductive and performance problems in sows, older pigs and hens (McDonald et al., 2002).

Potential constraints

Variability

Soybean meal is a very consistent product and one of the least variable protein sources for animal nutrition (Smith, 1986). However, genetics, growing conditions, storage conditions and processes cause variations in its

composition and nutritional quality. Because soybean meal can be included in large amounts in animal diets, small changes in quality might translate into important changes in animal performance, therefore, it is necessary to monitor its quality very closely (Van Eys et al., 2004).

Antinutritional Factors

Soybean seeds contain antinutritional factors. Soybean meal usually undergoes several heat treatments that destroy heat-labile antinutritional factors (particularly trypsin inhibitors and lectins) but it is necessary to assess whether the meal was adequately processed. Inclusion of soybean meal in broilers (21 day-old) diets containing low levels of TIA - trypsin inhibitor activity (1,8 mg/g vs. 4.8 mg/g) resulted in higher dietary digestibility coefficients for DM, N, energy, and aminoacids (AA) (Dourado et al., 2011).

Both under- and over-processing of soybean meal has been shown to depress average daily gains in broilers (Perilla et al., 1997). In pigs, performance of swine fed soybean meal will be depressed if it has not been adequately processed to inactivate the anti-quality factors (Grala et al., 1998). Underheating, that may result in the incomplete destruction of antinutritional factors, is verified by the urease test, which determines residual urease activity and is an indirect indicator of active trypsin inhibitors. Overheating causes Maillard reactions that decrease the concentration and availability of heat-sensitive amino acids, particularly lysine (van Eys et al., 2004). Overheating also suppresses phytate degradation in the rumen and leads to lower availability of dietary phosphorus (Konishi et al., 1999). Several methods assess overheating, including KOH protein solubility, Protein Dispersibility Index (PDI) and Nitrogen Solubility Index (NSI). Soybean meals adequately heat-processed should have PDI values between 15 and 30%, KOH solubilities between 70 and 85% and a urease index of 0.3 pH unit change or below. Residual antitrypsic activity can also be directly measured by reference methods, but the procedure is less adapted to routine quality control (van Eys et al., 2004).

Goitrogens and oestrogens

Soybean meal may contain goitrogenic substances. Soybean meal is goitrogenic for monogastrics and it has been shown to be responsible for the goitrous calves born to cows receiving soybean meal as the major source of supplementary protein (Hemken et al., 1971). Soybean meal contains 1 g/kg of genistein, which has oestrogenic properties (McDonald et al., 2002).

Non-Starch Polysaccharides (NSP)

The addition of 40 g/kg NSP to a commercial broiler diet decreased weight gain, feed efficiency and apparent metabolizable energy (AME) by 28.6, 27.0 and 21.2%, respectively (Choct et al., 1995). The antinutritive effects of NSP in poultry and pigs might be due to their physicochemical properties. In particular, soluble viscous NSP depress the digestibilities of protein, starch and fat (Smits et al., 1996). NSP content of soybean meal is approximately 61 and 103 g/kg (dry matter basis) for soluble NSP and insoluble NSP, respectively (Bach Knudsen, 1997). NSP increase microbial activity (fermentations) and may cause intestinal disorder. Birds cannot degrade α-1:6 galactoside, and the addition of enzymes could alleviate this problem (Leeson et al., 2005; Zanella et al., 1999). Enzyme addition (xylanase, protease and amylase) in poultry and pig diets is a good way to limit NSP issues (Dourado et al., 2011).

Phytates and mineral availability

Though soybean meal has a relatively high content in phosphorus, much of it is present in the form of phosphorus-phytate, a poorly digestible complex for monogastric animals. Most of phosphorus is thus excreted in manure, which raises growing concern about the effects of phosphorus upon the eutrophication of surface waters (Waldroup et al., 2008). Phytates also link to zinc whose availability is then low in soybean meal (Blair, 2007). Pigs fed on soybean meal should receive 50 mg/kg zinc, whereas the recommendation is 18 mg/kg for pigs fed casein (animal protein) as the source of protein in the diet (NRC, 1998 cited by Blair, 2007).

GM soybean meal

The potential health issues of genetically-modified soybean and other GM foods have been the matter of considerable debate. While most studies have failed to show deleterious side-effects to GM soybean use (EFSA GMO Panel, 2008), these varieties remain controversial, and are subject to legal authorisation in some countries. In the EU, for example, only 15 soybean varieties are allowed to be used as feeds (GMO Register, 2016).

Soybean meal in fish

Because of its global availability and cost, soybean meal is potentially considered as the most pertinent protein source as an alternative to fish meal (Brown et al., 2008). The most commonly used products in aquaculture are toasted soybean meals. Depending on availability, dehulled and non-dehulled soybean meals are used, as well as unground soybean cakes in several tropical and/or developing countries.

The general limitations with regard to the use of soybean products in diets for aquatic animals are due to the relatively high carbohydrate, low crude fat and crude protein levels, and the lower levels of sulphur-containing amino acids, compared to those found in fish meal. Phytic phosphorus is not available to fish, and also interfers with the absorption of other micronutrients. The presence of antinutritional factors in the seeds is also a matter of concern, though these are normally destroyed in toasted soybean meal.

Soybean meal is highly palatable to most warm water fish (Lowell, 1998; Akiyama, 1991). In different species of salmonids, partial replacement of fish meal by soybean products has been demonstrated (Kaushik, 2008). A commonly observed adverse effect with soybean products in the feeds for Atlantic salmon is related to enteritis (Baeverfjord et al., 1996; Bakke-McKellep et al., 2007), the exact cause of which has so far not been identified. Enterocytes morphology modifications have been found in sharpsnout sea bream fed on diets containing soy bean meal (Ferrara et al., 2015). In European sea bass, soy bean meal was found to affect the activity of intestinal brush boarder enzymes, in particular the activity of γ-glutamyl transpeptidase was significantly higher in the upper section in fish that consumed the control diet compared with fish that consumed the soy derivate diets, while in the soy derivate diets the activity of alkaline phosphatase was significantly lower than its activity in the control diet (Tibaldi et al, 2006).

Crustaceans (shrimps and prawns)

Soybean meal has been used to feed marine shrimp since the 1980s (Akiyama, 1991). By proper feed formulation using soybean meal along with other plant protein sources, it was possible to develop a “fish meal free” diet for rearing marine shrimp under pond culture conditions (Amaya et al., 2008). Fish meal can be totally replaced with soybean meal and distillers' by-products in the feeds for freshwater prawns (Tidwell et al., 1993).

Since soy extract and fish meal are responsible for a negative environmental impact, alternative protein sources must be found that are environmentally friendly, economical, have good nutritional value, no factors antinutritional.

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The word “insect” derives from Latin “*insectum*” with a notched or divided body, literally “cut in sections”, infact the insect body is divided in three sections. Plinius traslated the Greek word “*entomos*”, used for the first time by Aristoteles. The term was first documented in English in 1601 in Holland’s traslation of Plinius (Harpe and McComak, 2001).
The insect is a class of animals within the arthropod group that have:

- chitinous exoskeleton to protect them from the environment
- three parts body (head, thorax and abdomen)
- three pairs of jointed legs
- compound eyes
- two antennae

In addition, insects are cold blooded, undergo metamorphosis to be able to adapt to seasonal variations, reproduced quickly, have a large population and do not need parental care; finally the respiratory systems consist in networks of tracheal tubes, for this reason are tolerant of air and vacuum pressure, high-altitude flight and radiation (FAO 2013).

At the present there are about one million species of insects known, but it is estimated that the total number of species ranges from about 6 to 10 million, which represents potentially more than 90% of the Earth's animal forms (FAO 2013).
Jongema (2012) at Wageningen University conducted an inventory of Edible insect in the world, and estimated that there are about 1900 species of Edible insects (previously studies published by Defoliart et al., 1997 and Ramos-Elorduy in 1997 reported espectly 1000 and 1681 edible insect).
The Table 1 shows the inventory of the edible insect in the different areas of the world.

Table 3.1: Inventory of the edible insect from different authors

Authors	**Year publication**	**Number of species decribed**	**Country/continent/ region of the world**
Van Huis	2005	250	Africa
Ramos- Elorduy	2008	549	Mexico
Cerritos	2009	177	Mexico

Chen	2009	170	China
Young-Aree and Viwatpanich	2005	168	Lao's people Democratic republic, Myammar, Thailand and Vetnam.
Paoletti and Dufour	2005	428	Amazon

Insects, besides being an excellent protein source from a nutritional and economic point of view, are also important for other reasons.
Insects play an important role in the pollination (for exemple the honey bees), biological control and decomposition of organic material, thus making essential tasks for maintaining the ecosystem.
These animals can also be used as organisms capable of reducing livestock manure, such as pigs, by mitigating the bad odor. *Hermetia illucens* larvae meal can be used to transform manure into fertilizer and protein, for this reason this specie it also calls "*latrine larvae*" (FAO, 2013).
Insects have been used for thousands of years in the traditional medicine. Flyworms (*Hymenoptera*), for example, were used to wipe wounds from necrotic tissue and honey products such as propolis, royal jelly, and honey have been utilized for their healing properties. Botulinum toxin, contained in bee (Apis mellifera) poison, has also recently been used in medicine for aesthetic surgery.
The natural color of insects has been used for centuries by different cultures, for example the Aztecs used a red colorant made from the cochineals (*Dactilopius spp., Karmes vermilio)*, this dye is still used today in the food industry (indicated as E 120) and in the field of cosmetics.
Silk has been used for centuries because it is a soft fabric but at the same time durable and long lasting.

The entomophagy: the practice of eating insect
The entomophagy is intending the practice of eating insect. More than 2 billion of people eat insects in 113 countries in all the world (McEvilly,2000).
The food practice is influenced by culture nad historically and religious beliefs. The entomophagy is cited throughout religious literature in Christians, Jewish and Islamic faiths (FAO, 2013).

Bodenheimer in 1951 described the history on entomophagy. In the Middle East, 800 years b.c. servants were thought to have carried locusts arranged on sticks to royal banquet in the palace of Asurbanipal. In Greece the cicadas were considered a delicacy by Aristotele (384-322 years b.c.).
In the second century b.c., Diodorus called people from Ethiopia Acridophagi ore eaters of locusts and grasshoppers (*Acrididae* family, *Orthoptera* order). Plinius, a natural philosopher and naruralists author of *Naturalis Histora*, speaks about a "cossus", a dish higly coveted by Romans; in according to Bondenheimer (1951); cossus is the larva of longhorn beetle (*Cerambycs cerdo*), which live in oak trees.
Generally it is assumed that the practice of eating insect takes place exclusively in the countries of tropical area; this assumption is not completely true because they are consumed also in the countries partially or fully in temperate zones such as China (Feng and Chen, 2003), Japan (Mitsuhashi, 2005) and Mexico (Ramos Elorduy, 1997). There are also large differences between the countries to consider edibles or not some species of insect (Meyer- Rochow, 2005).
The insects are consumed more in the tropical area of the world because:

- The insect tends to be larger in the tropics than the temperate zones, which facilitates harvesting; however, it is not possible to generalize this trend (Gaston and Chown, 1999). The body size is affected by the insect metabolism, but how different body size occur is not completely known (Gaston and Chown, 1999). The insect (like humans) require oxygen and produce carbon dioxide as a waste product; instead of lung the insects species use a series of tubes called tracheal system. The gases are mainly exchanged throughout the body by diffusion, which happens faster at higher temperatures, allowing for the production of bigger insects in warmer climates (Kirkpatrick, 1975). Shear and Koukalova-Peck in 1990 shows that the insects has a much larger body during the late Palazoic period (some fossils as large as 1 meter) because of high atmospheric temperatures.
- In the tropical areas the insects often congregate in significant numbers, so a largest number could be collected during a single harvest. For example locusts swarms settle for the night, so the harvest is very easy early in the morning; winged termites, during the nuptial flight when the first rains fall after the dry season, emerge

from termite mounds in a large numbers (Madsen and Kirkman, 1988).

- In the temperate zone the insects during the winter hibernate to survive cold winters, during this season it is not possible found and harvest the insects; instead in the tropical area it is possible observe many species all the seasons (FAO,2013).
- For many species, in the tropical areas the harvest is predictable (except for locusts swarms), the local people where and when to harvest a wide range od insects species (FAO, 2013).
- Choo, Zent and Simpson (2009) observed that palms weevils are found in palms that have fallen, or that have been felled deliveratory to tigger beetles to lay eggs; Bamboo caterpillars could be found in sterms of bamboo: many insects prefer specific plants or tree species.

In many poor countries the insects are considered not only a delicacy, but also an important food source (they are rich in protein, fat, vitamin, mineral); the mammals meat is exclusively for the rich people in many tropical countries. In the Wersten world the insects are considered disgusting and dangerous for the human health (Looy et al., 2014).
Wiegel et al. (2016) studied the impact of Small-Scale Cricket Farming on Household Nutrition in Laos.
Malnutrition is the largest single risk factor for the burden of disease in developing countries, with women and children being especially affected (World Food Programme, 2016). In Laos, the rates of malnutrition, with nearly 30% of the population under-nourished, are among the highest in Southeast Asia (World Food Programme, 2013). Edible insects could make a significant contribution to improving malnutrition due to their excellent nutritional values, which have been described in various studies (Rumpold & Schlüter, 2013).
While Laos has a long tradition of entomophagy and more than 90% of Lao people eat insects, insect consumption has declined over the last decade and is infrequent, with most eating insects only a few times per year (Barennes, Phimmasane, & Rajaonarivo, 2015).

Wiegel et al. (2016) showed their results of an applied research project in Laos, which introduced small-scale cricket farming to 20 rural households and assessed how the cricket production affected household nutrition. Overall, cricket farming was found to be successfully adopted. Crickets were

eaten by all family members, regardless of age and gender. When consumed, crickets provided sufficient micronutrients and protein to improve nutrition. To increase the nutritional impact, production has to be stabilized and adapted to provide a more continuous supply of crickets (Wiegel et al., 2016).

Not only in humans but in many animal species the insects could be suitable for nutrition. Since the 1970s insects have been evaluated for feedstuff in poultry (it is reported by Bokonyi and Gal, 2010 that chickens normally eat worms found in the soil and can synthesize chitinases, enzymes that hydrolyze chitin) and others food-producing animal. Most of this data considered the dried insect meals (Finke, 2000), not intact insect, the evaluation of the quality protein is very important such as the aminoacids evaluation and experimental trials (on poultry and laboratories rats) (Finke, 2000).

More recently the nutritional content of selected species of cultured insects has been studied because of their role as food for captive insectivorous reptiles, birds, and mammals kept either in zoos or as pets by hobbyists (Barker, 1997). Most of these studies are concentrated to analyze the moisture, ash, crude protein, ether extract, mineral (such as calcium and phosphorus) (Barker et al.,1998).

Generally, this insectivorous pets are fed on one or two species of insects (mealworm and locusts), and they are likely to be more prone to nutritional deficiencies, especially in the insects are rearing on standardized substrate. In order evaluate what species are suitable for the zoo pets, Finke (2000) published a study on this regard.

Adult house crickets, house cricket nymphs (*Acheta domesticus*), superworms (*Zophobas morio* larvae), giant mealworm larvae, mealworm larvae and adult mealworms (*Tenebrio molitor*), waxworm larvae (*Galleria mellonella*), and silkworm larvae (*Bombyx mori*) were analyzed for dry matter, crude protein, crude fat, ash, acid detergent fiber (ADF), neutral detergent fiber (NDF), minerals, amino acids, fatty acids, and vitamins. Earthworms (*Lumbricus terresstris*) were analyzed for moisture, crude protein, crude fat, ash, ADF, NDF, minerals, amino acids, and vitamins A and D3. Proximate analyses were variable, with wide ranges found for moisture (57.9–83.6%), crude protein (9.3–23.7%), crude fat (1.6–24.9%), ADF (0.1–7.4%), NDF (0.0–11.5%), and ash (0.6–1.2%). Energy content ranged from a low of 674 kcal/kg for silkworms to 2,741 kcal/kg for waxworms. Using an amino acid scoring pattern for rats, the first limiting amino acid for all invertebrates tested was the total sulfur amino acid

(methionine and cystine) that are essential aminoacids and play an important role expecially during the growth of the animals. These data collected by Finke (2000) provide a basis for determining nutrient intake of captive insectivores, and will aid in the development of "gut-loading" diets to provide captive insectivorous animals with appropriate levels of necessary nutrients.

Mass production of edible insects and process technologies

The majority of edible insect are collected in the wild up to today (Laos, 2010). The rearing of insects is practiced for at last 7000 years for the sericulture (silk production), the production of shellac, then for the apiculture (the honey) and for the production of medicinal products.

In 1936 was documented the first mass production of screwworms in a factory on artificial diets had been accomplished paving the way for a sterile insect technique (SIT). A lot of researches has been conducted in order to evaluate the different rearing substrate (Singh, 1994; Singh and Moore 1985).

The traditional harvesting of insects is uncontrolled and unsuitable, the over collection contributes at the forest destruction and the extinction of some species (Schaebel,2010).

For marketing, the production of edible insects on an industrial scale is preferable to reduce cost production and preserve the environment. Actually, the mass production of edible insect in Europe is expensive and the prices are comparable to that of meat. The mealworms cost more than 30 euro per kg (http://www.webpoiler.nl/), based on rehidrated weight. The caterpillars (*Cirina forda*) in Nigeria is the most widely marketed edible insect and sells for about twice the price of the beef (Defoliart, 1999).

By contrast, Nakagaki and Defoliart (1991), compared different diets for rearing house cricket (*Acheta domesticus*) as a novelty food and calculated the feed costs per Kg of cricket produced to be from 0.17 to 2.05 euro; the crickets were sold for 24 to 48 euro/Kg, as a pet feed or a fish baits. This appears a wide profit margin. The authors are not taken into account the facilities and the manual labor. The consumer's acceptance (both humans and animals) is a very important point. The ideal candidate edible insects are selected based on their size, social behavior, safety, epidemic tendencies, reproductive and survival potential, nutritional benefits, potential for storage and marketability (Schabel,2010).

It is aimed for a high eggs production and hatchability, short duration of the larval stage, optimum synchronization of pupation, high weight of larvae

and pupae, high productivity and conversion rate, high potential biomass increases, ability to live in high densities, and high quality of protein (Rumpold, 2013). Others factors affecting the rearing process are: temperature, light/illumination, larvae population density, ovodeposition site, food composition and quality, microbiological safety (Peters and Barbos, 1977; Scriber and Slasky ,1981; Sharaby et al., 2010; Singh, 1982; Tchuinkam et al., 2011; Vantomme et al., 2012).
After harvested or reared in domesticated setting, the insects are sacrificied by:

- freeze-drying
- sun-drying
- boiling.

They can be processed and consumed in three differet ways:

- whole insect: generally in tropical countries are consumed whole, but some insects (grasshoppers and locusts) require to removal same body parts. In Lao people' s Democratic republic, the insect can be found in the market ready to eat snacks (FAO, 2013).
- in granular or paste forms: it is the most common method for processing a large variety of foods. The insects (such as meat and fish) can be processed in order to assume more palatable forms. They are often ground in a paste and addict to food and feed poor in crude protein.
- as extract of protein, fat or chitin, for fortified food and feed productions: in particular western consumers are reluctant to accept the insects as a protein source, because the insects never play an important role in their food culture. In the process of extraction, it is important that there is a knowledge of the properties of the extracted proteins (such as aminoacids profile, thermal stability, solubility, gelling, foaming and emulsifying capacity).

Recently a research group from Belgium (Van Campenhuot et al., 2017) studied the impact on blanching, industrial microwave drying and freeze drying on the nutritional quality, microbial quality and the crowing index on mealworm larvae.
Currently the larvae are blanched as a killing step and then frozen or freeze dried. This research group (Van Campenhuot, et al 2017) studied the possibility of the use of microwave as an alternative to freeze drying. The

authors described the impact on blanching, microwave, on nutritional quality and on the microbial quality and on the color of the larvae.
The researches described that the microwave drying is a valuable alternative to freeze drying for drying *Tenebrio molitor* larvae. In general a similar final product is obtained, except for the vitamin B12 content which is better preserved during the freeze drying. The advantages of the microwave drying are that the treatment time is shorter and that, in contrast to freeze drying, no browning of the product occurs during the storage afterwards. In this study the authors did not report the cost of the microwave processing, but the use of the microwave is chipper than the use of the freeze drying. The application of the vacuum drying the microwave treatment does not result in additional benefits, and hence, mealworm processors are advised not to invest in the vacuum option when considering the purchase of a microwave dryer (Van Campenhuot et al., 2017).

Food and feed safety
In the tropical countries the most edible insects are hervested from the nature (Defoliart, 1995); the insects considered edible in some areas may be considered not edible in others (Schabel, 2008).
In accordance to Schabel (2008):

- Some insects normally are considered edible but may be unhealthy on certain plants or originate from a polluted and a pesticide treated zone;
- the insects are safe for the most of consumers, but for some people eating insects is dangerous because may be causes allergic reaction;
- other species require special catch, processing, storage and transportation method to render and keep them safe.

In according to DeFoliart (1992) the people of not European countries eaten the insect for a long time, with little evidence to the contrary, any several health problem is attributed to insects consume.
People are regularly eating small amounts of insects unconsciously, but no serious complications have been observed, with the exception of individuals who react allergically (Mitsuhashi 2008).
In 2010, the Codex Alimentarius Commission (CAC) reported that food safety of edible insects has not been studied extensively, which may be due

to the fact that these insects are often treated as traditional foods of indigenous populations and rarely recognized as tradable food items.
The Codex Alimentarius Commission describes that insects are rich in nutrients providing a medium for growth of unwanted microorganisms under certain conditions (such as many meat production), especially making uncooked insects susceptible for microbiological hazards unless proper heat treatment or storage conditions are applied (Klunder et al., 2012; FAO, 2013). Some people have or may develop (food) allergies against certain species of insects. Probably the chitin (the major compound of the insect exoskeleton) is responsible of allergic reaction (Muzzarelli et al 2010).
Some insects may require other treatments before they are rendered edible (CAC, 2010). Insect proteins may display a cross-allergenicity with shrimps and house dust mites (Witteman et al., 1994; Leung et al., 1996; Reese et al., 1999; Houben 2012; Verhoeckx et al., 2013).

The pupae of African silkworms (*Anaphe venata*) contain a thyaminase and can cause a deficiency of thyamine, which is responsible of an ataxic syndrome (Nishimune et al., 2000). The insect can contain some toxic chemical such as a defense mechanism, and others are usually safe to eat, but can contain pesticides if they are harvest on pesticides treates-areas. The grasshoppers and the locust, in particular if they are harvesting, can causes problems if are consumed in large quantitative (FAO, 2013).
Toxic chemicals are acquired in 2 ways, either by autonomous production of defense chemicals (such as toxins and toxic metabolites) or by sequestering phytochemicals directly from the food plant (Duffey 1980; Wirtz, 1984; Berenbaum, 1993; Blum, 1994; Schabel, 2008). Defensive secretions that may be reactive, irritating, or toxic include, among many others, carboxylic acids, alcohols, aldehydes, alkaloids, ketones, esters, lactones, phenols, 1,4quinones, hydrocarbons, and steroids.
Phytochemicals sequestered by various insects include phenolics, flavin, tannins, terpenoids, polyacetylenes, alkaloids, cyanogens, glucosinolates, and mimetic amino acids (Wirtz, 1984). Some insects may contain chemicals in concentrations higher than acceptable levels for food consumption (Yen, 2009). For example, arsenic was accumulated in Bogong moth from agricultural sprays, such as the herbicide monosodium methylarsenate (Green et al., 2001), and selenium was accumulated in T. molitor (Hogan and Razniak, 1991). Food processing can also introduce toxic substances by chemical reactions of substrates of insects and other ingredients, such as heterocyclic aromatic amines, acrylamide,

chloropropanols, and furans (Dolan et al., 2010). A study published by Vjiver et al. (2003) showed that *Tenebrio molitor* reared on organic matter containing cadmium can accumulate this harmful metal. Lindqvist and Block (1995) showed that after the metamorphosis the insects lose a large part of cadmium.

In order to prevent health risks it is necessary that the common edible insects are rearing on pollutant free feed. For harvested insect is required a control on processing, handling and storage in order to prevent the contamination and to ensure feed and food safety. Schabel (2010) reported cases of botulism, parasitoeses, and food poisoning due to aflatoxins caused by eating insect. A direct zoonosis' by the insect to humans actually are unknown, but the insect could also transmit zoonotic agents such as bacteria, virus, parasites, fungi as a vector (Kruse, 2000).

Based on these studies on food safety of non-European insects, food safety issues should be identified and assessed before insects can enter the market in Europe (Spiegel et al., 2013).

Both chemical and microbiological hazards can be introduced or formed in concentrations that maybe harmful for public health. Feed for insects (food, vegetables, and waste products) can be contaminated with mycotoxins, natural toxins, heavy metals, veterinary residues (such as antibiotics), pesticides, and pathogens (Spiegel et al., 2013).

During rearing, insects may be able to convert or accumulate contaminants present in their feed, which can result in degradation or increase of the concentration of substances. The particular safety hazards depend on the insect species, their feed and environment, and production methods. Therefore, more attention should be directed toward the effects of these environmental and management conditions on the safety of insects destined for human or animal consumption (Spiegel et al., 2013).

Poma (2017) evaluated the chemical safety of the edible insects and insect-based food intended for human consumption, this study can contribute to the process of acceptance of insect as a possible protein source by the western people. In this study (Poma, 2017) the author analyzed the residual levels of different chemical compound:

- brominated and phosphorous flame retardants (BFRs, PFRs
- polychlorinated (PCBs)
- organochlorine compaunds (OCPs)
- dioxin and dioxin like PCBs
- pesticides

- metals (As, Cd, Co, Cr, Ni, Pb, Sn, Zn)

The author investigated the level of this chemical compounds on four species of edible insects (*Galleria mellonella, Locusta migratoria, Tenebrio molitor, Alhitobius diaperinu*s). The chemical levels measured in edible insects were compared with those found in other studies reporting the contamination level in the meat, fish and seafood, and the eggs at levels considered safe for human consumption, and they are similar; for this reason the author (Poma 2017) consider the insects and the insect safe to eat.

Opara et al. (2012) investigated on microbiological contamination of eible insects and showed *E. coli* and *K. Aerogenes* in freshly hervested, and Staphylococcus spp. in heat-processed pulm-grubs (*Rhynchophorus phoenicis*) collected in Nigeria; this contamination is attributed to improper processing and handling by healthy carriers of Staphylococcus spp.
Microrganisms are isolated from the gut and the body surface of *Musca domestica* coltured on fresh fish (Banjio et al., 2005) as pathogens (*Staph. aureus, P. aeroginosa, A. tamarii, B. cereus*) and not pathogens bacteria (B. subtilis and Staph. Faecalis).
Similar results are collected by Banjio et al. (2006) from african rhinoceros beetle (*Oryctes monocerus*): the authors isolated: *S. aureus, P. aeruginos, B. cereus* (pathogens) and *B subtilis and B. firmus*.
A study recently published (Kashiri et al., 2017) was focused on natural contaminating microorganism and inoculated *E. coli* (O157: H7) in black soldier fly larvae, in order to assess the usefulness of high hydrostatic pressure in controlling pathogenetic microorganism that potentially can contaminate the larvae. The best model describing inactivation curves for E coli was studied. The results in the study showed that high hydrostatic pressure is an excellent method to hygienenize black soldiers fly larvae at the same time that the inactivation the pathoenetic bacteria take in exam (*E. coli*) (Kashiri et al, 2017).

The legislation of insects as a "novel food".

For "novel food" is intending new food and feed (or ingredient) and all the products that not showed a significant consume before the emanation of the European Regulation 258/1997; the insects are not mentioned as novel food in this law.
Before the adoption of the new novel food regulation, there was legal uncertainty on the regulatory classification of edible insects. Except for

Regulation 834/2007/EC on organic production, in the European legislation no mention was expressly made to insects as food or feed. Considering this legal lack, insects and insect-based food were considered by most to fall within the scope of the novel food Regulation (EU 258/1997), although this qualification was far to be undisputed and not consistently implemented in EU member states (Paganizza 2016).
The classification of insects as novel food has been clarified through the adoption of Regulation (EU 2015/2283, which replaces Regulation 258/97. Recital (8) of the new regulation expressly states that:

"*it is appropriate to review, clarify and update the categories of food which constitute novel foods. Those categories should cover whole insects and their parts*".

Following this review, all edible insects can be deemed to fall within the novel food category under article 3, 2 (a), (v) that encompasses:

"*food consisting of, isolated from or produced from animals or their parts*".

The Reg (EC) 999/2001 banned officially the use of processed animal (both vertebrate and not) protein (in the regulation was banned indirectly the use of insect meal) in the European Union, in order to prevent the diffusion of BSE. After a first attempt in 2007, the Commission presented its new proposal on novel foods in 2013, approved by the European Food innovations; so the European Commission has issued a new regulation on novel foods including insects and processed animal proteins.
The European Regulation 56/2013 (approved since the first June 2013) providing that aquacultured animals can be fed with animal proteins derived from non-ruminant and with compound feed containing such feed materials. Although, following this amendment, the use of insects should be allowed, the impossibility to fulfill the slaughtering requirements, put insects outside the scope of this provision.
Following the issuing of a favorable opinion of EFSA with regard to the new food, a new regulation was adopted on 15 November 2015 (2015/2283), that abrogated the previously regulation 258/1997.
One of the main innovations in the (new) novel food legislation is that Regulation (EU) n. 2015/2283 explicitly brings insects under its scope, regulating their consumption for the first time in Europe. In order to assess

the safety aspects of edible insects, the Commission has asked for advice from EFSA. According to EFSA' report more scientific data and further information is needed to provide a complete risk assessment of using insects as food and feed (Lotta et al, 2017).
At the same time, FAO and other non-profit organizations highlight the potential benefits of insects as food and feed, with particular reference to the environment and the food security**.**
The novel food status of edible insects implies that they are subject to safety assessment and pre-market approval before being placed on the market. The new Regulation provides two different procedures that can be used to place edible insects in the European market. In both cases, the applicant is required to submit a set of information to the Commission that may involve the European Food Safety Authority (EFSA) in the food safety assessment.
The faster way to place edible insects on the European market is the notification procedure set in the article 14 for traditional food from a third country. Although insects are deemed to be food without a long history of consumption in Europe, it is a matter of fact that they have been widely consumed in Asia, Africa and South America.
Whilst the notification procedure presents the advantage of requiring a lower amount of information than those required under general rules (e.g. it is not required scientific evidence demonstrating that the novel food does not pose a safety risk to human health) and a faster timing, applicants cannot require the protection of data submitted through the dossier.
A new law it is attending before the first January of 2018 in order to clarify and obtain more information about the rearing substrates and the health risks connected to the edible insects (Van Huis, 2014).
In many European countries insects have already been marketed for the human consumption:

- One of the most innovative European countries is the Belgium, indeed it is possible to found in a supermarket a wide variety of products with insect (tomato, carrot and chocolate containing mealworm).
- In Holland are in sold a mealworm burger was a being at Jumo stores in the Netherlands. This product in it self was one of the reasons for the review of the laws.
- There are quite a few insect food companies starting up in the UK such as "Eat Ento", as well as others selling a whole range of products like "Bug Grub and Grub".

- One of our favorite French producers of insect based products are the young and dynamic "Insectéo team". They sell "apero" style naturally flavored insects.
- In Switzerland was completely against the introduction of insects as food into the market. However, in late February the government had a change of heart and stated that, the citizens is considering the possibility of introducing some insect species on the food market in Switzerland.

In Italy there are some entrepreneurs interested to produce insects for human consumption, young consumers are curious to taste them then the adults, and the men are more interested to eat insects than the women. (http://www.ilfattoalimentare.it). At present, Italian legislation does not allow to breed, transform, or sell for food purposes; in 2018, European legislation will come into force that will uniformize the use of insects in the various countries of the Union. The Bozzaotra brothers produce silkworms for sale as medical devices for the treatment of acne and dermatitis in Switzerland (because the legislation is more permissive than Italy). "Italbugs" a company born in Italy at Expo 2015, who made the first "panettone" (cake that the Italians consume mainly in the Christmas period) with silkworm flour, moved to the Netherlands to start marketing insect food products. The founder of the "Microvita" company started to produce insects for the biological fight in the 1980s, later insects to be used as fishing lures or feeding reptiles, birds and other pet animals; and now breed insects that can also be used for human consumption.

The most important insect species in Animal nutrition

The most studied species in animal nutrition are (Makkar et al 2014):

- black soldier larvae fly (*Hermetia illucens*)
- housefly maggot meal (*Musca domestica*)
- mealworm (*Tenebrio molitor*)

Black soldier fly (*Hermetia illucens*)

Black soldier larvae fly or Hermetia illucens (Linneus,1758) it is a Diptera (such as Musca domestica).Despite Hermetia illucens it is native to the tropical and subtropical areas of the American continent, the development of international transport about 70 years ago allowed the black soldier fly to reproduce in other hot and temperate areas all over the world(Leclercq et al

1997), according to Diener et al (2011) today the distribution area of Hermetia illucens is between 45 ° N and 40 ° S.The life cycle in ideal condition lasted 2 munth, but Harduin and Mahoux 2003 observed that the life cycle can be lasted up to 4 mounth if the organic matter available is not enough.
The black soldier larvae is whitish and it it 27 mm in lengh and 6 mm in with, the weigh at the and of larval stage is about 200 mg. Hermetiaa illucens larvea can feed from 25 to 500 mg of fresh organig matter per day and can it a big variety of organic material; in accoding to Harduin and Mahoux 2003, Diener et al 2011 and Van Huis et al 2013 blach soldier fly larvae can eat:

- Fruits
- Vegetables
- Coffee bean pulp
- Distillate grains
- Fish offals
- Animal manure
- Human excreta

At beginning of last decade of Twenty Century, rearing this species of insect on organic waste in orer to produce organic biomass rich in protein and fat, biodiesel and chitin production (Makkar et al 2014, Diener et al 2011, and Van Huis 2013).
The high content of crude lipids in the black soldier fly can be converted into biodiesel: 1 000 larvae growing on 1 kg of bovine manure produce 36 g of biodiesel, porcine and poultry manure produce respectively 58 g and 91 g of biodiesel (Li et al. 2011).
Harduin and Mahoux (2003) decribed that the larvae at the and of larval stage (it is call prepupae) start to accumulate reserve of feed in the gastrointestinal tract, after that stops to eat and moving, and search a dry pupation site (Diener et al 2011). The pupae stage last 14 days in ideal condition, but it is described that can be lasted up to 5 mounth (Harduin and Mahoux 2003).
The adult Hermetia illucens are black and are 15- 20 mm in lengh. The adult female 2 days after the end of pupae stage start to deposit the eggs near organic matter (it is the feed substrate to larvae after the hatch) in according to Diener et al (2011). Adult insects do not feed and use fat reserves accumulated during the larval phase to produce energy.

Black soldier fly does not feed at adult stage, are resistant to extremely temperatures (but the duration of the life cycle it depends to the rearing temperature) and do not need oxygen. Moreover, black soldier larvae fly is not dangerous for the human and animals life because it is not a biological vector of disease. Additionally, the larvae modify the microflora of manure, potentially reducing harmful bacteria such as *Escherichia coli* O 157:H7 and *Salmonella enterica* (Erickson et al., 2004) It fas been suggested that the larvae contain natural antibiotics (Newton et al.,2008).
Some authors proposed different substrates to rearing the black soldier fly.

Table 3.1. Inventory of the edible insect from different authors

Authors	**Year of publication**	**Substrate**
Sheppard et al	1994	Poultry manure
Barry et al	2004	Food wastes
Newton et al	2005	Pig manure

The production of black soldier pre-pupae also solved problems related to manure management. In addition, Sheppard et al. (1994) demonstrated how colonization of poultry and poultry manure by the soldier fly could reduce the common fly population (94-100%). The soldier fly also makes manure more fluid and therefore less suitable for domestic fly larvae, and its presence is believed to inhibit the deposition of eggs of this species (Sheppard, 1983).
Soldier fly larvae are able to convert the residual proteins of manure and other nutrients into most valuable biomass (eg animal feed). In this way they reduce the concentration of nutrients and most of the manure waste.
In cattle farms, it has been observed that larvae reduced 61-70% available phosphorus and 30-50 percent nitrogen (Sheppard, Newton and Burtle, 2008). In a field study conducted in Georgia (USA), digestion of lean weeds by H. illucens larvae resulted in 71% nitrogen, 52% phosphorus and potassium reduction of 52%, and for aluminum, boron, cadmium, calcium, chromium, copper, iron, lead, magnesium, manganese, molybdenum, nickel, sodium, sulfur and zinc have decreased by 38-93%
Therefore, larvae are able to reduce the potential pollution by 50-60% or more. The bad smells produced by the decomposition of manure are reduced

or eliminated by the digestion by soldier fly larvae. This is because the species arieggia and dries the manure reducing the smells.

In addition, larvae can modify manure microflora by potentially reducing harmful bacteria (Erickson et al 2004, Liu et al 2003). For example, larval activity significantly reduced Escherichia coli 0157: H7 and Salmonella enterica in hen fowl (Erickson et al., 2004). Sheppard, Newton, and Burtle (2008) suggested that larvae contain natural antibiotics similar to those contained in larvae of the green fly (Lucilia sericata) used in larval therapy (asticotherapy) for the purification of human wounds, a method increasingly practiced due to of prevalence of drug-resistant bacterial infections (Sherman and Wyle 1996).

The use of Hermetia illucens in animal feed should be taken seriously into consideration, not least because of their reduced environmental impact (Newton et al., 1977; Sheppard et al., 1994). Dried prepaints contain 42% Proteins and 35% fat (in relation to the dry substance) (Newton et al., 1977). Live forage contains 44% of dry matter and can be easily preserved for long periods of time. As complete feed ingredients have allowed a good growth in pollen (Hale, 1973), pigs (Newton et al 1977), trout iris (Oncorhynchus mykiss) (St-Hilaire et al. 2007), catfish (Ictalurus punctatus) (Pimentel et al., 2004), tilapia (Oreochromis aureus) (Sheppard et al., 2008). In the case of iridescent trout larvae may replace 25% fish meal and 38% fish oil.

In addition to being used as a fish food, insects can be bred on the fish itself. Among the organic waste products that can be given to the larvae, we find the fish offal (interiors, etc.). Compared with manure-fed larvae, the lipid content increased in this case by 30% and omega-3 fatty acids by 3%; Both increases occurred within 24 hours.

Chemical constituents

It is possible consume Black soldier larvae fly alive, dried and ground (this is the better formulation for the inclusion in animal diets), or chopped. The hermetia illucens larvae contain 40-44% of cude protein, the amount of fati s extremely variable because it depend of the diet: The hermetia illucens rearing on oil rich food waste contain42-49% of crude lipids (Barry et al 2004), larvae fed on poultry, swine and cattle maure contain respectively15-25%(Arango Gutierrez et al., 2004), 28%(Newton et al., 2005), and 35% (Newton et al., 1977) of fat. The housefly (Musca domestica) have more

crude protein and less crude lipids of hermetia illucens. Also the ash content it is variable because depends of the larval stage (it is higher in prepuae) the rane is 11-28%. For the high level in Ca (5-8%) and P (0.6-.0.8%)(Newton et al., 1977;Arango Gutierrez et al., 2004; St.Hilarie et al., 2007b) hermetia illucens larvae meal is suitabe for the animals at rapid growth such as broilers.

The fatty acids composition of larvae depends on the fatty acid composition of the diet, The lipids of larvae fed on cow manure contained 21% of lauric acid, 16% of palmitic acid, 32% of oleic acid and 0.2 % of omega-3 fatty acid, while this proportion were respectively, 43of lauric acid, 11 of palmitic acid,12.3 of oleic acid, larvae fed 50% fish offal and 50% cow manure. Total lipid contant also increased from 21% to 30% dry matter. Feeding black soldier fly larvae with a diet made of wastes containing desirable omega-3 fatty acid is therefore a way to enrich the final biomass (St.Hilarieet al.,2007).

Table 3.2: Chemical composition (%) of black soldier larvae fly collected by Makkar et al (2014)

Crude protein	42,6 ±1.0(41.1, 43.6)
Crude fibre	7.0
Ether extract	26.0 ±8.3(15.0,34.8)
Ash	20.6±6.0(14.6,28.4
Gross energy	22.1

Arango Gutierez et al. (2004), Newton et al. (1977), St Hilaire et al. (2007)

Table 3.3: Mineral content of Hermetia illucens larvae meal

Ca (mg/Kg)	75.6±17.1(50.0,86.3) g/kg
P(mg/Kg)	9.0±4.0(6.4,15.0)g/Kg
K(mg/Kg)	6.90g/Kg
Na(mg/Kg)	1.30 g/Kg
Mg(mg/Kg)	3.90 g/Kg
Fe(mg/Kg)	1.37 g/Kg
Mn(g/Kg)	246mg/Kg
Zn(g/Kg)	108 mg/Kg
Cu(g/Kg)	6 mg/Kg

Arango Gutierez et al. (2004), Newton et al. (1977)

Table 3.4: Aminoacids composition of back soldier fly

Aminoacids	g/16 g nitrogen
Alanine	7.7±0.8(6.9,8.8)
Arginine	5.6±0.3(5.3,6.1)
Aspartic acid	11.0±1.8(8.5,12.5)
Cysteine	0.1
Methionine	2.1±0.3(1.7,2.4)
Lysine	6.6±0.9(6.0,8.0)
Isoleucine	5.1±0.5(4.7,5.6)
Leucine	7.9±0.6(7.1,8.4)
Phenylalanina	5.2±0.4(4.6,5.6)
Threonine	3.7±1.7(1.3,4.8)
Tryptophan	0.5
Glutamic acid	10.9±2.4(8.7,13.5)
Histidine	3.0±1.0(2.3,4.5)
Proline	6.6(5.5,7.7)
Serine	3.1±1.9(0.3,4.2)
Tyrosine	6.9±0.7(6.0,7.7)
Valine	8.2±1.4(6.4,9.1)

Newton et al. (1977), Sealey et al. (2011), St Hilaire et al. (2007).

Housefly maggot meal and housefly pupae meal

The housefly (*M. domestica*, Linneus 1758) is most common fly (*Diptera*) species, The housefly, is very different from the black soldier fly both biologically (also feeds on the adult stage) and the distribution area (in fact it is ubiquitous); as in the adult stage it often nourishes manure can be, unlike the fly black soldier, vector of infectious diseases.

The interest to rearing Musca domestica in controlled condition has been investigated since 1960s (Calvert et al., 1969; Miller and Shaw, 1969). Musca domestica it is able to grow on different substrates and turn wastes in a biomass reach i protein and fat. Pretorius et al (2011) demonstrated that poultry fed musca domestica larvae meal rearing on poultry letter do not had negative effect on the animals, in addition was investigated the use of maggot fly on fish and crustaceans.

For the development of Musca domestica the ideal temperatures are less of 25°C. The Normally, the housefly eggs hatch after 8-12 h, generally, the larval stage lasts about 5 days and the pupal stage 4-5 days. This 10 days cycle can be shortened to 6 days under controlled conditions. Adult female

ovodeposit 500-600 eggs under natural conditions, the number of eggs is less under controlled conditions (200).
The adult fly feeds mainly on decaying organic matter, so It needs liquefy the food by regurgitating droplets of saliva, (this is also the meccanism to transmitting pathogens).
Harduin and Mahoux (2003) described that less 500 g of manure is possible feed 1500 housefly larvae, so from small amount of organic matter it is possible obtain a large population of larvae. The housefly generally is rearing on poultry manure (Akpodiete et al.,1997, Harduin and Mahoux, 2003), but are reported in literature other materials such as rotten fruit and animal offal (Odesaya et al., 2011). Other substrates mentioned in the literature include: pig manure (Viroje and Main,1988; Zhu et al.,2012), cattle blood and wheat bran (Aniebo et al., 2008), cattle blood and gut contents (Dankwa et al., 2002), cattle gut and rumen content (Ekoue and Hadzi,2000), fish guts (Ossey et al., 2012) and a mixture of eggs content, hatchery waste and whest bran (Ebenso and Udo, 2003).

Chemical constituents of housefly larvae meal
Housefly maggots are a source of protein and lipids, such as black soldier fly. Che chemical composition is variable infact the crude protein content varies between 40-60%, the Lipid contents is between 9-26%, it is important observe that the older larvae contain more lipids and less protein than the young (Inaoka et al.,1999; Aniebo et al.,2008, Aniebo and Owen,2010).
P contents in housefly maggots are similar order as in black soldier fly larvae but Ca levels are lower by about 15 times. Lysine content in housefly maggots, similar to that in black soldier fly larvae, is also high (from 5 to 8.2g/100 g CP with average of 6.1g/100 g CP). The fatty acid content is influenced to substrate composition with fatty acid composition being one of the first observed changes in the larvae in the response to changes in substrate composition. For istance, larva fed milk powder, sugar and layer manure had a fatty acid profile suitable for broiler growth (Hwangbo et al.,2009). Palmitoleic acid level (17.1%) in houselfy maggot is much higher (4-15 times) than that in mealworm and house cricket, while that of linoleic acid is much lower in housefly maggots compared with those in the other to insect.

Chemical constituent of housefly pupae meal
It is also called as housefly pupae. In general, housefly pupae meal contains higher crude protein, higher crude fibre, and lower lipids than housefly

larvae meal. An average crude protein (71%) and crude fibre contents (15, 7%) have been observed, Ca and P values of 5.2 and 17.2g/Kg DM are most similar to those in housefly larvae meal. Lysine content in housefly pupae (5.5g/100g CP) is slightly lower than that in housefly larvae. The same has been the pattern in sulphur containing amino acids (methionine and cysteine). The fatty acid composition of housefly pupae meal and housefly larvae also appear to be similar.

Table 3.6: Chemical composition of housefly maggot meal (%)

Crude protein	50.4±5.3(42.3,60.4)
Crude fibre	5.7±2.4(1.6,8.6)
Ether extract	18.9±5.6(9.0,26.0)
Ash	10.1±3.3(6.2,17.3)
Gross energy	22.9±1.4(20.1,24.4)

Adesine et al (2011), Adewolu et al (2010), Akpodiete et al (1997), Aniebo et al (2008), Aniebo and Owen (2010), Atteh and Ologbenia (1993), Awoniyi et al (2003), Bangbose et al (1999), Calag et al (1981), Fasakin et al (2003) Gohol et al (1982), Hwangho et al (2009), Ocio and Vinanas (1970), Odesanya et al (2011), Ogunnji et al 2009), Okah and Onwja (2012), Sogbesan et al (2006), Teguia et al (2002), Zuidof et al (2003).

Table 3.7: Mineral content of housefly larvae meal

Ca (mg/Kg)	4.7±1.7(3.1,8.0)
P(mg/Kg)	16.0±5.5(9.7,24.0)
K(mg/Kg)	5.7±3.5(1.0,12.7)
Na(mg/Kg)	5.2±2.4(2.8,8.6)
Mg(mg/Kg)	3.4±4.0(0.7,11.5)
Fe(mg/Kg)	1.0±0.44 (0.28,1.37)
Mn(g/Kg)	91±114(40,349)
Zn(g/Kg)	27.0±6.0(18.0,36.0)
Cu(g/Kg)	119.0±118(43,256)

Bangbose et al (1999), Calag et al (1981), Fasakin et al (2003) Gohol et al (1982), Hwangho et al (2009), Odesanya et al (2011),Zuidof et al (2003), Pretorius et al (2011).

Table 3.8: Aminoacids composition of housefly maggot meal.

Aminoacids	g/16 g nitrogen
Alanine	5.8±1.0(4.4,7.6)
Arginine	4.6±0.7(3.7,5.8)
Aspartic acid	7.5±1.5(4.5,8.5)
Cysteine	0.7±0.2(0.5,1.0)
Methionine	2.2±0.8(1.3,3.7)
Lysine	6.1±0.9(5.0,8.2)
Isoleucine	3.2±0.5(2.3,3.7)
Leucine	5.4±0.6(4.5,6.4)
Phenylalanina	4.6±0.8(3.7,5.9)
Threonine	3.5±07(2.0,4.1)
Tryptophan	1.5(1.4,1.5)
Glutamic acid	11.7±1.8(8.9,15.3)
Histidine	2.4±0.8(1.0,3.6)
Proline	3.3±0.7(2.5,4.7)
Serine	3.6±0.5(2.6,3.9)
Tyrosine	4.2±0.4(3.7,5.1)
Valine	4.0±1.1(1.3,4.9)

Akpodiete et al (1997), Aniebo et al (2008), Hwangho et al (2009), Odesanya et al (2011), Zuidof et al (2003), Pretorius et al (2011), Odesanya et al (2011).

Table 3.9: Acidic profile of housefly maggot meal

Fatty acid	% fatty acids
Lauric acid (12:0)	0.4(0.2,0.6)
Myristic acid (C14:0)	2.8±0.3(2.6,3.2)
Palmitic acid (C16:0)	29.6±4.6(26.4,34.9)
Palmitoleic acid(C16:1)	13.3±7.5(5.6,20.6)
Stearic acid (C18:0)	3.2±1.4(2.2,4.8)
Oleic acid(C18:1)	18.7(18.3,19.2)
Linoleic acid(C18:2)	16.4(14.9,17.8)
Linolenic acid(C18:3)	2.1

The fatty acids composition of Musca domestica are available in literature, they are decribed by Calvert et al (1969), Pretorius et al (2011) and Saint-Hilarie et al (2007a,b), this data are collected by Makkar et al 2014.

Mealworm (*Tenebrio molitor*)

Mealworms are the larvae of two species of darkling beetles of the Tenebrionidae family, the yellow mealworm beetle (Tenebrio molitor Linneus 1758 and smalles and less common dark or mini mealworm beetle (Tenebrio obscurus) described by Fabricius (1792).

Mealworm beetles are indigenous to Europe and now distribuated worldwide.

T. molitor is a pest of the grain, flour and food stores, but often so much importance since their population are quite small (Ramos- Elordoy et al., 2002). Mealworms ere easy to and feed and have a valuable protein profile. For these reasons, they are produced industrially as a feed for pets and zoo animals, including birds, reptiles, small mammals, amphibians and fish. They are usually fed live, but are also sold canned, dried or in powder form (Aguilar-Miranda et al.,2002; Harduin and Mahoux, 2003; Veldkamp et al., 2012). The life cycle of T. molitor is variable lenght, from 280 to 630 days. Larvae hatch after 10-12 days (at 18-20°C) and become mature after a variable number of stages (8 to 20), typically after 3-4 months (at ambient temperature), but the larva stage can last to 18 months. The mature larva is of a yellow-brown color, is 20-32 mm long and weighs 130-160 mg.

The pupal stage normally 7-9 days at 25°C and lasted up to 20 days at lower temperatures, the adult in natural conditions are alive for two- three mounth. Mealworms are omnivorous and can eat all kind of plant meterials as well as animal products such as meat and feathers (Ramos – Elorduy et al.,2002). They are tipically fed on cereal bran or flour (wheat, oats, maize) supplemented with protein sources such as soybean flour, skimmed milk powder or yeast. Fresh fruit and vegetables (carrot, potatoes, lettuce) are also included which provide moisture (Aguilar-Miranda et al.,2002; Harduin and Manoux, 2003). The diet should be balanced to contain about 20% protein in dry matter basis (Ramos- Elorduy et al.,2002).

Mealworms are able to utilixe small amounts of water contained in dry feeds but the productivity of water deprived mealworms slow (one generation per year). It is preferable to provide them with a souce of water for better productivity and in order to prevent cannibalism.

Relative humidity is linked positively with feltility and adult activity. It is necessary to monitor fresh feeds as they may turn moudly (Harduin and Mahoux, 2003). Mealworm have the ability to recycle plant waste materials of low quality in high- quality feed rich energy, protein and fat in a relatively short time. Mealworms have been shown to able to detoxify zearalenone by party metabolizing it to alpha-zearalenol. There was no risk of zearalenone

accumulating in mealwarm larvae so such an extent theat they could affect animal which ate them (Hornung, 1991).

Chemical constituents

They contain high amounts of crude protein (47-60 %)and fat (31-43%), expecially if they are rearing on oil rich substrate.

The Ca content is lower than the other insect species, in addition the the Ca: P ratio is very low. For this reason feeding the animals exclusively of tenebio molitor causea a syntomatic bone desease (Klasing et al., 2000). Notably the Ca content can be manupulated using Ca fortified diets. The essential amino acids composition of the meal is good. Fatty acids compoition of mealworm is closer to that of housefly maggot meal and house cricket.

Two fatty acid differ substantially: lauric acid is much lower and linoleic acid much higher in mealworm that in black soldier fly larvae.

Table 3.10: Chemical composition (%) of Tenebrio molitor larvae meal (Makkar et al 2014)

Crude protein	52.8±4.2(47.2,60.3)
NDF	12.0±3.5(7.4,15.0)
ADF	6.5(6.4,6.6)
Ether extract	36.1±4.1(31.1,43.1)
Ash	3.1±0.9(1.0,4.5)
Gross energy	26.8±0.4(26.4,27.3)

Baker et al (1998), CIRAD (1991), Finke et al 2002), Klasing et al (2000), Jones et al (1972), Martin et al (1976).

Table 3.11: Mineral content of Tenebrio molitor

Ca (mg/Kg)	2.7±1.9(0.3,6.2)
P(mg/Kg)	7.8±3.7(4.4,14.2)
K(mg/Kg)	8.9(8.5,9.3)
Na(mg/Kg)	0.9
Mg(mg/Kg)	2.3±0.4(2.0,2.8)
Fe(mg/Kg)	57.0±32.0(26.0,110.0)
Mn(g/Kg)	9.0±4.0(6.0,14.0)
Zn(g/Kg)	116.0±24.0(83.0,136.0)
Cu(g/Kg)	16.0±1.0(15.0,18.0)

Baker et al (1998), CIRAD (1991), Finke et al 2002), Klasing et al (2000), Jones et al (1972), Martin et al (1976).

Table 3.12: The aminoacidic composition of *Tenebrio molitor* Larvae meal (% of crude protein)

Alanine	7.3±1.0(6.2,8.2)
Arginine	4.8±1.0(3.8,5.6)
Aspartic acid	7.5±1.7(5.6,8.8)
Cysteine	0.8±0.0(0.8,0.9)
Methionine	1.5±0.4(1.3,2.0)
Lysine	5.4±0.8(4.6,6.1)
Isoleucine	4.6±0.5(4.1,5.0)
Leucine	8.6±1.8(7.4,10.6)
Phenylalanine	4.0±0.4(3.5,4.3)
Threonine	4.0±0.5(3.5,4.4)
Tryptophan	0.6±0.5(0.0, 0.9)
Glutamic acid	11.3±1.1(10.2,12.4)
Hystidine	3.4±0.2(3.2,3.6)
Proline	6.8±0.2(6.6,7.0)
Serine	7.0±3.5(4.9,11.1)
Glycine	4.9±0.9(3.9,5.6)
Tyrosine	7.4±0.3(7.1,7.8)
Valine	6.0±0.6(5.5,6.6)

Finke et al (2002), Jones et al (1972).

Table 3.13: Fatty acid composition of mealworm

Lauric acid (C12:0)	0.5±0.5(0.0,1.0)
Myristic acid (C14:0)	4.0±2.1(2.3,6.4)
Palmitic acid (C16:0)	21.1±6.7(16.1,28.7)
Palmioleic acid (C16:1)	4.0±1.8(2.8,6.1)
Stearic acid (C18:0)	2.7±0.4(2.3,3.1)
Oleic acid (C18:1)	37.7±8.7(27.7,43.3)
Linoleic acid(C18:2)	27.4±4.0(23.1,31.0)
Linolenic acid (C18:3)	1.3(1.1,1.4)

Finke et al (2002), Jones et al (1972).

The use of the insects as feed

Ruminants

In Europe in according to the regulation 999/2001 in order to prevent the trasmission risk of TSE" trasmissible spongiform encephalitis", it is not possible fed the ruminants on insect meal. In literature are not available studies on the use of Hermetia illucens, Musca domestica and Tenebrio molitor in these species. Joselevich et al (2004) studied the effect of silkworms on the ruminants and their results showed that the silkworms pupae present a favourable aminoacidic profile and highly undegradable protein, was also observed that the this insect showed a high fat content for the ruminant nutrition.

Pigs

Hermetia illucens larvae meal was found to be a suitable ingredient in growing pig diets, being especially valuable for its amino acid, lipid and Ca contents.

However, its relative deficiency in methionine + cystine and threonine requires the inclusion of those amino acids for the preparation of balanced diets. The ash content of the meal is also high and this requires attention. The diets containing the larvae meal were as palatable as a soymeal based diet (Newton et al., 1977). Dried black soldier fly prepupae meal was fed to early weaned pigs as a replacement (0, 50, or 100%) for dried plasma (meal in the diet: 0% during phase 1, 2.5% during phase 2, and 5% during phase 3), with or without amino acid supplementation. Without amino acid supplementation, the 50% replacement diet gave slightly better performance during phase 1 (+4% gain, +9% feed efficiency). However, the 100% replacement diets did not perform as well as the control (overall performance reduced by 3 to 13%). Additional refinement (cuticle removal and rendering) may be necessary to make black soldier fly prepupae meal suitable for early weaned pigs (Newton et al., 2005).

A recent study (Danicke et al 2016) on piglet that were fed on Hermetia illucens larvae meal at different inclusion the diet (2.5%, 4%, and 10%) administrated ad libitum for five weeks and feed intake, weight gain, feed efficiency and faecal consistency were determined weekly. Animals accepted diets with Hermetia meal inclusion up to 10% readily and displayed the common growth curve of weaned piglets. After five weeks pigs were slaughtered and blood and tissue samples taken. Diet showed no significant impact on average daily gain and live weight as well as feed intake and feed efficiency. Faecal consistency was also not changed in animals receiving Hermetia meal compared to those fed the control diet. Overall, piglet's

performance was comparable for diets including up to 10% Hermetia illucens protein meal.

Velten et al. (2017) studied the response of piglets due to amino acid optimization of mixed diets with 75% replacement of soybean-meal by partly defatted insect meal (Hermetia illucens). indicate that diet Hermetia illucens 75% with amminoacids addition yielded similar results for all zoo-technical parameters under study as compared to the control diet. Diet Hermetia illucens 75% with the basic level of aminoacidic fortification tended to slightly lower growth, feed intake, feed and protein conversion ratio, respectively. However, no significant effect was observed. Accordingly, the composition of gut microflora was also not significantly influenced by the dietary treatments. The autors (Velten et al 2017) observed that partly defatted meal of Hermetia illucens is a promising alternative protein source for replacing SBM in diets for piglets.

There is limited information on the use of housefly maggot meal for pig feeding. In Russia, sows and their offspring were fed a diet containing processed housefly maggot meal with no adverse effect on piglet performance, health and organoleptic properties or on the sows' physiology and breeding performance (Bayandina and Inkina, 1980). In Thailand, weaned pigs were fed on soybean based diet supplemented with 10% maggot meal to replace fishmeal and provided isoenergetic and isoproteic diet. This diet had no negative effect on body weight gain or feed conversion efficiency (Viroje and Malin, 1989). In Nigeria, early weaned pigs were fed 10% of a 3:1 mixture of dried rumen contents and maggot meal in the diet replacing 10% wheat offal without any adverse effect on performance (Adeniji, 2008).

Rabbits

Dried silkworm meal could totally replace soymeal in balanced diets for growing rabbits without adverse effects (Carregal and Takahashi, 1987). Silkworm meal at 7% in diet was included in the control diet for a study that evaluated a new variety of rapeseed meal in China (Liu et al., 1987), suggesting that the silkworm meal is a traditional ingredient in rabbit feeds in China.

Recently, Gasco et al. (2017) evaluated the use of Tenebrio molitor oil as a fat source in partial and total replacement of soybean oil. The results obtained from this trials put in evidence that hermetia illucens fat and Tenebrio molitor oil used as partial or total substitute of soybean oil can be used as feed ingredient in rabbit diets without impacting growth performance and slaughter traits (Gasco et al 2017).

Rodents

Makkar et al (2014) reported that maggot meal could be included at 5% in the diets of young rats as a partial replacement for fishmeal in isoproteic and isoenergetic diets for optimal weight performance and nutrient utilization (Bouafou et al., 2011a). However, the authors reported that the inclusion of 10% maggot meal was responsible of fibrosis in the liver and the kidneys (Bouafou et al., 2011b).

Aquaculture

Several experiments have shown that black soldier fly larvae could partially or fully substitute for fishmeal in fish diets. However, additional trials as well as economic analyses are necessary because reduced performance has been observed in some cases, and the type of rearing substrate and the processing method affect the utilization of the larvae by fish.

Chopped soldier fly larvae grown on hen manure fed to channel catfish alone or in combination with commercial diets resulted in similarbody weight and total length as on the control diets (young catfish refused whole larvae but consumed chopped ones). Aroma and texture of channel catfish fed larvae were acceptable to the consumer (Bondari and Sheppard, 1981).

A later study was less favourable: replacement of 10% fishmeal with 10% dried soldier fly larvae resulted in slower growth over a 15-week period for sub-adult channel catfish grown in cages. However, the replacement did not reduce growth rate significantly when channel catfish were grown in culture tanks at a slower growth rate. Feeding 100% larvae did not provide sufficient DM or CP intake for good growth for channel catfish grown in tanks. Chopping of the larvae improved weight gain and increased feed consumption in channel catfish, but resulted in lower feed efficiency.

Greater larvae waste was observed in the chopped larvae fed tanks than in the whole larvae fed tanks, and chopping was considered to be unnecessary in channel catfish feeding (Bondari and Sheppard, 1987). A comparison between menhaden fishmeal and black soldier fly prepupae meal showed that the latter could be advantageous as a replacement for fishmeal provided it was also supplemented with soybean meal in order to obtain isoproteic diets. It was shown that an inclusion rate higher than 7.5% of black soldier fly prepupae meal was unnecessary (Newton et al., 2005). In yellow catfish, 25% replacement of fishmeal by black soldier fly larva powder produced no

significant difference in the growth index and immunity index when compared with those in control group (Zhang et al., 2014a,2014b).

Chopped black soldier fly larvae (grown on hen manure) fed to blue tilapia alone or in combination with commercial diets resulted in similar performance (body weight and total length) as the control diets. Aroma and texture of tilapia fed larvae were acceptable to the consumer (Bondari and Sheppard, 1981). In a later experiment, feeding dry black soldier fly larvae as the sole component of the diet did not provide sufficient dry matter or protein intake for good growth for tilapia grown in tanks. Chopping of the larvae however improved weight gain by 140% and feed efficiency by 28% when it was used as the sole component of the diet (Bondari and Sheppard, 1987).

Dried ground black soldier fly prepupae reared on dairy cattle manure enriched with 25–50% trout offal could be used to replace up to 50% of fishmeal protein in trout diets for 8 weeks without significantly affecting fish growth or the sensory quality of trout fillets, though a slight (but non-significant) reduction in growth was observed (Sealey et al., 2011). In a 9-week study, replacing 25% of the fishmeal protein in rainbow trout diets with black soldier fly prepupae meal (reared on pig manure) did not affect weight gain and feed conversion ratio (St-Hilaire et al., 2007a).

A control diet containing 200 g/kg fishmeal (FM) was stepwise replaced by insect meal (black soldier fly larvae) at 25%, 50% or 100% FM replacement. Insect meal containing diets performed equally well as the FM group and increase in feed conversion efficiency was observed. Histology did not show any differences between any of the dietary groups and sensory testing of fillets did not reveal any significant difference (Lock et al., 2014). However, these authors did caution that the method of preparation of insect meal could impact performance.

Juvenile turbot accepted diets containing 33% defatted black fly soldier larvae meal (as a replacement of fishmeal) without significantly affecting feed intake and feed conversion. However, specific growth rate was lower at all the inclusion rates and higher inclusion rates decreased the acceptance of the diet. This resulted in reduced feed intake and lower growth performance. The presence of chitin might have reduced feed intake and nutrient availability and therefore reduced growth performance and nutrient utilization (Kroeckel et al., 2012).

The use of housefly maggots as supplements in the diets of tilapia and catfish species (Clarias gariepinus and Heterobranchus longifilis) has been reported in Nigeria (Madu and Ufodike, 2003).
There have been a number of experiments in Nigeria on the use of housefly maggots in the diets of African catfish, mostly C. gariepinus. The results are generally positive though the inclusion of maggot meal should be limited to 25–30% because performance tends to decrease when higher inclusion rates are used.
Nile tilapia fed a 4:1 mixture of wheat bran and live maggots had a better growth performance, specific growth rate, feed conversion ratio and survival than fish fed a diet containing only wheat bran (Ebenso and Udo, 2003). In another experiment, maggot meal was included at 15–68% in the diet, replacing fishmeal. The best performance and survival was obtained at a level of 25% maggot meal (replacing 34% fishmeal) in the diet. Maggot meal was beneficial to fish growth and performance and no adverse effects on the haematology and homeostasis were observed. However, to enhance the fatty acid profile in fish, adequate sources of n − 6 and n − 3 fatty acids should be included in the diet (Ogunji et al., 2007, 2008a and b).

Fresh and dried mealworms have been found to be an acceptable alternate protein source for the African catfish. Replacing 40% of fishmeal with mealworm (dried and ground) resulted in growth performance and feed utilization efficiency similar to that obtained with the control diet (both control and test diets being isoproteic). Catfish fed isoproteic diets with up to 80% replacement of fishmeal with mealworm meal still displayed good growth and feed utilization efficiency. Catfish fed solely on live mealworms had a slight depression in growth performance but fish fed live mealworms in the morning and commercial catfish pellets in the afternoon grew as good as or better than fish fed the commercial diet. Live and dried mealworms were found to be highly palatable. Catfish fed mealworm-based diets had significantly higher lipids in their carcass (Ng et al., 2001).
A study with gilthead sea bream juveniles fed diets containing mealworm at replacement levels of 25% and 50% of fishmeal protein showed that up to 25% inclusion of the insect meal in diet did not lead to adverse effects on weight gain and final weight, while 50% level induced growth reduction and less favourable outcomes for specific growth rate, feed conversion efficiency and protein efficiency ratio. The whole body proximate composition analysis did not show any differences between treatments. These results showed that the substitution of fishmeal protein in diets for

gilthead sea bream juveniles is feasible up to 25% without adverse effects on growth performance and whole body proximate composition (Piccolo et al., 2014 and 2017).
Mealworm added in a diet (containing 45% CP) at levels of 25% and 50% by weight (as a replacement of fishmeal) showed that mealworm can be used at an inclusion level of up to 50% without a growth performance reduction, leading to a saving of fishmeal (Gasco et al., 2014a).
A study showed that up to 25% inclusion of mealworm in the diet (as a replacement of fishmeal; all diets being isoproteic) did not lead to adverse effects on weight gain, while at 50% level of the mealworm induced reduction in growth, specific growth rate and feed consumption ratio. Protein efficiency ratio, feed consumption and body composition were not affected. On the other hand, mealworm inclusion influenced the fatty acid composition of body lipids (Gasco et al., 2014b).

Poultry
Broilers
Hale et al (1973; cited by Newton et al.,2005), fed chicks on a diet containing black soladier larvae meal (replace soybean meal) and observed a better weight gain in the broilers fed insect meal than the control group, and the broilers fed soybean meal consumed much feed, this observation suggested that the feed conversion efficiency in the broilers fed Hermetia illucens larvae meal was higher than the the control group.
Ballitoc and Sun (2013) studied the effects of Tenebrio molitor larvae meal (at inclusion level of 0 - 0.5-1-2- and 10%), the broilers fed the highest Tenebrio molitor inclusion presented a higher FCR, higher live body weight gain and the result on carcass traits resulted variables.
Schiavone et al (2014) and De Marco et al (2014) studied the apparent ileal digestiblity of broilers fed Tenebrio molitor and Hermetia illucens al 25% of a basal diet (constituated by soybean meal). The digestibility of dry and organic matter was higher in the control group than the insect meal diets, and the digestibility of crude protein and energy resulted highest in the control group, intermediate in the Hermetia illucens diet and lowest in Tenebrio molitor diet.

Other poultry species
Makkar et al (2014) reported a study on Japanese quails (Cotornix japonica Japonica). In India, Japanese quail were fed with various diets in which grasshopper meal (O. hyla) gradually replaced fishmeal. For a range of

growth parameters, the best results were obtained with the diet in which 50% of fishmeal was replaced with Oxya meal. Fecundity (i.e. the number of eggs laid per female) was significantly higher compared with the control treatment (Haldar, 2012).
Recently (Loponte et al., 2017) studied the use of two insect meals (from *Hermetia illucens,* HI and *Tenebrio molitor,* TM larvae) at two levels of soybean protein substitution (25 and 50 %) on barbary partridge in order to investigate the effect of on growth performance, blood profiles and carcass traits. From the results the feed conversion ratio (FCR) of the barbary partridge fed Tenebrio molitor larvae meal are more effective than the birds fed on Hermetia illucens larvae meal. It is described that the prebiotic effect of chitin reduced the albumin to globulin ratio and this result was not affected by the amount of chitin intake.

Laying hens

Calag et al (1981) showed that the laying hens fed on maggot larvae meal (replace the soybean meal) are not affected by dietary treactment egg production and shell streght. The 100% of replacement had negative effect on the hen day production because it was showed a decrease the production of the eggs (Agumbiade et al., 2007).
Akpodieke et al (1998), showed that the yolk cholesterol and the calcium content of the eggs producted by laying hens fed on maggot meal was higher than the control group, and is described that decrease at increased level of maggot meal in the diet, for this reason the use of maggot meal as protein source in the diet can reduce the cholesterol content in the eggs.
Also the mealworms could be suitable in laying hens: Giannone et al (2003) decribed that *T. molitor* and *T. mauritanius* could be a suitable alternative protein source in laying hens. Dried ground mealworms in replacement to fish meal in the diet of laying hens and resulted in 2.4% higher egg-laying and they obtained a good quality of the eggs.
Maurier et al (2016) conducted a trial on laying hens fed on *Hermetia ilucens* reared on vegetarian waste (such as pasta and waste convenience food industry) because the insects are able to use the waste and transform that in a good nutritional value. The author administrated two diets (H12 and H24, 12 and 24 g on the 100 g of feed), replacing 50 or 100% the soybean cake. Maurier et al.,2016 described that the group H24 presented the albumen weight lower than the control and H12. There are not differences reported in

the eggs production and feed intake, so Hermetia illucens larvae meal could be suitable in laying hens.

Al Qazzaz et al (2016) conducted a study on Arabic strain hens, the inclusion in the experimental diets was 5 and 1 %. The results showed that feed intake, weight gain, laugh unit, and hatchability not affected by dietary treatment. There was a significant improvement in hen day eggs production and hens house production. The feed conversion ratio, egg weight, shell ratio, shell tichness, egg yolk color, fertility and egg mass were affected by dietary treatment of *Hermetia illucens* larvae meal. There are no differences in appearance for texture, taste and acceptance of the eggs of hens. The odor was not affected by dietary treatment. The studies previously published, even if are limited, showed that the insect larvae meal could be suitable in laying hens diet.

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IV
PAPs – Processed animal proteins

At the beginning of this century, changes in European Union (EU) regulations made to prevent spreading of transmissible spongiform encephalopathy (EU 2001, 2003; Woodgate & Van Der Veen 2004) banned the animal by-products such as the most type of processed animal protein from feeds (the fish meal was banned in the ruminant but permitted in the other farming species).

Recently, the decreasing risk of TSE (transmissible spongiform encephalitis) and the changes in the industrial processing of the animal by-products, led the European Commission to reform the regulation, and the processed animal proteins (of non-ruminant origin) has been permitted in aquaculture species since 1st June 2013 (EU, 2012).

EFSA's decision of 2015, not only regulates the introduction of the novel ingredients in the European Union but also the use of new production technologies that would change their nutritional value (Regulation UE 2015/2283).

The animal by-products

It is estimated, according to Alm (2012), that during the slaughter and the meat processing, a quote variable from 32 to 48% of the body weight is discharged and not available for human consumption (the portions not considered edible depending on the species: in the chickens is not edible the 32% of the body weight, 38% for the pigs, 46% in the cattle and 48% in the small ruminants).

These residual parts, that the humans normally do not consider edible, represent the category of animal by-products and they include:

- blood
- bones
- feathers
- hides
- fat trims
- viscera.

The materials and the products originated from the food production process and not good for the human consumption, could be used to other purposes, for example as organic fertilizer, as animal feed, soil improvers, technical products for leather or chemical industry (Jedrejeck et al, 2016).

The European regulation in order to prevent the risk linked to the possible use of the animal by products and the derived products is considered one of the most stringent in the world (Jedrejeck et al, 2016).
For safety reasons, the management of the animal by-product is regulated by the European Union. The first regulation was approved fifteen years ago (Regulation 1774/2002), actually the regulations in force are the regulations 1069/2009 and 142/2011. The European legislation with regard to the food legislation includes the regulations 853 and 854/ 2004 and the animal by product are consider not good for human consumption.
In the European regulation are reported the rules for the collection, transport, storage, handling, processing, placing on the market, import/export and transit of the animal by products and the products derived from them (Farrar, 2010).
The animal by products, according to regulation 1069/2009, are classified into three categories based on the risk level that they present.
In the category 1, are comprised the animal by products that present a highest risk, and they include in particular the animal residue that represent a risk of TSE transmission or infected with zoonotic pathogens (responsible of diseases transmissible to humans), or animal by product derived from livestock exposed to contaminants (for example pesticides or heavy metal). The disposal of the European regulation in case of animal by product included in the category 1 is the incineration in authorized plant or bury in approved landfill.
The category 2 includes high risk animal by-product, less than the category previously cited, containing antibiotics residues, the imported products that are considered not good by the veterinarian control, manure or certain products derived from the slaughterhouse, and the animals killed or died outside the human food chain. According to European legislation (EU 1069/2009) the disposal is the incineration and/or rendering, the burial an approved landfill site, used in chemical industry, used as organic fertilizer or biogas production. It is not possible use the animal by product comprised in the category 2 to feed purposes.
The materials included in the category 3 are considered at low risk, are derived from slaughterhouses or from meat processing industries and includes the part of the livestock body that are not considered edible for humans (hides, horn, skin, hair, feather, bones, blood, viscera), or for commercial reasons. In this category are included also the former foodstaff and the catering and kitchen wastes. The European legislation (1069/2009) admits the recycling of this animal by products in very different way: they

could be incinerated and rendering, buried in approved landfill, composting anaerobic digestion, used as feed ingredient for livestock and pet animals.

Processed animal proteins (PAPs)

The processed animal proteins (PAPs) derives from animal by-products derived from slaughtered healthy animals, they are included in the category 3 of the animal by products EU 1069). The European legislation describes the processing methods in order to make the not edibles part of the livestock animals good to use for feed purposes. There are different kind of processed animal proteins:

- blood meals
- meat meals
- bones meals
- horn meals
- feather meals

The fish meals are also included in the category of processed animal proteins. Since the 1st June 2013 the PAPs derived from poultry and pork slaughtered can be used as ingredients in aquaculture. The fish meals can be used not only to feed farmed fish, but also as ingredients in horse, pig and poultry diets. Moreover, the fish meal is the only PAP authorized in ruminant. The processed animal proteins could represent a good alternative protein source because they show an excellent nutritional value and a considerable protein amount. The chemical composition of the PAPs depend on the source of the animal by-products used, and also on the processing methods adopted (for example for the hydrolysed feather meal, the temperature and the pressure adopted may affect the nutritional value (Jedrejeck et al., 2016). They are highly and easily adsorbed by the animals. They are rich in essential amino acids, in particular the amount of lysine is generally adequate to the animal's requirements, this essential amino acid playing an important role in the growth of the different animal species (Wang and Parsons, 1998). On the contrary, the vegetable protein sources are often poor in certain essential amino acids, and a supplementation of free amino acid in the diet in order to render it balanced is often required (Jedrejecket al., 2016). It is possible to obtain the energy from the fat contained in the processed animal protein (except from the blood meal, due to its low fat content), poultry and pork processed proteins shows the highest amount of fat (about 16%).

In addition, they are rich in calcium and phosphorus and in B12 vitamin. In the livestock animals the P is present in not edible part, for this reason the P in the processed animal protein is very high, especially in the pork PAP, high protein (about 5%). The P in processed animal protein is more digestible than the plants, in fact, the poultry can digest 62% of P contained in meat and bones meal, and only the 42% in soybean meal and 33% in rapeseed meal (Jedrejck et al.,2016).

The nutritional values of the most important PAP according to Alm (2012) are reported in the table below.

Table 4.1: Chemical composition of some processed animal proteins (Alm, 2012)

%	Moisture	Ash	Crude protein	Fat	Phosphorus
Blood meal	4-7	2-3	90-95	1	0.2-1
Feather meal	6-8	4-10	80-85	7-11	0.5
Poultry PAP (high ash)	5-7	17-20	60-63	12-15	2-3
Poultry PAP (low ash)	4-6	10-15	65-68	13-16	2
Pork PAP (High ash)	5	30-35	45-50	13-16	5-7
Pork PAP (low ash)	5	22-30	55-65	12-16	3-4

Blood meal

The blood collected during the slaughter of various livestock animals (except for the ruminant blood, according to European legislation), can be used to feed purposes, because it is classified in the category 3 of animal by products (Leg EU 1069/2009).

For safety reasons, blood must be heated to be used in animal feeding: a minimal temperature of 100°C for 15 min is necessary in order to destroy potential pathogens (salmonella, mycotoxins, prions) (Göhl, 1982). It is

recommended to avoid feeding a species with blood meal from the same species.
In the European Union, blood meal has been banned from feeding to animals since 2000 (Council Decision 2000/766/EC), though since 2006 blood products from non-ruminants have been authorized for use in aquaculture (Médale et al., 2009).
From the blood it is possible to produce two types of animal by product: blood meal (that derives from whole blood) and the blood products. The blood products derive from the slaughterhouse equipped with a system to separate the blood and they include dried/frozen liquid plasma, dried whole body, dried/frozen red cells. The blood meal can be used as ingredient in the feeds only in aquaculture.

Processing
Blood is a highly perishable product and must be processed as soon as possible after slaughter. Blood meal can be prepared by a small-scale operation. Blood meal is hydroscopic and needs to be dried to less than 10-12% moisture and stored in a dry place in order to preserve it. There are different ways to prepare blood meal: solar drying, oven drying, drum drying, flash drying, spray drying. The drying method is important because there is an inverse relationship between the amount of heat applied and protein digestibility. Particularly, lysine content and lysine availability decrease when the amount of heat increases (Batterham et al., 1986). Overcooked blood meals are darker, due to the destruction of the haemoglobin, and less palatable.
In order to prevent transmissible diseases, it is important to apply a correct temperature for a sufficient time, to kills the pathogens microorganisms. The inlet temperatures are between 160 °C and 300 °C and the minimum time of exposure between 10 and 30 seconds; the outlet temperatures are between 70 °C and 90° C. The blood meal contain the highest amount of protein compared to the other animal by-products (90-95% of crude protein), but it is poor in essential amino acids (except for the lysine).
Solar and oven drying (small scale production)
Solar drying is well suited for small-scale operations or when advanced technical equipment is not affordable. Blood is collected in large pans and slowly boiled while stirring constantly. When moisture is sufficiently reduced (10-12%), blood meal is spread on a clean cemented surface and then sun-dried. It can also be oven-dried. The blood may be spread on

milling offal, rice bran or other plant products for better drying and result in a complete feed.
For large scale operations the 3 processes detailed below are used.

Drum drying
The raw blood is finely comminuted to form a free-flowing slurry that is then deposited onto the descending side of the top of a heated drier drum and formed into a film by one or more spreader rolls. The film is rapidly dried and scraped in the form of a dried sheet which can either be flaked or pulverized to provide a high grade blood meal product. Vapours above the drying cylinder are scrubbed before being released to the atmosphere and represent the only effluents from the process (Overton, 1976).

Ring and flash drying
The blood is dispersed into the high velocity venturi section of the system. The blood first comes into contact with the hot drying airstream and the bulk of the evaporation occurs. The product is then dried as it is conveyed up through the drying column. The presence of a "manifold" or "internal classifier" in the ring drying system is what differentiates it from the flash dryer (GEA, 2009a; GEA, 2009b).

Spray drying
The blood is spray dried as whole blood, or after separation into plasma and red albumin (GEA, 2010). Blood products must be dried at low temperatures in order to prevent heat coagulation (GEA, 2009a; GEA, 2009b). Spray dried blood meals are also called spray dried blood powder or blood flour (Dipanjali Konwar et al., 2005).

Spray-dried porcine plasma is prepared as follows: the blood from slaughtered pigs is added to an anticoagulant (generally sodium citrate) and then centrifuged to remove erythrocytes. The plasma obtained is subsequently spray-dried and used for feeds production of (van Dijk et al., 2001).

Products resulting from the 3 processes have an overall higher quality than sun-dried blood meals since the duration of the heating period is lower than with cooking. Proteins and amino acids are better preserved and lysine content is higher (Cromwell, 2009).

In order to remove the water present in the blood (65% of fresh blood is made up of water), it can be used the addition of 1 % of unscaled or the 3% of scaled lime. This method removes the dry matter by the 10-15%, which includes many materials. The blood sometimes need to be stored prior to being processed and dried. The raw blood can be stored for one week by adding 3% of sulphuric acid or an equivalent amount of another acid, and storing to 72 hours before-drying (Divakaran, 1987; Divakaran, 1988).

Nutritional attributes

Blood meal contains mostly protein (about 90-95% DM) and small amounts of fat (less than 1% DM) and ash (less than 5% DM), though non-industrial blood meals may include other materials and thus be richer in ash. Unlike other animal protein sources, blood meal has a poor amino acid balance.

Its lysine content is relatively high (7-10% DM) which makes it an excellent supplementary protein to use with plant-derived feed ingredients that are low in lysine. However, its isoleucine content is very low (about 1% DM), so diets for monogastric animals must be formulated to contain enough isoleucine for the level of performance desired (Piepenbrink et al., 1998; Maiga et al., 1996).

Pepsin digestibility has been shown to be a good test for assessing the availability of the protein fraction of blood meal (Hegedüs et al., 1989). Blood meal is rich in iron (more than 1500 mg/kg DM).

The blood meal generally is not very palatable for the livestock animals, and in particular if the blood is overcooking (the taste depends on the free haemoglobin), it is not a good idea to add the blood meal more than the 5-6% in the diet, because they can reduce the feed consumption. (Martinez-Llorens et al., 2008). It is important that the animal has an adaptation period to get animal used to eating blood meal.

The nutritional values of blood meal are showed in the table 4.2.

Table 4.2: Nutritional values of the blood meal (Feedipedia)

Main analysis	Unit	Avg	SD	Min	Max	Nb	
Dry matter	% as fed	93.8	2.6	87.5	98.5	124	
Crude protein	% DM	94.1	3.9	84.9	100.0	142	
Crude fibre	% DM	0.5	0.4	0.1	1.5	18	
NDF	% DM	12.8				1	
Ether extract	% DM	0.8	0.7	0.1	3.1	25	
Ether extract, HCl hydrolysis	% DM	1.8	1.5	0.3	7.8	42	

Ash	% DM	3.0	1.5	1.6	7.3	116	
Gross energy	MJ/kg DM	24.1	2.2	17.7	24.9	14	*
Minerals	Unit	Avg	SD	Min	Max	Nb	
Calcium	g/kg DM	1.3	1.0	0.3	4.5	44	
Phosphorus	g/kg DM	2.2	0.9	0.8	3.9	51	
Potassium	g/kg DM	3.8	3.4	1.8	10.5	7	
Sodium	g/kg DM	4.5	2.2	2.1	9.4	11	
Magnesium	g/kg DM	0.2	0.0	0.2	0.2	7	
Manganese	mg/kg DM	1				1	
Zinc	mg/kg DM	24	2	21	27	8	
Copper	mg/kg DM	6	0	5	6	5	
Iron	mg/kg DM	2186	298	1459	2732	22	
Amino acids	Unit	Avg	SD	Min	Max	Nb	
Alanine	% protein	7.9	0.4	6.7	8.7	23	
Arginine	% protein	4.2	0.3	3.5	4.7	31	
Aspartic acid	% protein	10.7	0.7	9.8	12.1	22	
Cystine	% protein	1.1	0.2	0.7	1.4	26	
Glutamic acid	% protein	9.5	0.4	8.8	10.2	22	
Glycine	% protein	4.5	0.4	4.0	5.6	25	
Histidine	% protein	6.2	1.0	4.9	8.4	31	
Isoleucine	% protein	1.1	0.4	0.6	2.0	49	
Leucine	% protein	12.1	1.0	10.1	13.9	49	
Lysine	% protein	8.7	0.7	7.1	10.3	54	
Methionine	% protein	1.2	0.2	0.9	1.5	31	
Phenylalanine	% protein	6.9	0.5	6.0	8.0	30	
Proline	% protein	4.0	0.4	3.4	4.7	13	
Serine	% protein	4.9	0.4	4.4	5.6	23	
Threonine	% protein	4.7	0.4	3.8	5.6	49	
Tryptophan	% protein	1.4	0.4	1.0	2.1	10	

Tyrosine	% protein	3.0	0.5	2.2	4.1	16	
Valine	% protein	8.5	0.7	7.4	9.7	31	
Ruminant nutritive values	Unit	Avg	SD	Min	Max	Nb	
Nitrogen digestibility, ruminants	%	74.7				1	
a (N)	%	22.5				1	
b (N)	%	44.1				1	
c (N)	h-1	0.050				1	
Nitrogen degradability (effective, k=4%)	%	47					*
Nitrogen degradability (effective, k=6%)	%	43		40	43	2	*
Pig nutritive values	Unit	Avg	SD	Min	Max	Nb	
Energy digestibility, growing pig	%	89.3					*
DE growing pig	MJ/kg DM	21.5					*
Nitrogen digestibility, growing pig	%	90.7	2.9	89.0	94.0	3	
Poultry nutritive values	Unit	Avg	SD	Min	Max	Nb	
AMEn cockerel	MJ/kg DM	13.5	0.8	12.1	14.2	6	*
AMEn broiler	MJ/kg DM	13.5					*
Fish nutritive values	Unit	Avg	SD	Min	Max	Nb	
Energy digestibility, salmonids	%	60.4		31.9	89.0	2	
Nitrogen digestibility, salmonids	%	62.2		29.4	95.0	2	

Avg: average or predicted value; SD: standard deviation; Min: minimum value; Max: maximum value; Nb: number of values (samples) used.
The asterisk * indicates that the average value was obtained by an equation.

Blood meal in fish

Blood meal is a good quality ingredient for fish and has been tested successfully in many fish species. Spray-dried blood meal can be used also as a binder in fish feeds.
In gibel carp (Carassius auratus gibelio) (Yang et al., 2004), African catfish (Clarias gariepinus) (Goda et al., 2007) and tilapia (El Sayed, 1998), spray-dried blood meal can replace 50 to 75% of the fish meal, and in rainbow trout (Oncorhynchus mykiss) up to 100% (Watanabe et al., 1998; Médale et al., 2009).
However, the fish meal substitution with blood meal was found to lead to lower growth performances in tilapia (El-Sayed, 1998). Blood meal is well digested by the humpback grouper (Cromileptes altivelis) (Laining et al., 2003) and its gross energy is well digested by the rohu (Labeo rohita) (Noreen et al., 2008). A maximum of 5% blood meal in the diet is recommended for gilthead sea bream (Sparus aurata) as sensorial alterations may occur at a 10% inclusion rate (Martinez-Llorens et al., 2008).

Feather meal - Hydrolysed feather meal (FEM)

An estimated 140,000 t of waste are produced annually by hatcheries that generate commercial broilers, laying hens, and turkeys (Das et al., 2002). Included in this material are infertile eggs, dead embryos, egg shells from hatchlings, and unsalable chicks.
Traditional means of disposal for this material have been landfills or broadcast land application as fertilizer. These methods have become less acceptable as the volume of the waste and its concentration in location increased. As the volume of waste grew larger, the land mass required for proper application also increased, raising fuel expenditures for transportation to application sites, making land application increasingly less economical.
Landfills in the vicinities of the hatcheries began rejecting the larger quantities of material without the assessment of large tipping fees. Finding practical techniques for recycling the nutrients in hatchery waste has become a high priority.
Feather meal is an animal by product comprised in the category 3 (Reg EU 1069/2009), it is derived from the poultry slaughterhouse and it is one of the first animal by-product used is animal nutrition (the first review published dates back to 1944). The processing method, in particular temperature and pressure applied, is very important because it can affect significantly its nutritive value (Jedrejeck et al, 2016).

Feathers represent from 5% to 7% of the body weight of most domestic fowls. While they bring many benefits to birds, they become a waste product during poultry processing. Feathers, which are comprised of about 90% keratinaceous protein, have poor digestibility characteristics and deficiencies of several essential amino acids (Onifade et al., 1998), including lysine (2.5% of CP in feathers), methionine (0.6% of CP in feathers), and histidine (1.1% of CP in feathers). Despite the challenges they present, feathers can be washed, hydrolysed to improve digestibility, dried, and ground to yield hydrolysed feather meal (Ockerman and Hansen, 2000). Part or all the blood from the processing facility may be mixed with the feathers and may contribute up to 10% to the DM in the final product.

Processing

Although crude feather meal contains a high percentage of protein, it mainly exists of keratin that is unavailable for fish. Raw feathers contain mainly keratins that contain disulfide bounds that without processing are unavailable for fish and other animals. Steam together with pressure can break the disulfide bonds whereby the protein and amino acids become available. A fine balance between sufficient hydrolysis and over-processing makes feather meal more or less digestible. Therefore significant differences in apparent digestibility of protein have been observed among feather meals from various origins.

Pressurized cooking of feathers is the primary method of processing used in preparing feather meal. Some bacteria have been identified that produce a feather digesting enzyme, that will convert the protein fraction into a digestible form (Shih, 1993). Pepsin digestibility is used as method of assessing the quality of feather meal. Normally a pepsin digestibility of 75 % is considered to be a minimum value to assure that the feather meal has been adequately processed.

Co-extrusion with SBM is one means of processing hatchery waste (Lilburn et al., 1997). Lactic acid fermentation followed by extrusion or drying and grinding is another (Deshmukh and Patterson, 1997). If hatchery waste is cooked, dried, and ground, the resulting meal is known as poultry hatchery by-product (PHB). Research has shown that all the resulting products can be successfully incorporated into diets for both laying fowl and broilers (Wisman, 1964; Vandepopuliere et al., 1977; Deshmukn and Patterson, 1997;

Lilburn et al., 1997). In addition to supplying energy and protein, PHB and similar products also supply Ca. The mineral availability of PHB was similar

to bone meal or limestone (Lilburn et al., 1997) and it resulted in good egg shell quality in laying hens (Vandepopulier et al., 1977). These same qualities might make it useful in diets for lactating livestock or in any diet in need of Ca and protein supplementation; however, research in this area has yet to be documented.

Potential constraints

Feather meals needs to be tested (pepsin digestibility) to assure that it has been processed properly. Care need to be taken to select other supplemental protein sources that will complement to poor amino acid profile of the feather meal, when formulating rations.

Ruminants

Feather meal is particularly appealing for ruminant diets because a large portion of the protein is RUP- rumen undegradable protein and is available for digestion in the lower gastrointestinal tract. In situ, experiments indicated that about 70% of the CP in feather meal escapes degradation as compared to only 25% of the CP in soy bean meal (Goedeken et al., 1990a; Klemesrud et al., 1998; Klemesrud et al., 2000).

Calves

Feeding a combination of feather and blood meals resulted in the best growth in calves (Blasi et al., 1991). When feather meal was incorporated into liquid supplements to replace a portion of the CP provided by urea, average daily gain and reproductive performance was improved in mature beef cows (Pate et al., 1995). Calves fed feather meal as their primary supplemental protein didn't respond to when supplemental lysine or methionine was fed (Klemesrud et al., 1998). Steers fed FEM or other protein sources gained weight faster than steers fed a diet containing urea as a source of supplemental N (Goedeken et al., 1990).

Dairy cattle

Feather meal has been found to be an effective supplemental protein source for lactating dairy cattle (Harris et al., 1992). Combination of feather meal and blood meal was shown to be acceptable as a protein source in dairy cattle (Johnson et al., 1994). Feather meal can also be utilized in dairy rations. A FM and BM mix added to diets for lactating dairy cows increased milk production, milk protein, and solids-corrected milk yield when the diets contained 17.6% CP (Grant and Haddad, 1998) but resulted in decreased production when diets contained 19.6% CP. These authors offered no

explanation for the reduction in milk production observed at the higher protein level and it seemed to defy their finding that efficiency of use of metabolizable protein improved with the addition of FM at both protein levels.

Small ruminants

Feather meal was found to increase ADG when it replaced soybean meal and urea in sheep diets (Punsri, 1991). When FEM was utilized as a supplemental protein source in place of all or a portion of soy bean meal in diets for lambs, weight gains increased linearly as FEM increased (Thomas et al., 1994). Supplementation with feather meal showed no effect on straw digestion in lambs (Thomas et al., 1994). Wool fibre diameter and sulphur content of wool didn't differ in lambs fed feather meal (Thomas et al., 1994). Cozzi et al. (1995) observed similar weight gains and feed to gain ratios (F:G) among growing and finishing lambs fed diets containing SBM or a mixture of 56% SBM, 22% FEM, and 22% blood meal. Lactating dairy goats responded well to the inclusion of a FM and BM mix which supplied 30% of supplemental N in their diets (Andrighetto and Bailoni, 1994).

Pigs

Swine feeding trials found that when feather meal replaced soybean meal that ADG and FC declined (Duangsmorn Sinchermsiri et al., 1989). High levels (5 and 7.5 %) of dietary feather meal decreased digestibility of DM and CP, decreased loin eye area, decreased FC and decreased feed intake in swine (Rachan Buaban, 1988). Feather meal when fed up to 10% of diet didn't was not found to affect DDM or DCP of the diet in swine (Rachan Buaban et al., 1989). In growing-finishing swine rations feather meal could provide up to 25 % of the dietary protein with significantly affecting performance (Khajarern et al., 1982b). No difference in performance was observed when up to 4 % feather meal was fed to swine 0-4 weeks of age and up to 8 % could be fed to the 4 to 8 week old age group (Khajarern et al., 1982b). Levels up to 10 % of feather meal in the diets of swine didn't affect total diet DM or CP digestibility, but as feather meal increased the Biological Value of the dietary CP decreased (Rachan Buaban et al., 1989).

Poultry

Broilers and laying hens

Pullets fed feather meal was found to grow satisfactorily, the addition of methionine was found to improve performance (Khajarern et al., 1982a).

The effect of supplementing feather meal with 0.2 to 0.5 % methionine was found to increase carcass quality in broilers and egg weight and shell thickness in layers (Miranda et al., 1981)

Ducks

Feather meal can provide up to 50 % of the supplemental protein for young growing duckling, 100 % for older growing ducks and 50 % in ducks that are laying (Sucheep Suksupath, 1980).

Rabbits

Digestion parameters were similar in does fed FeM and BM to those of does fed meat meal and milk yield was not compromised. Milk yield and body weight were also maintained in a trial conducted by Lu et al. (1990).

Fish

The use of processed animal protein have been largely studied in fish nutrition, in particular in Tilapia and in salmonids (Bureau et al 1999; Tacon et al., 1999), before their prohibition at the beginning of the XXI Century in according to EU Regulation 169/2001.

Tacon et al (1983) observed that a meat and bone meal hexane-extracted or a meat and bone meal: blood meal included in a diet for tilapia (*Oreochromis mossambicus*) at 4:1 ratio with the addiction of methionine, histidine and lysine could replace fish meal protein up to 50% (Tacon et al , 1983). Hydrolysed feather meal supplemented with methionine, histidine and lysine could replace only 30% of FM protein (Tacon et al., 1983).

Davies et al (1989) demonstrated that the optimum meat and bone meal: blood meal ratio was 3:1 or 2:3 ratio, and this ratios could replace the fish meal completely in the *O. mossambicus* diets. When the blood meal where used as a total replacement of fish meal the growth rate was comparable to the control diet.

Other two authors (Mansour, 1998; al Sayed 1998) described that the Nile tilapia fed on meat and bone meal and poultry by product meal as a single dietary protein source produced a significant retard on growth rate and on feed efficiency.

Tacon et al (1983) on Nile tilapia, Viola and Zaher (1984) on Monzambique Tilapia and Davies et al. (1989) on tilapia hybrids found that diets containing hydrolysed feather meal showed poor growth performances, presumably due to poor digestibility and unbalances of essential amino acids.

Bureau et al (1999) tested the digestibility of 20 types of processed animal proteins in rainbow trout, they observed that the processing cycle (as the

dried equipment) could affect the quality and as a consequence the digestibility and the fish growth performance and reported a significant difference of digestibility in the feather meals, blood meals and meat and bone meals used for their trial.

Bureau et al. (2000) evaluated the feather meal used alone or in combination with corn gluten meal and blood meal in replacement of herring meal in diets. The authors found that the inclusion of up to 15% feather meal in the diet was possible without affecting growth, feed efficiency, nitrogen or energy gains of the fish. The authors concluded that feather meal has a possible alternative source in rainbow trout diets.

Trzebiatowski et al (1982) carried out an experiment on carps, feather meal was found to be between poultry by-product meal and blood meal in its feeding value.

Table 4.3: Chemical composition and nutritional value of feather meals (Feedipedia)

Main analysis	**Unit**	**Avg**	**SD**	**Min**	**Max**	**Nb**
Dry matter	% as fed	92.1	1.9	88.3	95.7	107
Crude protein	% DM	85.7	5.0	73.8	96.5	118
Crude fibre	% DM	0.9	0.6	0.3	2.9	18
NDF	% DM	55.8	1.9	53.8	57.5	3
ADF	% DM	6.5	2.9	2.0	11.7	10
Lignin	% DM	5.5	2.2	4.1	8.0	3
Ether extract	% DM	6.7	2.5	2.5	13.6	46
Ether extract, HCl hydrolysis	% DM	9.5	1.8	4.8	12.9	57
Ash	% DM	5.5	3.8	1.3	16.0	115
Total sugars	% DM	0.3	0.2	0.2	0.6	4
Gross energy	MJ/kg DM	23.5	0.4	22.7	24.0	18
Minerals	**Unit**	**Avg**	**SD**	**Min**	**Max**	**Nb**
Calcium	g/kg DM	12.7	4.1	3.6	16.8	22
Phosphorus	g/kg DM	8.2	1.9	2.6	8.8	22
Potassium	g/kg DM	1.3	0.2	1.0	1.5	10

Sodium	g/kg DM	1.3	0.2	1.0	1.4	10
Magnesium	g/kg DM	0.9	1.3	0.4	4.5	10
Manganese	mg/kg DM	16	6	7	21	7
Zinc	mg/kg DM	142	20	106	157	7
Copper	mg/kg DM	10	1	7	11	6
Iron	mg/kg DM	625	213	246	833	6
Amino acids	**Unit**	**Avg**	**SD**	**Min**	**Max**	**Nb**
Alanine	% protein	4.6	0.3	4.1	5.3	19
Arginine	% protein	6.7	0.4	5.6	7.4	24
Aspartic acid	% protein	6.7	0.2	6.5	7.0	19
Cystine	% protein	4.3	0.3	4.0	5.0	23
Glutamic acid	% protein	10.6	0.9	8.6	11.6	19
Glycine	% protein	7.3	0.5	6.1	8.3	21
Histidine	% protein	0.8	0.2	0.5	1.4	24
Isoleucine	% protein	4.9	0.4	3.5	5.3	25
Leucine	% protein	8.0	0.5	7.3	9.2	26
Lysine	% protein	2.1	0.2	1.7	2.6	27
Methionine	% protein	0.7	0.1	0.6	1.0	26
Phenylalanine	% protein	4.7	0.4	3.9	5.4	25
Proline	% protein	9.4	0.3	8.8	10.0	17
Serine	% protein	11.4	0.9	8.5	12.0	19

Threonine	% protein	4.6	0.4	3.7	5.3	26
Tryptophan	% protein	0.6	0.1	0.5	0.8	7
Tyrosine	% protein	2.5	0.3	2.1	3.3	18
Valine	% protein	7.2	1.1	5.1	8.1	25
Ruminant nutritive values	**Unit**	**Avg**	**SD**	**Min**	**Max**	**Nb**
OM digestibility, Ruminant	%	76.8	4.1	72.0	82.7	6
Energy digestibility, ruminants	%	82.6	1.0	71.0	82.6	4
DE ruminants	MJ/kg DM	19.4	0.5	15.9	19.4	4
ME ruminants	MJ/kg DM	13.3	0.5	13.3	14.5	4
Nitrogen digestibility, ruminants	%	74.1	5.9	69.0	85.2	6
a (N)	%	15.8				1
b (N)	%	48.3				1
c (N)	h-1	0.055				1
Nitrogen degradability (effective, k=4%)	%	44				
Nitrogen degradability (effective, k=6%)	%	39		28	39	2
Pig nutritive values	**Unit**	**Avg**	**SD**	**Min**	**Max**	**Nb**
Energy digestibility, growing pig	%	88.7				

DE growing pig	MJ/kg DM	20.8				
MEn growing pig	MJ/kg DM	18.9				
NE growing pig	MJ/kg DM	11.6				
Nitrogen digestibility, growing pig	%	72.1		71.1	73.0	2
Poultry nutritive values	**Unit**	**Avg**	**SD**	**Min**	**Max**	**Nb**
AMEn cockerel	MJ/kg DM	12.5	0.5	12.5	14.4	5
AMEn broiler	MJ/kg DM	11.7				
Fish nutritive values	**Unit**	**Avg**	**SD**	**Min**	**Max**	**Nb**
Energy digestibility, salmonids	%	63.7		57.4	70.1	2
Nitrogen digestibility, salmonids	%	64.4		58.0	70.8	2

Avg: average or predicted value; SD: standard deviation; Min: minimum value; Max: maximum value; Nb: number of values (samples) used.
The asterisk * indicates that the average value was obtained by an equation.

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V
In vitro Crude protein digestibility of Tenebrio molitor and Hermetia illucens insect meal and its correlation with chemical composition traits

Before starting the experimental animal trials, an in vitro experiment was performed in order to evaluate the correlation between in vitro crude protein digestibility coefficents of the insect meal from larvae of *Tenebrio molitor* and *Hermetia illucens* and their chemical composition characteristics as well as to develop regression equations able to estimate the in vitro crude protein digestibility from proximate analysis of the insect meals.

The nutritional aspects of the insect-based feeds should be carefully evaluated in view of the few availability of data on this topic in the literature and their high variability. There are many conditions that can affect the nutritional composition of the products obtained from insect as the species, the substrate used for rearing. This latter aspect strongly affects the amount of lipids and the mineral in the insect. As an example, black soldier fly (*Hermetia illucens*) larvae can contain from 15 to 50 % of lipids if reared on poultry manure or oil rich substrate (Makkar et al., 2014).

The developmental stage, given the existence of mute and metamorphosis, is also very important in these terms, because it depends on the value of chitin.

Chitin, discovered by the French chemist and the pharmacist Henri Braconnot in 1811, is one of the major components of the insect exoskeleton, of the cell wall of fungus, of the hydrophilic peristalsis and is also present in the epidermal cuticle or in other structures superficial of many other invertebrates. After cellulose, chitin is the most abundant biopolymer present in nature.

From a chemical point of view chitin is a polysaccharide, consisting of several units of N-acetylglucosamine (N- acetyl -D-glucos-2-amine) bonded to each other with a β-1,4 bond, the same as glucose units that form cellulose. Chitin can therefore be considered as a cellulose in which a group of acetylamine has been substituted for the hydroxyl group on each of the two monomers.

Chitin is generally not digested by monogastric animals, which is why it affects the digestibility of insects; To solve the problem of its indigestion, chitinase can be used, a hydrolytic enzyme capable of breaking the glycidic linkages present in chitin, added to the diet.

Chitin can have a positive effect on the functioning of the immune system. By administering insects to chicken, the use of antibiotics can be reduced (FAO, 2013). This is very important considering that the non- appropriate use of antibiotics in animal production is considered one of the condition responsible of the development of antibiotic-resistant bacterial strains which can lead to human infections due to antibiotic resistance to bacterial strains. As an example, in the Netherlands, studies have been conducted on patients with severe blood and urinary tract infections. In one out of five of these patients, the infection was sustained by BSBL (broad spectrum beta-lactamase) bacteria, genetically identical to bacteria found in chickens. Bacterial strains containing BSBL produce enzymes that result in resistance to antibiotics such as penicillin and cephalosporins. Two bacteria most commonly produce BSBL enzymes: Escherichia coli and Klebsiella pneumoniae. Approximately 35 percent of isolated human cases contained BSBL genes that could be associated with chickens (FAO, 2013).

The use of antibiotics is higher in the Dutch poultry industry than in any other European country; Consequently, the prevalence of BSBL is correspondingly high. The study also revealed that almost all (94 percent) chickens in Dutch supermarkets and poultry farms are infected with BSBL bacteria, probably due to the common use of antibiotics in their diet. Other research is needed to ascertain whether to feed chickens with insects (containing chitin) may, by strengthening the immune system, render unnecessary the use of antibiotics (Van Hall et al, 2011).
The nutritional values and properties of insect larvae meal was investigated only in the recent years. Studies previusly published, have focused on human nutrition (Banjio et al, 2006), the results demonstrate a good nutritional property.
The use of insect in animal nutrition has been less studied, but many species of insect can be rapresent in the next future a standard ingredient in poultry (Ravindran and Brair, 1993; Wang et al., 2005; Ojewola et al., 2005; Oyegoke et al.,2006), in fish (Gasco et al., 2014 a and b) and in other species (St. Hilaire et al., 2007; Ng et al., 2001).
The main objective of this study was to evaluate the correlation between in vitro crude protein digestibility coefficents of insect meal from *Tenebrio molitor* and *Hermetia illucens* and their chemical composition characteristics as well as to develop regression equations able to estimate the in vitro crude protein digestibility from proximate analysis of insect meals.

Material and Methods

Twelve samples of insect meals were analyzed at the laboratory of animal feed of the department of Veterinary Medicine and Animal Production of University of Napoli Federico II.

The samples, 6 insect larvae meal of Tenebrio molitor and 6 of Hermetia illucens larvae meal, were obtained from different producers:

- Gaobeidian Shannon Biology C.O., Ltd., Shannong, China (one samples of *Tenebrio molitor)*
- Kreca, The Netherlands (three samples of *Tenebrio molitor* from different batches)
- Enviroflight L.C.C,OH, USA(two samples of *T. molitor*, and four of *Hermetia illucens* from different batches)
- Laboratory of Entomology, Wageningen University, The Netherlands (Two samples of *Hermetia illucens* from different bathes).

The samples were analysed according AOAC (2004) using the following methods:

- Dry matter (DM, procedure number 943.01)
- Ash (procedure number 924.05)
- Crude protein (CP, procedure number 954.01)
- Ether extract (EE, procedure number 920.39)
- Neutral detergent fibre (NDF, procedure number 973.18)
- Acid detergent fibre (ADF, procedure number 2002.04)
- The amount of protein linked to acid detergent fibre (ADIP).

The chitin has been estimated by the acid detergent fibre amount corrected for the ash content (Bernard and Allen, 1997). The aminoacid represents from the 14.2 to 68.8% of the ADF residue by weight, suggesting that the ADF represents the both protein and che chitin protein linked (Finke et al., 2007). For this reason, the estimation of the chitin in insect meals was done as follows:

chitin (%)= ash free ADF (%)- ADIP (%)

In order to simulate the digestion of the insect meals throught the stomach and the small intestine of monogastric animals an in vitro trial was performed.

The in vitro assay was a two steps method developed to maximize the hydrolysis of the animal protein peptide bonds with minimal enzyme usage (Qiao et al., 2004).

The samples were ground to pass a 1 mm screen (Brabender Wiley mill, Brabender OHG Duisburg, Germany) and accurately weighed (0.5 g/ samples, five replications for each samples) in 150 ml glass jars, without preliminary defatting.
All the enzymes were from porcine origin and obtained from Sigma- Aldrich (S. Louis, MO, USA).

First step

- Fresh pepsine (Enzyme Commission number 3.4.23.1, 250 U/mg solid) solution was preparated (10 mg/ml) in pH citrate buffer solution (0.1 µM) to avoid pepsine precipitation that occurs in pH 2 citrate buffer solution.
- An aliquot of fresh pepsin solution was immediantly transferred in to each 150 jar to make desired concentrations of pepsin solution (0.25% of pepsin related to protein content of the sample)
- The final volume in each test jar (20 ml), was obtained a pH 2 citrate.
- Jar were incubated at 38°C for 24 h under continus stirring.

Second step

- fresh **trypsin** (Enzyme Commission number 3.421.4,1000 BAAE units/mg solid) -**enriched prancreatin** (Enzyme Commission number 232.468.9, 8x USP specifications) was prepared (trypsin 2 mg/ml, pancreatin 10 mg/ml , ratio: 1:5)in pH 8 phosphate buffer solution (0.1 mol/L)
- An amount of 30 ml of phosphate buffer solution was added to each jar and, after adjasting the pH at 7 by adding 0.1 M NaOH, the trypsin +pancreatin was inoculated (7.5 enzyme protein relative to substrate protein, final substrate concentration 5 mg/mL)
- The digestion was continued for 96 h more under continus starring. All buffers contained 0.06%(wt/v) sodium azide to prevent microbial growth.

The lenght of this incubation times correspond the time needed to loose over 95% of the enzymes activity, in order to maximize their efficacy, thus allowing for animal enzyme usage.
To correct the results for a possible amount of nitrogen in the reagent used in the trial, three tubes were incubate without substrates and followed the same digestion process than the other samples. At the end of digestion

samples were filtered (Whitmann 401) and the residual material were submetted to CP Analyses in according to AOAC (2004).
The calculation of in vitro digestibility coefficient was obtained from the following formula:

CPd= [CPs-(CPr-CPb)]/CPs*100

CPd= crude protein digestibility
CPs=crude protein content of samples (before the digestion)
CPr= crude protein content of residual material after digestion
CPb= crude protein amount of the tubes without substrates (blanks)

Statystical analysis

The differences between the average values of chemical composition and crude protein digestibility of mealworm (Tenebrio molitor, Linneus 1758) and black soldier fly (Hermetia illucens, Linneus, 1758) were analysed by t-test.
The coefficients of correlation between the crude protein digestibility and parameters of chemical composition were estimated using a PROC CORR procedure (SAS, 2000).
Prediction equations of crude protein digestibility from chemical analysis of insect meal samples were developed by a multiple stepwise regression analysis, using the REG procedure of SAS (2000). Only linear models were tested and it was assumed that there was no interaction among variables.

Results

Table 5.1: Chemical composition of the insect meals used in this trial

	DM	ASH	CP	EE	NDF	ADF	ADIP	CHITIN	CPd
TM 1	96.0	3.60	52.2	28.4	11.7	7.95	2.80	5.51	66.3
TM 2	95.8	3.67	51.8	29.8	11.6	7.52	2.72	4.80	66.7
TM 3	99.0	6.36	59.0	16.6	48.7	10.9	4.19	6.73	65.5
TM 4	99.2	6.49	58.8	17.1	52.5	11.4	5.10	6.34	66.2
TM 5	98.2	3.51	57.6	28.9	18.9	10.3	3.96	6.37	65.8

TM 6	99.0	3.74	57.4	28.9	19.4	10.5	3.30	5.15	66.2
HI1	95.1	9.88	52.0	11.3	25.8	8.41	3.06	4.75	67.1
HI2	94.8	9.96	51.8	11.3	34.4	8.80	3.65	4.50	67.3
HI3	98.8	6..43	58.8	12.9	5.99	4.89	1.96	2.93	67.6
HI4	98.9	6.52	58.4	11.6	6.03	4.69	1.83	2.86	68.7
HI5	95.9	4.72	49.9	29.0	18.3	8.30	3.32	4.98	66.8
HI6	95.9	4.64	50.5	28.4	19.2	8.53	3.03	5.50	66.0
Average values									
TM	**97.9**	**4.56[a]**	**56.1**	**24.9**	**27.1**	**9.77[a]**	**3.68[a]**	**5.75[a]**	**66.1[a]**
HI	**96.6**	**7.02[b]**	**53.6**	**17.4**	**18.3**	**7.27[b]**	**2.98[b]**	**4.25[b]**	**67.3[b]**
P value	0.106	0.028	0.254	0.119	0.339	0.035	0.036	0.012	0.022

The table 1 shows the nutritional-chemical characteristics of the 12 samples of insects examined (DM, Ash, CP, EE, NDF, ADF, and ADIP chitin), the *in vitro* coefficient of digestibility of proteins (CPd) and the average content for *Tenebrio molitor* and *Hermetia illucens* samples for all the criteria listed above.

It is possible to put in evidence from the results obtained:

- The variability of the dry matter (DM) is quite low (the values are between 94.8 and 99.2%);
- The ash content is higher in *Hermetia illucens* samples than *Tenebrio molitor* ones.
- The pencentage of crude protein (CP) shows major variability: the highest amount reported is 59%(TM3) and the lowest is 49.9%(HI5);
- The content in the ethereal extract of the samples tested was quite different, it is possible observe the lowest values in the samples HI1 and HI2 (11.3%) and the highest in the TM2 (29.8%)
- The NDF values are included between 5.99%(HI3) and 52.55%(TM4);
- The TM4 sample report the highest amount of ADF (5.10%), the lowest values of ADF is showed by the sample HI4;
- The crude protein digestibility is higher in Hermetia illucens samples than Tenebrio molitor ones;
- Tenebrio molitor samples reported higher values in ADIP and chitin compared to the samples of Hermetia illucens.

Table 5.2: Correlation coefficient between crude protein digestibility (CPd) and the chemical characteristics of the insects samples from *Tenebrio molitor*.

CPd		0.429	0.190	-0.459	-0.137	0.017	-0.747	0.036	-0.891
	0.396	0.719	(0.360)	(0.796)	(0.975)	(0.035)	(0.951)	(0.017)	
DM			0.617	0.582	-0.622	0.544	0.586	0.684	0.657
		0.192	0.195	(0.187)	(0.090)	(0.103)	(0.137)	(0.157)	
Ash				0.649	-0.993	0.981	0.687	0.599	-0.009
		0.162	(<0.001)	(0.005)	(0.137)	(0.214)	(0.099)		
CP					-0.667	0.777	0.989	0.859	0.633
			(0.148)	(0.069)	(<0.001)	(0.028)	(0.178)		
EE						-0.979	-0.687	-.0902	0.004
			(0.001)	(0.132)	(0.014)	(0.999)			
NDF							0.705	0.935	0.174
				(0.053)	(0.006)	(0.741)			
ADF								0.853	0.841

				(0.031)	(0.033)				
ADIP									-0.442
					(0.381)				

A,B,C Pvalue <0.01; a,b,c Pvalue <0.05.

In the table 5.2 are reported the correlation coefficient between crude protein digestibility (CPd) and the chemical characteristics of the insect samples from *Tenebrio molitor*, its is possible to affirm:

- Crude protein digestibility is negatively correlated correlated (P<0.05) to ADF and the chitin contents;
- The ash amount is positively correlated to NDF (P<0.01) and ADIP (P< 0.05) and negatively to ether extract (P<0.01);
- The crude protein showes a positive correlation to ADF(P<0.01) and ADIP (P<0.05);
- The ether extract is negative correlated to NDF (P<0.01) and ADIP (P<0.05);
- The NDF is positively correlated to ADIP(P<0.01);
- The ADF showes a positive correlation (P<0.05) to ADIP and chitin.

Table 5.3: Correlation coefficient between crude protein digestibility (CPd) and the chemical characteristics of the insect's samples from *Hermetia illucens.*

CPd		0.799	0.157	0.941	-0.699	-0.625	0.901	-0.791	-0.992
	(0.057)	(0.776)	(0.005)	(0.103)	(0.184)	(0.001)	(0.061)	(0.001)	
DM			-0.418	0.602	-0.221	-0.752	-0.677	-0.661	0.657
		(0.410)	(0.114)	(0.674)	(0.093)	(0.121)	(0.138)	(0.131)	

Ash				0.609	-0.793	0.604	0.220	0.280	-0.078
		(0.987)	(0.059)	(0.204)	(0.675)	(0.551)	(0.973)		
CP					-0.614	-0.756	-0.966	-0.922	-0.973
			(0.195)	(0.082)	(0.002)	(0.009)	(0.001)		
EE						-0.016	0.416	0.343	0.655
			(0.976)	(0.414)	0.501	(0.158)			
NDF							0.888	0.923	0.688
				(0.018)	(0.009)	(0.131)			
ADF								0.966	0.941
				(0.002)	(0.005)				
ADIP									0.846
					(0.034)				

A,B,C Pvalue <0.01; a,b,c Pvalue <0.05

The table 5.3 reported the correlation coefficient between crude protein digestibility and the chemical composition of *Hermetia illucens* larvae meal samples, it is possible to observe:

- The crude protein digestibility is negatively correlated (P<0.01) to ADF and chitin and positively to crude protein amount (P<0.01);
- The NDF has positive correlation to ADF(P<0.05);

- The ADF is positive correlated (P<0.01) to ADIP and chitin;
- The ADIP is correlated positively to chitin (P<0.05).

Table 5.4: Regression equations for estimation of in vitro crude protein digestibility of Tenebrio molitor larvae meal

	Variables	**R^2**	**RSD**	
Intercept	**Chitin**	**ADIP**		
68.195	-0.3325		0.7945	12.06
(0.49003)	(0.0845)			
72.425	-0.2672	0.0845	0.9213	6.29
(0.4959)	(0.0674)	(0.0385)		

In the table 5.4 are showed the regression equation to estimate the crude protein digestibility (CPd) of *Tenebrio molitor* larvae meal samples from chemical composition characteristics.

Table 5.5: Regression equation for estimation of in vitro crude protein digestibility of Hermetia illucens larvae meal

	Variables	**R^2**	**RSD**	
Intercept	**Chitin**	**CP**		
7.57	-0.907		0.9830	2.50
(0.2804)	(0.064)			
80.09	-14.12	-0.1243	0.9940	1.17
(3.62)	(0.191)	(0.0529)		

Table 5.5 reported the regression equations to predict the crude protein digestibility (CPd) of *Hermetia illucens* samples from chemical characteristics.
The chitin was the first parameter chosen, explaining the 79.45% of CP variability for *Tenebrio molitor* samples and the 98.30% for *Hermetia illucens*.
The ADF was added to model for Tenebrio molitor and it is reported a decrease of RSD (47.5%).

In the second step of the stepwise analysis, for *Hermetia illucens* larvae meal samples, was evaluated the crude protein and the RDS decrease was 1.17.

Discussion

The tables 6 and 7 show the results of chemical composition of our *Hermetia illucens* (HI) and *Tenebrio molitor* (TI) samples compared to the results of Sanchez Muros et al. (2014) and Makkar et al. (2014).

Table 5.6: Our results compared with the results previously published by Sanchez-Muros et al. (2014) and Makkar et al. (2014).

%	Sanchez-Muros et al (2014)	Makkar et al (2014)*	Our results
CP	38.9	42.1	53.6
EE	28.3	26.0	17.4
ASH	15.2	20.6	7.02
CF	---	7.0	---
ADF	---	---	18.3
NDF	---	---	7.27

*The results are reported on dry matter basis.

Data on HI obtained from our samples showed, compared to the data in literature, higher values of crude protein and a low content of lipid and ash. It is true that, as previously described, fat an ash are two parametrs with a high variability, tied to the subrate used for insect rearing, but protein content is, in general, less variable. So, it is probable that, among our samples there was some one "partially defatted". This reduces the amount of lipid and increases the concentration of the protein. Regarding the amount of ash, this result is certainly tied to the composition of the rearing substrate. The results of chemical analysis on Tenebrio molitor samples are in line with the other findings available in the literature.

The insect meal received at our laboratories were dehidrated, so the amount of dry matter resulted obviously higher than the larvae that not received any transformation process. The total amount of dry matter in Hermetia illucens fresh larvae is from 35 to 45% (Newton et al 2008) and Tenebrio molitor 40% (Makkar et al 2014).

Our results has shown that the amount of crude protein and crude lipids are extremely varius:

- the minimum amount of crude protein in the sample number 5 of Hermetia llucens larvae meal (49.9%) and the maximun in the tird sample of Tenebrio molitor (59.0%);
- the highest value of ether ectract reported in our results was 29.8% (Tenebrio molitor 2) and the lowest amount was 11.3%(Hermetia illucens 1 and 2).
- The protein and fat content in the insect are extrimely variable because it depens:
- to the developmental stage (for example in case of *Hermetia illucens* the larvae at final stage accumulate the feed in the gastroenteric tract that the it will consume during the adult life, so the amount of ether extract in this stage could be higher than the initial larval stage, in according to Hardouin and Mahoux, 2003);
- the most important factor is the substrate, in our case we can suppose that Hermetia illucens received at our laboratories was rearing on poultry manure, because it is described in literature (Arango Gutierrez et al 2004) that the range of ether extract of black soldier larvae fly rearing on this substrate was about 15-28%, and in addition because it is the most common substrate, our result (from 11.3 to 29 %) are in accoding to Arango and Gutierrez et al (2004).

Unfortunately, we did not have any information by the producers regarding the rearing substrate and on the exact larval stage.
Our results showed that *Tenebrio molitor* larvae meal had a higher chitin amount than *Hermetia illucens* larvae meal (5.75% vs 4.25% respectively). The are not many studies that reported the chitin amount of insect, so it was possible to compare only the chitin amount of Hermetia illucens larvae meal to Finke et al. (2012) results. The amount of chitin estimated in TM samples (5.75 %) is in line with the results reported by Finke (2007) (4.98 %), while the chitin estimated in HI samples resulted slightly underestimated in comparison to the results of Finke (2007) (4.25 vs. 5.41 %, respectively).

Our results showed that in the chemical composition there are a variable amount of crude fibre, NDF and ADF, and this is in agreement to other authors (Finke et al 1984, Finke et al 2002, Pennino et al 1991, Barker et al

1998). It is known that in the plants the NDF is composed of cellulose, hemicelluloses and lignin, and the ADF consisting of cellulose and lignin (van Soest and Robertson, 1977). In the insect are unknown the components that make up the NDF faction (Finke, 2007).
Finke (1984, 2002) and Barker et al. (1998) suggested that the fibre in insect is the chitin, because the chitin and the cellulose structurally are similar.

The in vitro crude protein digestibility coefficient of tenebrio molitor and Hermetia illucens larvae meal are respectively 66.1 and 67.3, they are in according to the values available in literature; the range of crude protein digestibility in insect species is from 45 to 66.9%, however the most of species is 60 % (Sanchez-Muros,2014). The crude protein digestibility of various vegetable protein sources is similar to the insects (57% in canned pinto beans, 66% in canned chickpeas, 52% in peanuts meal), and the whole wheat crude protein digestibility is 40%, lower than the insects range (Longavah et al ,2011); chitin negatively influences the crude protein digestibility of insect meals, for this reason it is very important the correct estimation of chitin content in animal nutrition. It is known that the chitin is not degradated by the pancreatic enzymes and absorbed by the small intestine (Vidanarachchi et al, 2010), thus has described by Shiavone et al (2014) and Bovera et al (2015) that it has a negative effects on protein digestibility in chicken broilers. Our results indicate that the chitin is the main fator affecting the in vitro protein digestibility for both insects meal.
Our results show that the crude protein digestibility is not correlated to amount of protein linked to acid detergent fibre, this suggests that propaply there are other proteins that affect the crude protein digestibility, in order to confirm this suggestion are necessary other studies.
The NDF, in our trial, is not correlated to the crude protein digestibility, probably because this estimation is not adeguate to evaluate the chitin content of insect meal. This is a confirm in according to Finke (2002 and 2007) that the better analysis to quantify the chitin amount is the ADF.
There is a positive correlation beetween the crude protein level and the crude protein digestibility for black soldier larvae meal, this indicate that as the percentage of crude protein increases, also the crude protein digestibility increases, and this could be explain the nagative correlation between crude protein and ADF or protein linked to acide detergent fibre (ADIP).

V In vitro crude protein digetibility

It is possible to observe in our black soldier larvae meal results that as crude protein level increase, the ADF and the protein linked to ADF (ADIP) decreases, and his could be affect the crude protein digestibility.
Indeed our Tenebrio molitor results showed a positive correlation between crude protein and ADF and the protein linked to ADF; so the crude protein increase in the insect body is correlated to a increase of the cuticolar protein linked to exoskeleton, but this increase is unable to produce a significant correlation between the crude protein and the digestibility of crude protein.
This differences between Tenebrio molitor and Hermetia illucens could be attribuated to insect species.
The chitin was the first indipendent variable included n the model estimated by the STEPWISE procedure, to predict the in vitro crude protein digestibility from the nutritional value of *Tenebrio molitor* and *Hermetia illucens* larvae meal, this is a confirm of our suggestions even if its efficacy on crude protein digestibility estimation was stronger in *Hermetia illucens* larvae meal.
Anyway, the most important factor that affect the crude protein digestibility is the chitin.
The second variable included in the STEPWISE model was the protein linked to ADF for Tenebrio molitor, and the amount of crude protein for *Hermetia illucens* samples, because there was a different relationship between crude protein, ADF and protein linked to ADF.
The digestibility of crude protein of *Hermetia illucens* and *Tenebrio molitor* larvae meal are linked to the chitin amount. It is possible to observe that there was a negative significant correlation with ADF, but in the final equation next to the chitin, the protein linked to ADF was chosen for *Tenebrio molitor*, and the crude protein was chosen for Hermetia illucens larvae meal, to obtain an accurate estimation of crude protein digestibility from the chemical composition of insect meal samples. Because there are differences between the insect species, it is necessary further investigation on single insect species in order to find the estimation equation parameters.

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VI

Tenebrio molitor larvae meal as an alternative protein source in broiler

The situation of poultry production in Italy.

The modern poultry farming in Italy was born during the middle of the twenty Century, the intensive rearing of broilers was the first to develop, then was born the breeding of laying hens. Consumers during the 1950s were skeptical of the chicken grown under intensive breeding because initially the broilers were raised in a cage, and this allocation system restricted the movement of the birds, for this reason the meat resulted pale, soft and exudative.

During the 2016 in Italy the production of the broiler chicken increased of 5.1% compared to 2015 (the total production was 981.000 tons), also the demand of turkey meat increased during the 2016 of 331.000 (+ 5.8%) tons then the 2015. (UNAITALIA) Italian people eat 15.33 Kg of chicken meat during the last year and 4.44 Kg of turkey meat (in total the fowl meat eaten during the 2016 was 21.01 Kg) (UNAITALIA).

Data provided by "UNAITALIA" (based on ISTAT data) also show self-supply levels in the poultry sector: in fact, in Italy is produced the 105.5% of the chicken meat consumed, and even 122.9% of the turkey meat is produced, confirming the poultry a completely self-sufficient sector, guaranteeing the origin and quality of the product being brought to the table.

The pourposes of the experimental trial

In the presented study a meal obtained from larvae of Tenebrio molitor was used as complete replacement of soybean meal to formulate a diet for broilers; this diet was compared to a corn-soybean isoprotein and isocaloric diet. Along the experimental period lasted from 30 to 62 days of age, the following characteristics were studied:

- The growth performance (feed intake, live body weight, body weight gain, feed conversion ratio, protein efficiency ratio, energy efficiency ratio, and European efficiency ratio);
- Hematological profile;

- Carcass traits;
- Physical and chemical characteristics of the meat;
- Ileal apparent digestibility of the diets;
- Volatile fatty acid profile of the caecal content.

Materials and method

All the animals were treated humanely according to principles stated by the EC Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes.

Animals, ingredients and diets

The experiment was carried out in a private farm of Southen Italy (Caserta). A total of 80, 30 days old male Shaver brown broilers (average body weight 1.76 ± 0.19 kg) were homogenously divided into two groups (40 animals per group, each consisting of 8 replicates of 5 broilers). The groups were submitted to different dietary treatments: the group used as control fed a standard corn-soybean meal based diet, formulated to meet broiler requirements according to the Shaver Brown Management guide (2008) and was identified as SBM group. The second group fed a diet formulated to be isoprotein and isocaloric than the control diet but in which soybean meal was completely replaced by an insect meal obtained from larvae of Tenebrio molitor (purchased from Gaobedian Shannon Biology Co., Ltd, Shannong China); this group was named TML.

The broilers were housed in a semi-opened building and each replicate was placed in a floor pen (1.0 × 1.0 m^2/pen) furnished with rice hulls as a litter.

The trial was carried out in October 2014 from 30 to 62 d of age. The groups were fed on two isoproten and isoenergetic diets, whose ingredients and diets chemical-nutritional characteristics are reported in Table 1 and 2, respectively.

The chemical characteristics (dry matter, crude protein, ether extract, crude fibre and ash) of the two protein sources were analysed in the laboratories of the Department of Veterinary Medicine and Animal Production of the University of Napoli Federico II, according to AOAC (2004) and are reported in the Table 3. In the same Table also are reported data on essential aminoacid contents supplied by producers.

For the only insect meal, the percentage of chitin was estimated using the following formula (Marono et al., 2015):

chitin % = ash free ADF (%) – ADF linked protein (%)

where the ADF and the amount of protein linked to ADF were measured according to AOAC (2004).

The chemical characteristics of the diets were measured according to AOAC (2004); while the amount of aminoacids were calculated starting from scheduled values for each conventional ingredient (NRC, 1997). The Metabolizable Energy content for each diet was calculated taking into account the specific formula provided by NRC (1997); on this regard, for the insect meal the digestibility values of the nutrients were obtained from De Marco et al. (2014).

Table 6.1: Chemical- nutritional characteristics and aminoacidic profile of the protein sources used in the trial (soybean meal and *Tenebrio molitor* larvae meal).

	Soybean meal	***Tenebrio molitor* meal**
Dry matter %	90.92	93.90
Ash%	6.13	4.69
Crude protein%	44.51	51.93
Ether extract%	1.84	21.57
Acid detergent fibre%	4.79	7.20
Arginine g/100g protein	6.17	3.61
Hystidine g/100g protein	2.51	2.11
Isoleucine g/100g protein	4.60	2.63
Leucine g/100g protein	4.64	4.52
Lysine g/100g protein	2.83	1.68

Methionine+Cysteine g/100g protein	3.18	1.62
Threonine g/100g protein	3.43	2.71
Valine g/100g protein	4.09	3.72
Tryptophan g/100g protein	1.45	1.75

Table 6.2: The ingredients of the diets administrated to the broilers during the trials.

Ingredients (%)	Soybean meal diets (SBM)	Tenebrio molitor larvae meal (TML)
Maize grain	47.20	63.09
Soybean meal	44.75	---
Insect meal	---	29.65
Vegetable oil	2.20	-
Monocalcium phosphate	1.43	2.54
Celite	2.00	2.00
Calcium phosphate	0.74	0.94
Min Vit	0.64	0.64
NaCl	0.42	0.42
DL- methionine	0.27	0.37
Sodium bicarbonate	0.25	0.25
Fe	0.07	0.07
L-Lysine	0.03	0.03

MinVit mixture provides per kg of the diet: retinol 24 mg, α tocopherol 20 mg, menadione 2.3 mg, cholecalciferol 0.05 mg, riboflavin 5.5 mg, calcium

pantothenate 12 mg, nicotinic acid 50 mg, choline chloride 600 mg, cyanocobalamin 10 µg, pyridoxine 3 mg, thiamine 3 mg, folic acid 1 mg, d biotin 0.50 mg. Trace mineral (mg per kg of diet): Mn 80 Zn 60, Fe 35, Cu 8, Se 0.60.

In order to determinate the in vivo digestibility of the nutrient using the acid insoluble ash (AIA) method proposed by Vogtman et al. (1975) the celite (Sigma Aldrich) was added to each diet at 20g/Kg as an indigestible marker.

Table 6.3: The chemical nutritional value of the diets administrated to the broilers during the trials.

	Soybean meal (SBM)	***Tenebrio molitor* larvae meal (TML)**
Dry matter %	86.62	88.14
Crude protein %	20.30	20.19
Ether extract %	4.75	5.23
Acid detergent fibre %	4.11	3.93
Ash %	6.77	6.67
Lysine (g/100 of CP)	1.24	1.18
Methionine+Cysteine (g/100 of CP)	0.89	0.96
Tryptophane (g/100 of CP)	0.24	0.27
Threonine (g/100 of CP)	0.81	0.90
Arginine (g/100 of CP)	1.49	1.32
EM (MJ/Kg)	11.8	11.9

Growth performance

The broilers are weighed individually at 30 (at beginning of the trial), 37,45,53, and 62 days of the age.

The body weight gain (BWG g/d) was calculated for the periods:

- 30/37 d

- 38/45 d
- 46/53 d
- 54/62 d
- 30/62 d.

The feed consumption was recorded every week from 37 to 62 days of the age by weighing the amount of feed consumed for each group. The feed conversion ratio (FCR) was calculared for the same interval than body weight gain.
For the protein efficiency ratio (PER), energy efficiency ratio (EER) and European efficiency ratio (EEF) were considered the entire period of the experiment (from 30 to 62 days). The PER and the EER were calculated according to Kamran et al. (2008):

PER= weight gain(g)/total protein fed

EER= weight gain (g)*100/total of metabolizable energy intake

The European efficiency ratio was calculated according to Marcu et al. (2013):

EEF = (liveability*live weight Kg) *100/days of age * feed conversion ratio

Hematological traits
The last day of trial (62 days of age), blood samples were collected from the wing vein of two birds replicate (16 samples for group, a total of 32 samples), in collection tubes with and without heparin.
The blood samples without heparin were centrifugated at 1500 rpm for 15 min and the obtained serum was stored at -20°C before analysis.
The biochemical traits of the blood serum were analysed for:

- Total protein (g/l)
- Albumin (g/l)
- Globulin (g/l) (calculated by the difference total protein- albumin)
- Albumin/Globulin
- Cholesterol (mmol/l)
- Triglycerides (mmol/l)
- Aspartate aminotransferase (U/l)
- Alanine aminotrasferase (U/l)
- Gamma glutamyl- trasgerase (U/l)

- Alkaline phosphatase (U/l)
- Creatine kinase (U/l)
- Lactic dehydrogenase (U/l)
- Calcium (mmol/l)
- Blood urea nitrigen (mmol/l)
- Creatinine (mmol/l)
- Uric acid (mmol/l)

The hematobiochemistry parameters were determined using commercially avaible kits by Spinreact (Santa Caloma, Spain) using enzimatic calorimetric or kinetic methods according to the manifacturer's instruction. Spettrophotometric measurments were performed using an automatic biochemical analyser AMS AUTOLAB (Rome, Italy).
The heparinised blood was analysed for:

- Hematocrit
- Heamoglobin
- White blood cells (WBC)
- Red blood cells (RBC);

using an automatic blood analyser (ADVIA 120 Siemens, Munich, Germany).

Carcass traits
At 62 days of age two birds per replicate were randomly chosen (a total of 32 broilers, 16 each group) and, after recordered the life weight, are slaughtered in a specialized slaugther house.
The dressing perentage of the carcasses was estimated by weighing each carcass after bleeding and removal the feather, whole digestive tract, head, neck and legs.
The liver, spleen, kidney, lungs and the heart were removed, weighed and expressed as a percentage of live weight.
The yield of eviscerated carcass on broiler live weight was determinated.
All the tracts of the small (duodenum, jeiunum and ileum) and the large (sum of ceca lenght, colon and cloaca) intestine were misured. The full intestinal lenght was calculated and expressed as a percentage of broiler live weight.

Ileal apparent digestibility
After the length misuration, the tract of the ileum included from 20 mm after Meckel's diverticulum and 40 mm proximal to ileo- cecal junction was separated from the rest of the intestine, to avoid the contamination with the

content of the other intestinal tracts. Thus, the ileum content was collected (one pool from 2 broilers per replicate, 16 samples); after that, the samples are freeze-dried at -20° C until analysis.
The dried ileum digesta were analysed for chemical characteristics (dry matter, crude protein) according to AOAC (2004) and the acid insoluble ash (AIA) in the diets and in the ileal contents was measured according to Vogtman et al. (1975); thus, the apparent ileal digestibilities of nutrients were calculated according to this formula:

AIA=100 – 100 x [(% AIA in the diet/% AIA in the ileal content) x (% nutrient in the ileal content/% nutrient in the diet)].

Physical Criteria
The left breast and the skin of the broilers were also collected and, frozen at -80°C until analysis performed at laboratories of the Department of Agri-food production and Environmental Science of Firenze (Italy).
In order to determinate the value of cooking loss, the breasts were sectioned by a trasversal cut, weighed and vacuum packed in plastic bags. The plastic bags were boiled for around 14 min, until the cover temperature arrived at 79°C. The samples, removed from the plastic bags, cooled at room temperature and dried by a paper, were weighed again.
The cooking losses were determinated by the formula:

100 x [cooked sample weight (g) / raw sample weight (g)]

The pectoralis muscle pH was measured in two different sites, and the resulted average values were used for statistical analysis.
The WHC (water holding capacity) was measured by low speed centrifugation at 1500 rpm X 5 min in raw samples of breast (in triplicate).
The texture analyses were determinated using a Zwick Roell® 109 texturometer (Ulm, Germany) with Text Expert II software, equipped with a 200 N load cell.
The shear force of meat was measured by the Warner-Bratzler shear test, using a staight blade of 7 cm (perpendicular to muscle fibre diretion), at a crosshead speed of 30 mm/min to 50% of total deformation.
Maximum shear force, defined as maximum resistance of the sample to shearing in according to Veland and Torrisen (1999) was measured.

The meat color was measured on the external and internal sides of the left breast (raw and after cooking by boiling), and on the external side of the skin.
The color was measured by a Spectro-color®116 colorimeter (Bell Technology Ltd, Auckland, New Zealand), using the Spectral qc 3.6 software, according to the CIELab system (CIE, 1976).

In the CIE system:

- lightness (L*) is expressed on a 0 – 100 scale from black to white
- redness index (a*) ranges from red (+60) to green (-60) and yellowness index (b*) ranges from yellow (+60) to blue (-60).

After that, Chroma (color saturation) and Hue (taint) angle were measured (CIE, 1976) using the following formulae:

Chroma = (a*2 + b*2)1/2;

Hue = (tan-1 b*/a*)

Chemical-nutritional value of the feed and the meat from pectoralis muscle

The meat from the pectoralis muscle was homogenized and freeze-dried until analysis performed according to AOAC (2004) methods in order to determinate:

- Moisture
- Ash

The protein content was calculated by difference (Bovera et al 2012).
Total lipid extraction was performed according to a modified Folch et al. (1957) method. Total lipid content was quantified gravimetrically (gross weight minus tare). A quantity of 0.5 mL of lipid extract was weighed in a crucible and, after complete evaporation of chloroform in an oven at 105 °C for 5 minutes, lipid content was determined. The extracted lipids were utilized for the analysis of fatty acids profile.
FAME (Fatty Acid Methyl Ester) analysis was performed according to a modified method of Morrison and Smith (1964). A total of 3 mg of the extracted lipids was saponified at 95 °C for 40 minutes with 5 mL of 0.5 M KOH in methanol; after, fatty acids were hydrolysed by adding 2.5 mL of 2 M HCl and were extracted by adding 2.5 mL of petroleum ether.
Methyl esters were prepared by transmethylation using 2 mL of boron fluoride-methanol at a concentration of 14%. Methylated FAs were

dissolved in petroleum ether (40-60), dried, and finally resuspended in 1.5 mL of hexane.

FA composition was determined by liquid gas chromatography (LGC). A GC Varian 430 gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and a Supelco Omegawax™ 320 capillary column (30 m x 0.32 mm i.d., 0.25 µm film and polyethylene glycol bonded phase; Supelco, Bellefonte, PA, USA) was utilized.

The oven temperature was held at 90 °C for 2 minutes, increased to 175 °C over 5 minutes at a rate of 20 °C/min, increased to 220 °C over 13.75 minutes at the rate of 3 °C/min, and kept at 220 °C for 40 minutes.

Injector and detector temperatures were set at 220 °C and 300 °C, respectively. One microlitre of sample in hexane was injected into the column with the carrier gas (helium) at a constant flow of 1.5 mL/min. The split ratio was 1:20. Chromatograms were recorded with computing integrator software (Galaxie Chromatography Data System 1.9.302.952; Varian Inc., Palo Alto, CA, USA). Fas were identified by comparing the retention time of FAME with the standard Supelco 37 component FAME mix (Supelco, Bellefonte, PA, USA). Fas were quantified through calibration curves, using 1 mL of tricosanoic acid (C23:0) (Supelco, Bellefonte, PA, USA) as internal standard.

The fatty acid composition was used to determine quality indexes of lipid profile for the purpose of the evaluation of the fatty acid profile in terms of nutrition and health. The atherogenicity index (AI) and thrombogenicity index (TI) were calculated according to Ulbricht and Southgate (1991), while the hypocholesterolemic to hypercholesterolemic fatty acids ratio (h/ H) according to Santos-Silva et al. (2002), using the following formulas:

$$\mathbf{AI = ([C12:0 + (4 * C14:0) + C16:0]) (MUFA + n\text{-}6\ PUFA + n\text{-}3\ PUFA)}$$

$$\mathbf{TI = ((C14:0 + C16:0 + C18:0)) / ([(0.5 * MUFA) + (0.5 * n\text{-}6\ PUFA) + (3 * n\text{-}3\ PUFA) + (n\text{-}3\ PUFA / n\text{-}6\ PUFA)])}$$

$$\mathbf{h/H = ((C18:1c\ n\text{-}9 + C18:2\ n\text{-}6 + C20:4\ n\text{-}6 + C18:3\ n\text{-}3 + C20:5\ n\text{-}3 + C22:6\ n\text{-}3)) / ((C14:0 + C16:0))}$$

Moreover, the ratios PUFA n-6/PUFA n-3 and PUFA/SFA were calculated.

For the calculating of the quality indexes, the fatty acids concentrations were expressed as g / 100 g of fillet.

Caecal fatty acids

The caeca were tied at both ends, separated by sterile instruments from the rest of the gastrointestinal tract, placed in tightly closed plastic bags and put in pre-warmed thermos. After sampling, the material was transported as soon as possible (about 1 h) to the laboratories of the Dipartment of Veterinary Medicine and Animal Production of the University of Napoli Federico II.

In the laboratory, two quotes of caecal content (each about 5 ml) were used for volatile fatty acids (VFAs). After dilution of the samples with oxalic acid (1:1, v/v), the VFAs were analysed by a gas chromatography method (Thermo-Electron mod. 8000top, FUSED SILICA Gaschromatograph (ThermoElectron Corporation, Rodano, Milan, Italy) with OMEGAWAX 250 fused silica capillary column 30 m X 0.25 mm X 0.25 mm film thickness; analysis temperature, 125 °C; flame ionisation detector, 185 °C; carrier helium, 1.7 ml/min (Stanco et al., 2003).

Statistical analysis

Data were analyzed by one-way ANOVA using the GLM procedure of SAS (2002), according to the model:

$$\mathbf{Yij = m + Pi + eij}$$

Where **Y** is the single observation, **m** the general mean, **P** the effect of protein source (**i** = soybean meal or Tenebrio molitor larvae meal) and **e** the error. Differences among means were separated using Tukey's test (SAS, 2002) at $P < 0.05$.

Results

No morbility or mortality were observed during the trial in any group. During the experimental period were checked the enviroment paramethers inside the building:

- Average maximun temperature was 21 ± 2.2°C
- Average minimum temperature was 14± 1.9°C

- Average humidity was 65.3 ± 8.7%

Growth performance

The table 6.4 reported the values of the live body weight (Kg) recordered weekly during the trial.

Table 6.4 showed the results of body weight of the control group(SBM), insect group(TML)

Days (Kg)	SBM	TML	RMSE	Pvalue
30 d	1.76	1.76	0.043	0.9102
37 d	2.09	2.04	0.049	0.3371
45 d	2.51	2.49	0.032	0.6057
53 d	2.93	3.01	0.089	0.3376
62 d	3.37	3.47	0.110	0.3343

SBM: soybean meal; TML: Tenebrio molitor larvae meal; RMSE: root mean square error.

The table 6.5 showed the body weight gain(g/d) recordered weekly during the experimental trial.

Table 6.5: The body weight gain (g/d) recordered during the trial

Body weight gain (g)	Soybean meal SBM	Tenebrio molitor larvae meal TML	[1]RMSE	Pvalue
30-37 d	55.09	47.01	9.983	0.3778
38-45 d	59.81	64.04	3.473	0.2102
46- 53 d	60.24	73.62	13.87	0.3031
54- 62 d	55.51	57.91	7.585	0.7186
37-62 d	50.49	53.40	3.312	0.3386

SBM: soybean meal; TML: Tenebrio molitor larvae meal; RMSE: root mean square error.

The results reported in the tables 5 put in evidence that there are not difference stastistically significant between the conventional diet SMB) and the use of insect meal(TML).

Feed intake and feed conversion ratio
The table 6.6 showed the feed intake recordered weekly during the trial.

Table 6.6: Feed intake of the broilers fed soybean meal and insect meal based diets.

Feed intake	SBM	TML	RMSE	Pvalue
30-37 d	153.5	155.0	8.02	0.8296
38-45 d	205.8	203.9	10.07	0.8287
46- 53 d	236.0^{a}	213.7^{b}	9.09	0.0412
54- 62 d	235.9^{A}	197.1^{B}	10.84	0.0090
37-62 d	207.8	192.4	9.18	0.1096

Abbreviations; SBM: soybean meal group; TML *Tenebrio molitor* larvae meal group RMSE: root mean square error; ^{a,b} Pvalue < 0.05; ^{A,B} P value < 0.01.
Despite the feed intake was not different during the entire period of the experiment, the control group showed a higher feed intake than the TML group in the periods:

- **46-53 d** (P < 0.05)
- **54-62 d** (P< 0.01)

In the table 7 are reported the values of FCR.

Table 6.7: Feed conversion ratio of birds fed on soybean meal (SMB) and *Tenebrio molitor* larvae meal(TML)

FCR	SBM	TML	RMSE	Pvalue
30-37 d	2.91	3.32	0.352	0.4872

38-45 d	3.44	3.19	0.284	0.3476
46- 53 d	4.29[a]	2.92[b]	0.295	0.0262
54- 62 d	4.28[a]	3.47[b]	0.133	0.0365
37-62 d	4.13[a]	3.62[b]	0.182	0.0312

RMSE: Root mean square error.

The feed conversion ratio (FCR) was different between the group except in the periods:

- 30-37 d
- 38-45 d

During the following period TML group showed a more favourable FCR than the control group.

In the periods:

- 46-53 d (P-value <0.05)
- 54- 62 d (P-value <0.05)

Considering the entire period of the trial, the feed conversion ratio (FCR) was lower for the *Tenebrio molitor* larvae group (P < 0.05).

Table 6.8: Protein efficiency ratio, Energy efficiency ratio and European efficiency factor.

	SBM	**TML**	**RMSE**	**Pvalue**
PER	1.19[b]	1.37[a]	0.043	0.024
EER	8.64	9.02	0.221	0.7014
EEF	132.6B	156.2[A]	30.04	0.0062

From the data reported in the table 6.8 the PER (protein efficiency ratio) was higher in the Tenebrio molitor larvae group than the control (P <0.05), the EER (energentic efficiency ratio) was unaffected by dietary treatments

and EEF (European efficiency factor) was quite lower in the soybean group than in the insect group.

Hematological traits

The hematological traits at 62 days of age were not different between the groups.

Table 6.9: Hematological traits.

	SBM	TML	RMSE	Pvalue
Packed cell volumeI/l	0.35	0.43	0.1642	0.1834
Hematocrit%	32.93	32.61	0.7818	0.7277
Hemoglobin g/dl	9.86	10.2	1.565	0.5604
Red blood cells 10^6/mm	3.34	3.24	0.3626	0.5143
White blood cells 10^3/mm	23.71	22.0	3.304	0.1741

The concentration of protein, albumin and globulin in the serum samples of the group fed Tenebrio molitor was not different than the broilers fed soybean meal; except the albumin/ globulin ratio in the birds of the control group was higher than the insect group (0.44 vs 0.30, in the control and in the insect group respectively, $P < 0.05$). The haematic levels of AST and ALT were lower in the control than in the insect group. It is possible to observe that the uric acid amount was lower in the birds fed Tenebrio molitor larvae meal than the soyben meal group.

Table 6.10: blood serum enzymes

	SBM	TML	RMSE	Pvalue
Total protein (g/l)	30.2	31.0	6.45	0.7536
Albumin(g/l)	8.9	7.1	3.79	0.2325
Globulin (g/l)	21.4	23.9	4.85	0.1745
Albumin/Globulin	0.44^{a}	0.30^{b}	0.082	0.0487
Cholesterol(mmol/l)	2.27	2.44	0.471	0.0932
Triglycerides(mmol/l)	0.35	0.34	0.132	0.9438
AST (U/I)	178.6^{B}	195.5^{A}	15.93	0.0092
ALT(U/I)	46.7^{B}	82.1^{A}	14.67	<0.0001

GGT(U/I)	16.0	19.4	6.23	0.1538
ALP(U/I)	661.6	636.0	82.28	0.4091
Ca(mmol/l)	2.23	2.23	0.128	0.9210
BUN (mmol/l)	0.35	0.27	0.179	0.2820
Creatinine (µmol/l)	26.5	24.7	5.48	0.4309
Uric acid (mmol/l)	0.32[a]	0.25[a]	0.018	0.0227
CK (U/I)	4204	3384	1266	0.0929
LDH (U/I)	961.6	856.7	166.7	0.1025

Carcass traits

The following table showes the weight and the carcass traits of the broilers.

Table 6.11: Carcass traits.

	SBM[1]	**TML[2]**	**RMSE[3]**	***P* value**
Weight, g				
Live weight	3.376	3.469	341.5	0.470
Digestive system	357.3[b]	424.1[a]	67.5	0.015
Head	88.3	84.9	13.2	0.497
Dressed carcass	2.428	2.515	276.5	0.194
Carcass without internal viscera	2.313	2.361	241.6	0.601
Liver	59.7	58.1	6.82	0.521
Kidney	16.8	18.1	3.38	0.326

Hearth	16.9	18.5	2.20	0.071
Lungs	24.6	26.7	4.02	0.271
Spleen	4.40^{b}	5.57^{a}	1.20	0.041
Percentage on live weight. %				
Digestive system	10.4^{b}	12.7^{a}	2.49	0.021
Head	2.61	2.45	0.58	0.473
Carcass yield	71.2	73.2	8.37	0.379
Eviscerated carcass yield	68.5	68.9	8.07	0.901
Liver	1.77	1.67	0.18	0.146
Kidney	0.50	0.52	0.15	0.395
Hearth	0.50	0.55	0.24	0.239
Lungs	0.73	0.77	0.16	0.961
Spleen	0.12^{B}	0.16^{A}	0.05	0.008

In the table 6.11 are reported the carcass traits such as weight and as percentage of the carcass weight. The full gastrointestinal system of the

broiler fed Tenebrio molitor larvae meal has showed a greater weight than the ones fed soybean meal (+66.8 g. Pvalue <0.05). In addition, the digestive system expressed as percentage of live body weight in the broilers fed insects are higher than the control group (+2.3% compared to the control group. Pvalue <0.05). The weight and the percentage of live weight of the spleen was lower in the soybean group (- 1.17 g and -0.04 %, Pvalue <0.05) than the *Tenebrio molitor* group.
The following table (6.12) are reported the weight and the length of the full intestine and the single trailts of small and large intestine.

Table 6.12: length and weight of full intestine and single traits of small and large intestine.

	SBM[1]	**TML[2]**	**RMSE[3]**	***P* value**
Length. Cm				
Duodenum	38.7	43.0	7.63	0.120
Jejunum	87.6	89.2	18.7	0.818
Ileum	89.9^{b}	99.2^{a}	12.5	0.022
Colon	12.5	13.8	10.9	0.756
Rectum	3.16	4.08	1.49	0.096
Ceca	43.1^{B}	48.7^{A}	4.49	0.002
Full intestine	274.9^{b}	298.0^{a}	27.0	0.025
Weight. G				
Ceca	19.4^{B}	24.1^{A}	3.23	0.001
Right breast	238.7	235.7	42.9	0.852
Right thigh	362.3	375.0	55.2	0.543
Percentage on live weight. %				

Full intestinal length	8.14[a]	8.60[b]	1.10	0.048
Cecal weight	0.57[b]	0.70[a]	0.19	0.011
Right breast weight	10.3	9.94	1.43	0.467
Right thigh weight	15.6	15.9	1.91	0.703

The intestine total lenght is lower in the broilers fed soybean meal group than the *Tenebrio molitor* larvae meal group. The ileum length is higher in the Tenebrio molitor larvae meal (+9.7 cm. Pvalue <0.05) compared to the soybean meal group. The caecal weight expressed as percentage of live body weight was greather in the group fed tenebrio molitor larvae meal than the control.
The full intestine and the caecal weight expressed as a percentage of live body weight resulted greater in the birds fed Tenebrio molitor larvae meal than the control diet (the full intestine was +0.46% and the caecal weight resulted +0.13% than the control; Pvalue 0.05).

Physical criteria
The physical criteria of the shin and the left breast are reported in the following table (table 6.13). The muscolar PH and the cooked loss was greater in the group fed in the group fed insect than the soybean meal group.

Table 6.13: physical criteria of the meat

	SBM[1]	**TML[2]**	**RMSE[3]**	***P* value**
Left breast weight. G	221.1	214.5	36.2	0.627
Left breast skin weight. G	21.2	22.9	4.81	0.348
pH	5.95[B]	6.12[A]	0.14	0.004
Water Holding Capacity. %	95.5	95.9	1.41	0.462
Shear Force. N	143.0	140.3	7.54	0.337
Cooking loss. %	21.4[b]	23.6[a]	2.67	0.032

Skin color				
Lightness	60.7	62.8	6.15	0.382
Redness	4.66	3.77	0.36	0.332
Yellowness	13.2	14.2	0.54	0.445
Chroma	14.2	14.9	0.67	0.605
Hue	71.1	76.4	4.32	0.126
External breast color				
Lightness	44.2	44.0	7.25	0.926
Redness	1.18	1.07	0.05	0.249
Yellowness	0.78	0.69	0.02	0.386
Chroma	1.92	1.91	0.25	0.977
Hue	150.3	168.1	22.1	0.612
Internal breast color				
Lightness	41.9	40.5	3.25	0.673
Redness	3.12	2.98	0.41	0.263
Yellowness	6.31	5.86	0.36	0.636
Chroma	6.76	6.99	0.22	0.865
Hue	79.9	70.0	5.13	0.069
Cooked breast				
Shear Force. N	69.3	73.2	4.31	0.597
Lightness	68.9	68.3	5.26	0.918
Redness	3.25	4.63	0.29	0.497
Yellowness	10.9	10.8	0.45	0.897
Chroma	11.6	13.9	0.56	0.051
Hue	71.4	93.9	4.26	0.063

The data of the physical criteria of chicken breast showed that there are no differences statistically significant between the groups.

The chemical composition of the meat collected from the right breast showed is reported in the table 6.14.

Table 6.14: Chemical composition of the right breast.

	SBM	TML	RMSE	*P* value
Moisture. %	72.8	73.1	0.69	0.314
Ash. %	1.55	1.29	0.44	0.126
Protein. %	24.2	24.1	1.11	0.697
Lipid. %	1.38	1.51	0.24	0.143

SBM: soybean meal; TML Tenebrio molitor larvae meal. RMSE: root mean square error.

The absence of results statistically significant put in evidence that the chemical composition of the broilers fed on soybean meal diet are comparable to the ones fed on Tenebrio molitor larvae meal.

Fatty acids content of right breast

Table 15. Fatty acids content (as a percentage of total fatty acids) of the chicken breast.

	SBM	TML	Pvalue	RSD
Total fatty acids	0.6439	0.5968	0.2456	0.042
C12:0	1.37a	1.26b	0.002	0.115
C13:0	0.71a	0.65b	0.002	0.059
C14:0	1.98a	1.86b	0.040	0.180
C14:1n5	0.81a	0.76b	0.011	0.061
C15:0	0.80a	0.73b	0.001	0.064
C16:0	18.06	18.52	0.106	0.878
C16:1n9	0.94a	0.89b	0.001	0.048
C16:1n7	2.95	3.07	0.58	0.661
C17:0anteiso	0.77a	0.72b	0.002	0.049

C17:0	0.72a	0.67b	0.004	0.056
C17:1	0.82a	0.72b	0.041	0.156
C16:4n1	0.70a	0.59b	0.001	0.096
C18:0	6.52	6.54	0.856	0.490
C18:1n9	22.33	22.93	0.437	2.472
C18:1n7	2.88	2.79	0.105	0.190
C18:2n6	22.00	22.15	0.806	1.884
C18:3n6	0.68a	0.64b	0.006	0.050
C18:3n3	1.79	1.76	0.664	0.211
C18:4n3	0.56a	0.51b	0.001	0.045
C20:0	0.66°	0.60b	0.003	0.052
C20:1n11	0.38°	0.35b	0.002	0.028
C20:1n9	0.53°	0.50b	<.0001	0.021
C20:1n7	0.34°	0.31b	0.002	0.029
C20:2n6	0.67	0.62	0.090	0.096
C20:3n6	1.00	0.98	0.705	0.187
C20:4n6	4.48	4.49	0.987	1.125
C20:3n3	0.34°	0.31b	0.009	0.033
C20:4n3	0.33°	0.31b	0.004	0.029
C20:5n3	0.46	0.43	0.192	0.056
C22:0	0.02	0.02	0.283	0.005
C22:4n6	1.36	1.33	0.793	0.389
C22:5n6	0.43	0.42	0.678	0.115
C22:5n3	0.88	0.88	0.994	0.200
C22:6n3	0.004	0.004	0.319	0.001
SFA	31.62	31.57	0.891	1.083

MUFA	31.98	32.31	0.711	2.792
PUFAn6	30.62	30.61	0.983	2.339
PUFAn3	5.08	4.93	0.391	0.556
PUFAn1	0.70a	0.59b	0.001	0.096

Quality indexes				
PUFAn6/PUFAn3	6.0667	6.2597	0.277	0.562
Atherogenicity Index	1.0882	1.0573	0.159	0.069
Thrombogenicity Index	0.3542	0.3545	0.983	0.061
h /H	2.5921	2.5791	0.827	0.191

SBM: soybean meal; TML: Tenebrio molitor larvae meal; RSD: Residual Standard Deviation.

The total amount of lipids (showed in the table 6.15) and measured fatty acids were not different between the groups, as the percentages of saturated, monounsaturated and polyunsaturated n-6. n-3 and n-1 amounts. However, several differences were detected between the groups for the less consistent fatty acids and all were higher ($P < 0.05$) for TML group. All the quality indexes (n6/n3. atherogenicity and trombogenicity indexes. h/H) were unaffected by dietary treatment.

Table 6.16: Fatty acids content (as a percentage of total fatty acids) of the chicken abdominal fat.

	SBM	**TML**	**Pvalue**	**RSD**
Fatty acids (%)				
C12:0	1.31	1.27	0.545	0.136
C13:0	0.69	0.67	0.633	0.073
C14:0	2.01	1.80	0.056	0.185
C14:1n5	0.81	0.80	0.832	0.086
C15:0	0.77	0.74	0.464	0.069

C16:0	16.39	17.01	0.662	2.578
C16:1n9	0.99	0.95	0.304	0.083
C16:1n7	3.91	4.34	0.343	0.810
C17:0 anteiso	0.85	0.82	0.360	0.061
C17:0	0.69	0.67	0.435	0.055
C17:1	0.64	0.62	0.622	0.064
C16:4n1	0.63	0.61	0.510	0.067
C18:0	4.80	4.81	0.990	0.771
C18:1n9	27.85	27.49	0.709	1.799
C18:1n7	2.61	2.70	0.421	0.199
C18:2n6	26.30	25.76	0.680	2.378
C18:3n6	0.71	0.70	0.813	0.064
C18:3n3	2.28	2.53	0.083	0.256
C18:4n3	0.55	0.55	0.865	0.056
C20:0	0.64	0.63	0.591	0.053
C20:1n11	0.39	0.39	0.838	0.039
C20:1n9	0.56	0.54	0.502	0.037
C20:1n7	0.34	0.33	0.733	0.035
C20:2n6	0.50	0.48	0.490	0.041
C20:3n6	0.55	0.55	0.967	0.053
C20:4n6	0.67	0.69	0.799	0.108
C20:3n3	0.31	0.31	0.767	0.031
C20:4n3	0.31	0.30	0.749	0.032
C20:5n3	0.34	0.35	0.799	0.036
C22:0	0.02	0.02	0.952	0.004
C22:4n6	0.21	0.22	0.668	0.040
C22:5n6	0.12	0.12	0.926	0.016
C22:5n3	0.15	0.15	0.641	0.022
C22:6n3	0.05	0.05	0.471	0.008

SFA	28.17	28.42	0.878	2.981
MUFA	38.10	38.15	0.973	2.682
PUFAn6	29.05	28.51	0.693	2.485
PUFAn3	4.06	4.32	0.191	0.354
PUFAn1	0.63	0.61	0.510	0.067

Quality indexes				
PUFAn6/PUFAn3	7.181	6.616	0.111	0.615
Atherogenicity Index	13.125	13.270	0.915	2.460
Thrombogenicity Index	0.504	0.511	0.902	0.093
h/H	3.160	3.116	0.878	0.527

The lipid content and the fatty acid profile (table 6.16) of broiler abdominal fat was ot affected by the dietary treatment.

Volatil fatty acids

The volatile fatty acids were analysed from the broilers ceca and the results were expressed as absolute value (mmol/l) and as percentage of total measeured volatile fatty acids. It is interesting observe that the total amount of volatile fatty acids (VFA) was higher in the ceca of broiler fed tenebr molior larvae meal than the soybean meal group. and it is the same for the single volatile fatty acids measured. The volatile fatty acids (expressed as percentage of total VFA. acetate. Valerianic acid and propionate were higher in the group SBM (P <0.01).

Table 6.17: Caecal volatile fatty acids (mmol/l and percentage).

	SBM	**TML**	**RMSE**	**P-value**
	Mmol/l			
Acetate	6.64^{B}	11.13^{A}	2.83	0.0002

Propionate	2.64^{B}	4.49^{A}	1.27	0.0005
Isobutyrrate	0.26^{B}	0.48^{A}	0.19	0.0036
Butirrate	1.44^{B}	4.07^{A}	0.94	<0.0001
Isovalerianic	0.38^{B}	0.79^{A}	0.38	0.0072
Valerianic	0.27^{b}	0.41^{a}	0.15	0.0189
Total VFA	11.63^{B}	21.38^{A}	5.22	<0.0001
	% of total VFA			
Acetate	56.86^{A}	52.30^{B}	3.64	0.0023
Propionate	22.78^{A}	20.91^{B}	1.60	0.0040
Isobutyrrate	2.31	2.20	0.65	0.6642
Butirrate	12.23^{B}	19.19^{A}	3.02	<0.0001
Isovalerianic	3.49	3.55	1.23	0.9069
Valerianic	0.33^{A}	1.85^{B}	0.38	0.0022

The table 6.18 repoted the the apparent digestibility coefficients of dry and organic matter. and for crude protein.

Table 6.18: Dry matter. organic matter and crude protein ileal digestibility of broilers fed soybean or *T. molitor* larvae meal at 62 d of age (8 experimental units per group. each consisting of 2 birds)

	SBM	**TML**	**RMSE**	***P* value**
Dry matter. %	88.22^{A}	86.42^{B}	0.9478	0.008
Organic matter. %	88.69^{A}	86.89^{B}	1.007	0.011
Crude protein. %	87.35^{A}	80.20^{B}	0.6983	<0.0001

SBM: Soybean Meal group; TML: *Tenebrio molitor* larvae meal group; RMSE: Root Mean Square Error; A. B: $P < 0.01$

The ileal digestibility reported in thetable 18 of dry matter. organic matter and crude protein resulted higher in the birds fed soybean meal than Tenebrio molitor larvae meal (Pvalue <0.01).

Discussion

Growth performance

In our trial the inclusion of Tenebrio molitor larvae meal as a protein source in total replacemenet of soybean meal did not have negative effects on

growth performance of the broiler during the period from 30 to 62 days of age. Indeed, all the groups showed the same body live weight at the end of trial (3.37 and 3.47 kg. for SBM and TML group respectively).
Considering the period from 30 to 62 days of age (the entire period of the trial). the FCR and the PER were more favourable in the animals fed insect than the soybean diet. Probably this result is affected by the feed intake during the periods 46-53 and 54-62 d that was lower in the TML group.
Our results are in agreement to Ballotic and Sun (2013) who described a better FCR in broilers fed on a diet including the 10% of Tenebrio molitor than the control group (0% of *Tenebrio molitor* inclusion).
The feed intake during the total period of the trial (30-62 days) was similar between the groups and this seems indicated that *Tenebrio molitor* larvae meal diet had a good palatability also when the inclusion level was around 30% of the diet as in our trial. Previous studies showed that the 10% inclusion level of Tenebrio molitor in the diet had a satisfactory palatability for the broilers (Ramos- Elorduy et al 2002).
The European efficiency factor (EEF) is a parameter used by Van (2003) and Attia et al. (2012) in order to measure and compare the growing performance of broilers. Because we did not record any death. the most important factor in the EEF calculation was the FCR; the EEF resulted higher in TML group than SBM (156.2 vs 132.6. for TML and SBM group. respectively).
The EEF range values in broilers slaughtered at the age from 35 to 40 days are from the 200 and 225 units (Samarakoon and Samarasinghe, 2012). it is decribed by Samarakoon and Samarasinghe (2012) that the genetic type and sex can affect the EEF. our values resulted lower than the normal range because the EEF units decreased when the slaughter age increased because increased the fat deposition (Samarakoon and Samarasinghe. 2012).
Hematological traits
There are no differences between the groups regarding the blood concentrations of total protein, albumin and protein; however, the albumin/ globulin ratio was lower in the broiles fed *Tenebrio molitor* diet. It is decribed (Griminger and Scanes. 1986) that the high globulin concentration and the low albumin/globulin ratio in the birds indicate a better disease resistance and immune response. This result could be due to the chitin content of the insect meal. The chitin content of the insect meal used for this trial (64.2% of ash-free ADF. 4.62% as feed) was in line with the finding of Finke (2007). who indicated that the ADF fraction in insects contains an

amount of protein from 9.3% to 32.7% and the amount of chitin ranges from 2.7 to 49.8 mg/kg.

Considering that the level inclusion of insect meal in our diet was 30% and the feed intake during the experimental period was 192.4 g/d, it is possible to affirm that the the broilers during the experimental period ingestested 2.63 g/d of chitin. Recent studies published by Neyrinck et al. (2011a, 2011b) and Brownawell et al. (2012) indicated that the chitin is responsible of the rebalance of microbial community and improving the colonic function even if the diet shows a high fat amount.
Van Huis (2013) observed that using insects as an ingredient in the poultry diet may decrease the use of antibiotic drugs in poultry industry and this can be very important because of the adverse effects on human health as the drug-resistant bacteria strain. In addition, the chitin is not degradated and adsorbed by the small intestine and can be fermented by the bacteria in the large intestine. playing a prebiotic role.
Vidanarachchi et al. (2010) suggest that the chitin presents a batteriostatic property on *Escherichia coli, Vibrio cholera, Shigella dysenteriae and Bacteriodes fragile.*
This antibacterial property was described also by Khoushab and Yamabhai (2010). that reporting also an antifungal activity.
The mechanisms by which chitin plays its antimicrobial and antifungal role are not well known yet. Many authors have suggested hypothesis:

- Defence mechanism defence of host organisms (El Ghauth et al 1992). by inducing the accumulation of chitinases and other pathogenesis related proteins (El Ghauth et al 1992).
- Young et al decribed that there is an interaction beetween the chitin molecules (positively charged) and the bacterial surface (negatively charged).

This effect of chitin is responsible of a reduction of same infectivous agents duration and help the heath status of digestive system of the broilers fed insect meal diet.
A recent study (Islam et al., 2017) was conducted to evaluate dry mealworm (Tenebrio molitor) and super mealworm (Zophobas morio) larvae as alternatives to antibiotics in broiler chicks.
The authors described that dietary supplementation with Tenebrio molitor and Zophobas morio increased average daily gain and IgG and IgA levels in

broiler blood, and reduced feed conversion ratio, mortality and cecal E. coli and Salmonella spp. levels, (Islam et al., 2017).

In our results it is described an elevated level of AST in the bird fed on insect meal (the total increase was 8.3% more than the SBM group. even if it falls in the normal range for broilers); generally higher level of this enzime are linked to:

- vitamin E. selenium or methionine deficiencies (Gylstorff and Grimm, 1987).
- liver damage (particularly psittacosis or Pacheco's disease virus) (Goodwin et al.,1982; Rosskopf and Woerpel, 1984).
- pesticide and carbon tetrachloride intoxication (Lewandowski et al.,1986)
- and muscle damage.

To consider the AST parameter linked to a serius pathology. it is important know the level of other parameters (Lumeij and Westerhof,1987).
The lactid dehydrogenae and the creatinine kinase activity resulted normal. so we can exclude that the high AST level was linked to epatic or muscle damage. The activity of GGT. a specific enzyme localised of the cell membrane of bile ducts (Bovera et al., 2007). was a further confirm of liver damage absence. There are no diffrerence between the groups the blood contentration of triglycerides and cholesterol and the level are normal (in case of liver damage their concentation is high).
The alanine amino transpherase (ALT) concentration was also higher in the broilers fed Tenebrio molitor larvae meal (more than 75.7% comparated to control group) the alanine amino transpherase is not an important parameter in the birds. because the muscles and other many peripherical tissues can produce this enzyme (Attia et al., 2014).
Our results showed that the uric acid contentation was higher in the SBM group than the TML group (even if within the normal range). this ban be due to the higher protein intake. The feed intake of the SMB group during the last week of trial was higher in the control than the TML group (38.8 g/d more). The crude protein digestibility was lower due to presence of chitin which is found linked to numerous protein (Merzendorfer. 2014). The uric acid is the major nutrogenus waste producted by te avian species and it is an importan antioxidative agent (Harr. 2002; Jurani et al., 2004). the amount of ingested protein is directed linked to the seum acid concentation (Szaboet al..2005); for this reason any change in protein metabolism can affect in

serum uric acid concentrations (Piotrowska et al., 2011). Our results on BUN and creatinine concentrations not evidence any difference between the groups. for this reason it is possible affirm that the renal function of birds was unaffected by the dietary treatments; moreover, this two parameters fall in the normal range for the broilers (Attia et al 2014). so the renal function it was not damaged.

Carcass traits. apparent ileal digestibility and caecal volatile fatty acids

It is possible to observe some differences between the groups for carcass traits: the leght and the weight of the entire intestine and that of small intestine and caeca in the broiler fed on Tenebrio molitor larvae meal diet were higher than the birds fed on soyben meal.

There are not many studies on the effects of the insect meal diet on the gastrointestinal tract characteristics. Ballitoc and Sun (2013) showed that the 10% inclusion of Tenebrio molitor larvae meal in broiler diet was responsible of the increasing of small intestine weight and length compared to the control group (0% of inclusion). The effect of TML diet on the full intestinal lenght and weight could be due to the chitin amount (in our trial the chitin percentage was 4.62%).

The chitin, the second most abundant polysaccharide in nature (the first is the cellulose), is a polymer of N-acetyl-D-glucosaminerepresents the major structural component not only in insects but also in crustaceans and fungi (Khoushab et al., 2010; Bueter et al.,2013). Although these chitin-containing organisms have been suggested as novel animal feed resources, chitin has long been considered as indigestible fibers in the animal body (Bays et al.,2013).

The first chitinase discovered was acidic chitinase (Chia) is a protease-resistant major glycosidase in mouse gastrointestinal tract and that it digests chitin in the mouse stomach in physiological condition (Boots et al., 2001; Boots et al., 2005; Ohno et al., 2012).

However, the physiological role of Chia in other animals including poultry remains unknown (Tabata et al.,2017).

Tabata et al (2017) reported that Chia can function as a digestive enzyme that breaks down chitin-containing organisms in chicken gastrointestinal tract. The acidic chitinase mRNA is predominantly expressed in the glandular stomach tissue in normal chicken.

The authors (Tabate et al., 2017) showed also that chicken acidic chitinase has a robust chitinolytic activity at pH 2.0 and is highly resistant to

proteolysis by pepsin and trypsin/chymotrypsin under conditions mimicking gastrointestinal tract.

Acidic chitinase degraded shells of mealworm larvae in the presence of digestive proteases and produced N-acetyl -D-glucosamine (Tabata et al., 2017).

Thus, functional similarity of chicken Chia with the mouse enzyme suggests that chitin-containing organisms can be used for alternative poultry diets not only as whole edible resources but also as enhancers of their nutritional value (Tabata et al., 2017).

The chitin affects the development of the gastrointestinal traits because reduce the nutrient digestibility and has a prebiotical activity as confirmad by the high levels of VFA produced in the caecal contents of broilers fed insects and in particular of butyric acid.

Indees our results showed that the apparent ileal digestibility of the dry matter and the organic matter of TML diet were 2% lower than the control. and that of crude protein was 8.2% lower than the control. Our results are in agreement to Schiavone et al. (2014) because the authors found a decrease of nutrient digestibility and an increase of small intestine lenght and weight when the broiler were fed on Tenebrio molitor larvae meal (inclusion level 25%).

The increase of the volume and the weight of the gastrontestinal tract was linked to a compensatory mechanism to increase the amount of the feed intake as well as a surface available for nutrient absorption (Borin et al., 2006).

Despite the nutrient digestibility was lower in the TML group. the dietary treatment did no affect any growth performance (all the groups presented the same weight at slaughtered). at the contrary the FCR was more favourable in the TML group than the SBM. in agreement to Ballitoc and Sun (2013) at inclusion level of Tenebrio molitor of 10%.

The protein had the most significant reduction (87.35 vs 80.20 % in SBM and TML diet. respectively); the decrease of protein digestibility in TML diet could be due to to the protein linked chitin (the cuticle protein linked to the exoskeleton) in according to Bellicco et al. (2013).

Moreover, the insect meal protein had a low content in essential amino acids (methionine. cysteine. lysine. tryptophan) and the essential amino acids in the cuticle are different from that of the insect whole body (Finke. 2007); probably, the essential aminoacid level ingested by broilers of the insect group was sufficient to satisfy a correct growth.

Some authors (Hill et al., 2005; Dahiya et al., 2006; Pieper et al.,2008) suggest that when there are a low digestibility in a diet, the indigest portion of the feed remains in the gastrointestinal tract and affects the growth performance of the broilers as a substrate for intestinal bacteria.
Widyaratne and Drew (2011) described that the growth performance of the broilers fed low-protein diets are comparable to high-protein diets when highly digestible ingredients are used.
Our results showed also an increase in total volatile fatty acids in broiler fed Tenebrio molitor than soybean meal group (resulted 11.63 Mmol/l in the control group. and 21.38 Mmol/l in the insect meal group). In particular it is possible to observe a very important increase in butyric acid (+6.96 Mmol/l compared to the soybean meal group). Probably this increse is linked to the prebiotic role of chitin. On this regard Khempaka et al. (2011) described that the inclusion in broiler diet of shrimp head (chitin source) at 15 and 20% of inclusion as well as addiction of 1.9% of purified chitin increased the production of butyric acids in the ceca.
The butyric acid is one of the most important volatile fatty acids. for many reasons. At first because it is considered the most important enterocytes energy source (Bovera et al.,2010). In addition it is necessary also fot the suitable increase of the Gut-Associated Lymphoid Tissue (GALT). In according to MROZ et al. (2005). The butyric acid is also the mayor intestinal energy source even when other fuel sources (glucose and glutamine) are available and could stimulate the the growth of colon rectal and ileal mucosal cells (Montagne et al.. 2003. Topping and Clifton. 2001) and finally plays an important role into inhibition of *Salmonella* and *E. coli* colonization of the intestine (Van der Wielen et al., 2000).
Mahdavi et Toki (2009) described that a higher amount of butyrate promote the increases of nutrient for enterocytes blood flow thought intestine. as a conseguence help the tissue oxygenation and nutient transport and ossigenation.
Mroz (2005) observed that the blood flow stimulate the neural networks and chemoreceptors together has a direct effect on smooth muscle cells; This signals are importante because induce the growth factors production. as a consequence stimulating the growth of the different intestine traits (Mahdavi e toki. 2009).
The volatile fatty acids has a important role in broilers on bacteriostatic effect on bacteria such as Salmonella typhimurium. and thay not inhibit the beneficial bacteria such as Lactobacillus (Van der Wielen et al..2000).

Nisbet et al (1996) observed that there is a negative correlation between the propionate increase in caecal volatile fatty acids production and the colonization of Salmonella in chikens.
In the carcass traits results it is possible observe that the broilers fed on Tenebrio molitor diet presented a highest weight (5.57 g in TML and 4.40 g in SBM group) and incidence of the spleen (0.16% vs 0.12%. respectively for TML and SBM group). This increase probably could be due to the increase of immune system activity. The stress conditions plays and important role on the weight changes of broilers spleen. on this regard several authors (Awadalla et al., 1998; Kusnadi and Djulardi, 2011) reported a lower weight of the spleen in the stressed broilers than the control group.
The stress conditions stimulate a increase of plasmatic corticosterone level. that depressed the growth of lymphoid organs in the broilers (spleen or *Fabricii bursa*) in according to Siegel (1995).
Several authors reported that the speen weight can be low in the animals under stess conditions.

Physical and chemical criteria of meat

Our results showed that there are no differences in most physical meat an skin properties (such as color and tenderness). these data are very important because they could be influence the consumer acceptance of the meat. The color in particular is one othe the parameters that influences at the most the consumers because if the color varies from the "standard" the consumers will reject the product (Qial et al 2001).
Our results of raw (internal and external) and cooked meat and on the skin color indicated that the broiler meat fed Tenebrio molitor larvae meal could be accepted willingly by the consumers.
The pH values and the cooking losses are higher in the broiler fed TML than the broiler fed on soybean meal; that indicate a decrease quality of the meat obtained by feeding insects; however the pH values fall in the normal range for both the groups (5.95 vs 6.12. for SBM and TML group respectively). Breast meat of the broilers it classified as pale (PSE) if the pH values are lower than 5.7. or dark (DFD) id the p His higher than 6.2 in according to Flether et al (2000). The breast meat of the broiler analysed in this trials colud be considered normal. and the higher values (but it falls in the normal pH range) reported fot TML group colud be ascribed to a lower amount in muscle glycogen. The values of cooking losses are in line with other results available in literature fot broiler breast (Hashim et al..2013) even if they were higher in the broilers fed on *Tenebrio molitor* diet than the soybean meal group. The WHC was not affected by dietary treactment.

Unfortunately, it was not possible for technical problem to analyse the fatty acid profile of the diets or of the two main sources of protein used in the present trial, so it was not possible to link the changes observed in the meat and in the deposition fat to correspondent changes in diet. However, even if in the breast meat several differences are recorded between the single fatty acids, the total of saturated, monounsatured and polyunsaturated fatty acids were unaffected by dietary treatments. In addition, atherogenicity and thrombogenicity indexes were also not different between group indicating that insect meal from Tenebrio molitor is not able to modify the nutraceutics properties of the poultry meat. The same consideration on SFA, MUFA, PUFA, AI and TI can be applied to abdominal fat even if in this case the differences between the single fatty acids are very low.

Conclusions

The use of meal from Tenebrio molitor as main protein source in broiler in total replacement of soybean meal along a period from 30 to 62 days of age had no significant effect on most of growth performance, carcass traits (such as dressing out) and chemical and physical properties of meat, the latter important for marketing purposes. The intestinal length and weight as well as the absolute and relative spleen weight were higher in broilers fed TML in comparison to SBM and this can be attributed to the effect of chitin which reduces nutrient digestibility and act as a prebiotic. Tenebrio molitor larvae meal could be a suitable alternative protein source for chicken broilers also when used as principal protein source in the diet.

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VII
Laying hens fed on *Hermetia illucens* larvae meal

As underlined in the previous section of this thesis, there are several evidences in literature on the use of insect meals in broiler production, and this is possible due to short cycle of production but also to the relative simple system of management that allow to make an experiment without much "complication". Regarding laying hens, to make an experimental trial is more difficult, not only due to the length of the productive cycle but also because if we want to reproduce conditions similar to that of intensive caged systems, the only way is to work on an intensive farm. Probably for this reason, in literature are not so much studies on the use of insect larvae meal in laying hens.

All the animals were humanely treated according to the principles stated by the EC directive 2010/63EEC, regarding the protection of animals used for experimental and other scientific purposes. The experimental procedures were approved by the Ethical Animal Care and Use Committee of the University of Napoli Federico II (Prot. n. 2017/ 0017676).

Materals and Methods

Animals and experimental conditions

The experimental trial was carried out in a private laying hens farm, located near Caserta, in Southern Italy, and lasted 21 weeks (from 24 to 45 weeks of age of the hens), from September 2015 to February 2016.

A total of 108 Lohman Brown classic laying hens were equally divided in 2 groups (54 birds per group); the average weight of the hens was 1.78 ± 0.15 kg. The hens were housed in the same building in modified cages (800 cm^2 /hen) under controlled temperature and humidity conditions.

For each group the animals were divided in 3 cages (18 bird per cage) and each cage was equally divided in 3 parts (6 hens/ replication); in total there were 9 replications for group.

The feed and the water were administered manually and appropriate separations were placed in order to control the feed intake for each replication. Similar separations were located in the line of the eggs collection to control the egg production per each replicate. The cycle light/ dark was 15:9 hours.

After two weeks at the beginning of the trial (adaptation period) at the new diets (when the laying hens are 26 weeks of age), the collection of the data was started.
The feed intake was recorded weekly per replicate weighing the amount of the feed distributed and that residual and scattered. The feed intake was expressed as individual grams per day.
From 26 to 45 weeks of age the number of the eggs produced and eggs weight were registred for each replicate every week.
The eggs were assigned to a weight class, as follows:

Table 7.1: Eggs weight class

weight range	**Class**	**Abbreviation**
Up to 52 g	Small class	S
53-62g	Medium class	M
63-73g	Large class	L
More than 73 g	Extra large	XL

Per each replicate of each group, eggs mass was calculated by multypling eggs weight by eggs production percentage and feed conversion ratio (FCR), was calculated as gram of feed consumption per day divided by gram of egg weight per day.

Ingredients and experimental diets
The groups were fed 2 isoproteic and isoenergetic diets, differing for the ingredient used as main protein source:

- The control group fed a corn- soybean meal based diet and was indicated as SBM group
- in the other group a defatted meal from Hermetia illucens larvae (Hermetia Deuschald GmbH CO HG, Amtsgerch, Postdam, Germany) completely replaced the soybean meal. This group was indicated as HILM group.

The diets were formulated to meet the hen's requirement (Lohman Brown classic Managment Guide, 2011). The ingredients and the diets were analysed in according to AOAC (2004) using the following methods (the values of chemical composition are showed in the tables 7.2 and 7.3):

- Dry matter (DM, method number 943.01)
- Ash (method number 924.05)
- Crude protein (CP, method number 954.01)

- Ether extract (EE method number 920.39)
- Neuthal detergent fibre (NDF, method number 2002.04)
- Acid detergent fibre (ADF, method number 973.18)

The data on aminacidic profile and some mineral content (Ca, P and Na) of two protein sources were supplied by the respective producers, and used to correctly formulate the diets.

The metabolizable energy content of the two diets was calculated taking into account the chemical composition and the equations proposed by NRC (1997). For insect meal, the nutrient digestibility used in the calculation was obtained from our previous in vitro trial (Marono et al., 2015).

In the insect meal, the amount of chitin was calculated according to the following formula, proposed in our previous study (Marono et al.,2015):

Chitin (%)= ash free ADF (%)- ADF linked protein (%)

Table 7.2: chemical composition of Hermetia illucens larvae meal and soybean meal, used as a protein sources in the trial

Chemical composition (%)	Hermetia illucens lavae meal	Soybean meal
Dry matter	97.8	90.0
Crude protein	61.3	43.4
Ether extract	4.61	1.10
ADF	12.1	5.90
ADF linked protein	5.59	1.78
Ash	7.82	6.01
Ca	6.90	2.83
Total P	0.91	0.57
Na	0.12	0.16
Lysine (% protein)	4.05	2.92
Methionine (% protein)	1.30	0.61
Methionine+cysteine(% protein)	1.42	1.33
Isoleucine(% protein)	3.11	2.30
Tryptophan(% protein)	0.30	0.73
Valine(% protein)	5.02	2.11
Treonine(% protein)	3.23	1.74

Table 7.3 Ingredients included in the experimental diets.

Ingredients(g/Kg)	Hermetia illucens larvae meal diet(HIML)	Soyben meal diet(SBM)
Maize grain	653.0	583.0
Soybean meal	---	235.0
Insect meal	170.0	---
$CaCO_3$	80.0	80.00
Dehulled sunflower	50.0	50.00
Vegetable oil	10.0	15.00
Min Vit [1]	30.0	30.00
Monocalcium phosfate	5.00	5.00
Salt	2.00	2.00

[1] Provided 20 g of celite and 10 g of mineral and vitamin supplements. Per kilogram: vitamin A (retinyl acetate) 20,000 IU, vitamin D3 (cholecalciferol) 6,000 IU, vitamin E (dl-α-tocopheryl acetate) 80 IU, vitamin B1(thiamine monophosphate) 3 mg, vitamin B2 (riboflavin) 12 mg, vitamin B6 (pyridoxine hydrochloride) 8 mg, vitamin B12 (cyanocobalamin) 0.04 mg, vitamin K3 (menadione) 4.8 mg; vitamin H (d biotin) 0.2 mg, vitamin PP (nicotinic acid) 48 mg, folic acid 2 mg, calcium pantothenate 20 mg, manganous oxide 200 mg, ferrous carbonate 80 mg, cupric sulphate pentahydrate 20 mg, zinc oxide 120 mg, basic carbonate monohydrate 0.4 mg, anhydrous calcium iodate 2 mg, sodium selenite 0.4 mg, choline cloride 800 mg, 4-6-phitase 1,800 FYT, D.L. methionine 2,600 mg, canthaxanthin 8 mg.

Table 7.4: Chemical catacteristics of the experimental diets.

Chemical caracteristics	Hermetia illucens larvae meal diet (HILM)	Soybean meal diet(SBM)
Dry matter	90.5	90.1
Crude protein	17.9	18.1
Crude fibre	4.14	3.96
Ether ectract	4.26	4.33
ADF	3.82	3.45

ADIP	2.88	1.52
Ash	14.2	14.2
NDF	15.2	14
Ca	4.96	4.26
Total P	0.67	0.69
Na	0.30	0.19
Lysine	0.91	0.90
Methionine	0.64	0.55
Methionine+Cysteine	0.86	0.84
Isoleucine	0.79	0.77
Tryptophan	0.16	0.21
Valine	1.19	0.82
Treonine	0.64	0.63
ME Kcal/Kg	2.745	2.780

Table 7.5: Chemical characterization of H. illucens larvae meal and of the two experimental diets.

	Hermetia illucens	**Soybean meal diet**	**Hetmetia illucens diet**
12:0	32.34	1.36	7.89
14:0	7.38	1.63	2.85
16:0	13.09	12.95	12.91
16:1 n-7	3.59	0.94	1.53
18:0	3.48	3.23	3.42
18:1 n-9	12.02	20.55	18.98
18:1 n-7	2.50	3.11	3.22
18:2 n-3	7.33	36.58	29.73
18:3 n-3	1.56	2.67	2.95
20:0	1.27	1.46	1.39
EPA+DHA	1.14	1.13	1.10
ΣSFA	61.46	24.74	32.45
ΣMUFA	22.23	28.64	27.66
ΣPUFA n-6	10.10	39.31	32.44
ΣPUFA n-3	4.66	5.75	5.94
Total fatty acids	54.59	29.84	31.12

Total tocopherol	42.72	3.41	2.62
Cholesterol	ND	ND	ND
δ- tocopherol	1.35	0.39	0.74
γ- tocopherol	3.20	0.43	0.63
a-tocopherol	38.18	2.60	1.25
Retinol	0	0.23	1.6
Total carotene	2.15	1.70	6.83
Lutein	1.15	0.96	4.06
Zeaxanthin	0.96	0.69	2.59
β-carotene	0.04	0.05	0.18

Blood samples

Blood samples were collected from the wing vein of 2 bids per replicate (a total of 36 blood samples, 18 samples per group) in collection tubes with and without eparin.

The serum was separated by a centrifugation at 1500 rpm for 15 minutes and stored at -20°C until the analyses.

The blood collected in tubes with the eparin was analysed using an automatic analyzer (ADVIA 120 Siemens, Munich, Germany) for:

- Hematocrit
- Heamoglobin
- Blood cells count (white bllod cells, WBC; red blood cells RBC).

The differential cont of WBC (white blood cells) was performed on blood smears stained with May- Grunwald-Giemsa by counting 100 cells with an optic microscope.

All biochemical traits were analysed with the serum samples:

- Total protein
- Albumin
- Globulin
- Glucose
- Cholesterol
- Tryglicerides
- Aspartate aminotransferase
- Alanina aminostransferase
- Gamma glutamil trasferase
- Alkaline phosphatase

- Creatinine kinase
- Lactic dehydrogenase
- Lactate
- Blood urea nitrogen
- Creatinine
- Uric acid
- Calcium (Ca)
- Phosphorus (P)
- Magnesium (Mg)
- Iron (Fe)
- Chloride (Fe).

The biochemical analyses were determinated using a commercial kit by Spinreact (La Val d'en Bois, Girona, Spain), by enzymatic colorimetric and kinetic methods, according to manufacturer's instructions.
Spectrofotometric measurement were performed using an automatic biochemical analyzer (AMS AUTOLAB, Rome, Italy).
Globulin concentration was calculated by difference between total protein and albumin, in addition, the albumin/globulin ratio was calculated.

The egg quality determination

In order to determinate the quality, we collected 144 eggs (72 eggs each group) in 4 different days of deposition; the eggs were stored at -20°C until the analyses.
The eggs were analyzed at the laboratories of the Department of Agrifood Production and Environmental Science, University of Florence.
The weights of whole egg, shell, yolk and albumen were recorded.
The diameter of the yolk was measured manually. The yolk pH was measured in two different points by a pH meter (Columbus, OH, USA).
The yolk color measurement was determinated by a Dr. Lange Spectro-colour colorimeter (Keison International Ltd, Chemsfold, Essex, UK) equipped with a spectral qc 3.6 software, according to the "Commission internnationale de L'eclairage "(CIE) system (CIE, 2004) and expressed as:

- Lightness(L*)
- Redness(r*).

The yolk and the albumen were analized for:

- Moisture (AOAC, 2004, procedure number 943.01)
- Ash (AOAC, 2004, procedure number 924.05)
- Crude protein (AOAC, 2004, procedure 954.01)

- Lipids (Folch., et al 1957; modified)

The lipids content of the samples was determined and fatty acids (FAs) in lipid extract were trans-esterified to methyl esters (FAME) using a base-catalyzed trans-esterification followed by a boron trifluoride catalyzed esterification in according to Christie (1982).

The FA composition was evaluated by gas chromatography (GC) using a Varian GC 430 gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and a Supelco Omegawax™ 320 capillary column (30 m × 0.32 mm i.d., 0.25-µm film and polyethylene glycol-bonded phase; Supelco, Bellefonte, PA, USA), purchased from Agilent Technologies (Santa Clara, CA, USA).

Chromatograms were registred with computing integrator software (Galaxie Chromatography Data System 1.9.302.952; Varian Inc., Palo Alto, CA, USA) and fatty acids were identified by comparing the FAME retention time with the standard Supelco 37 component FAME mix (Supelco, Bellefonte, PA, USA).

In order to quantify the amount of fatty acids (thought calibration curves) are used tricosanoic acid (C23:0, 0.4 mg mL-1) (Supelco, Bellefonte, PA, USA) as internal standard.

The cholesterol content was determinated in the yolk with the methods described below:

- 0.2 mL of lipid exctract was added with 0.5 mL of 5α-cholestane (0.2 mg mL-1 prepared in chlorophorm) (Supelco, Bellefonte, PA, USA) as internal standard,
- After solvent evaporation, 5 mL of KOH (0.5 M in methanol) was added and then left in a 95°C bath for 40 minutes in order to promote lipid saponification,
- 4 ml of distilled water and 2 mL of n-hexane were finally added. The upper phase was then directly transferred into a vial for GC analysis.

A Varian GC 430 gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and a Supelco SACTM fused silica capillary column (30 m × 0.25 mm i.d., 0.25-µm film; Supelco, Bellefonte, PA, USA), purchased from Agilent Technologies (Santa Clara, CA, USA). One- µL of sample was injected with a split ratio 1:100 at 300°C. Oven temperature was programmed in order to rise from 130°C to 290°C in 8 minutes (20°C min-1) than left at 290°C for 11 minutes. Detector was set

at 300°C. Helium was utilized as carrier gas kept at a constant flow of 1.3 ml min.

The content of carotenoids and vitamin E in yolks and feeds was determined in the lipid extracts adjusting previously proposed methods. Lipid extracts were saponified following the same method described for cholesterol but avoiding the warm bath. Saponification indeed was performed by standing the samples at room temperature overnight. Then, unsaponifiable matter was resuspended in 200 µL of chlorophorm/methanol (1:1) solution.

Finally, 20 µL of each sample were quantified using a Prostar HPLC (Varian Inc., Palo Alto, CA, USA) equipment with UV-DAD and a C18 reverse phase column (ChromSep HPLC Columns SS 250mm × 4.6mm with ChromSep guard column Omnispher 5 C18). The mobile phases were (A) methanol: acetonitrile water (5:85:10), and (B) methanol: ethylacetate (70:30). The flow was 90:10 of mobile phase A and B respectively at 1 mL min-1 for 18 min followed by 50:50 (1 mL min-1) for 2 minutes followed by 0:100 at 1.5 mL min-1 for 10 min.

Carotenoids (lutein, zeaxanthin, and β-caroten) were detected at 450 nm, vitamin A (retinol) at 325 nm and tocopherols (α, γ, and δ) at 292 nm.

All the molecules were determinated by an external calibration curve, obtained from concentrations ranging:

- from 0.005 to 0.17 µg mL-1 t determinate the carotenoids,
- from 0.02 to 0.5 µg mL-1 retinol,
- from 0.01 to 0.97 µg mL-1 for tocopherols.

Results were expressed as mg kg^{-1} total lipid.

Effects of the diet of gastrointestinal trait

At 45 weeks of age all the hens were weighed and 18 per group (2 per replicate) were slaughtered. The full intestine was weighted and the different tracts of the small (duodenum, jejunum, ileum) and large (caeca, colon, cloaca) intestine were isolated, measured and weighted after elimination of the respective contents.

1. Small intestine

Brush border enzymes activity

Thus, the small intestine tracts were washed with cold buffered saline pH 7 solution, blotted with adsorbent paper, collected on alumionion foil and stored at -20°C in order to determinate the brush border membrane enzymes. Brush borer enzyme membrane were determined according to Shirazy-Beechey et al. (1991) with some modifications. The analyses were

performed at laboratories of Department od Agricolture, Food and Environmental Science, University of Udine.
All the steps in the procedure were performed at 4°C:

- 100 mg of the sample collected, were diluted 1:10 with a buffer (100 mM mannitol, 2 mM Hepes-tris, pH 7.1), added with MgCl2 at a final concentration of 10 mM, and crushed with a tissue-lyser (Tissue Lyser II, Qiagen, Germany) at 30 Hz for 1 minute
- Samples were centrifuged at 2,000 x g at 4°C for 10 min and the supernatant was tranferred in a new vial and centrifuged at 15,000 x g at 4°C for 10 min. The resulting supernatant was mantained at -20°C until the analysis of the BBM enzyme activity.

One unit (U) of enzyme activity is the amount of enzyme that transforms or hydrolyses 1 mole of substrate per minute. Specific enzyme activity was calculated enzyme activity (U)/ protein (mg).
The amount of total protein in the supernatant was determined according to Bradford et al. (1976) using Bradford reagent (Sigma-Aldrich cat. no. B6916), using the bovine serum albumin (Sigma-Aldrich cat. no. 0834) as standard.
The hydrolysis of sucrose and maltose by the mucosal enzymes maltase (EC 3.2.1.20), and sucrase (EC 3.2.1.48), was determined according to previous methods (Dahlquist, 1964; Uni et al., 1998) with some modifications. The method used are described below:

- 40 µl of the final solution were mixed with 40 µl of substrate (0.056 M maltose and 0.056 M sucrose) and incubated for 60 minutes at 37°C
- The reaction was stopped by the incubation in ice for 4 minutes. Then 3.5 µl of the reaction mixture were transferred in plate diluted 1:10 with glucose reagent (Sigma-Aldrich cat. no. G3293) and incubated for 5 minutes at 37°C.
- The absorbance was measured at 340 nm versus saline solution as reference in a plate reader (Quant, Bio-Tek Instruments, Inc., Vinoosky, Vermont, USA)

Intestine Alkaline phosphatase (IAP) activity was determined using a commercial kit (Paramedical, Pontecagnano Faiano, Sa, Italy). The method was:

- 3 µl of diluted extract (1:2) were added to 300 µl of the working solution in a well-plate at 37°C.

- The hydrolysis of p-nitrophenilphosphate by the mucosal enzyme, yielding p-nitrophenol and inorganic phosphate was measured by a plate reader (Quant, Bio-Tek Instruments, Inc., Vinoosky, Vermont, USA) at 405 nm as indicated by the manufacturer.

γ-glutamyl transpeptidase (GGT) activity was determined using a commercial kit (Paramedical, Pontecagnano Faiano, Sa, Italy). 24 µl of extract was diluted with 300 µl of the working solution in a well-plate at 37°C. The production of p-nitroaniline from γ-glutamil-p-nitroanilide by the mucosal enzymes was measured by a plate reader (Quant, Bio-Tek Instruments, Inc., Vinoosky, Vermont, USA) at 405 nm as indicated by the manufacturer.

Small intestine morphology

Other samples of intestinal trait (about 3- 5 mm) are collected from duodenum, jejunum and ileum were washed, and collected in eppendorf tube with a solution of PFA 4% overnight at 4°C. he day after they were washed for three times with a PSB solution, and after stored in ethanol solution (75%) at 4° C, the analyses were performed at laboratories of Department of Marine Biology, University of Ancona.

Apparent ileal digestibility

After the weight and the length measurements, the ileum was sepatated from 2 cm after Mekel's diverticulum to 4 cm proximal to ileocecal junction in order to avoid contamination of others intestinal contents and pooled per replicate (1 pool from 2 hens per replicate; 9 pools per group), the samples collected are immediatly frozen and then freeze- dried. The dried ileal content were ground to pass a 1 mm sieve and stored at -20°C antil the analysis. The apparent ileal digestibility coefficients of dry and organic matter, crude protein and ether extract were measured using the acid insoluble ash (AIA) method and the Celite was added to the diet at 2% as an internal marker according to Vogtmann et al., (1975). The apparent ileal digestibility of nutrients were determined using the following formula:

100 – 100 × [(% AIA in the diet/% AIA in the ileal content) × (% nutrient in the ileal content/% nutrient in the diet)].

2- <u>Large intestine</u>

Caecal Volatile fatty acids

The caeca were separated by sterile instruments from the rest of the gastrointestinal tracts, placed in tightly closed plastic bags and put in pre-warmed thermos. The samples were transported as soon as possible at the laboratories of the Department of Veterinary Medicine and Animal

Production (Napoli). In the laboratory, two quotes of caecal content (each about 5 ml) were used to determinate the volatile fatty acids (VFAs). After dilution of the samples with oxalic acid (1:1, v/v), the VFAs were analysed by a gas chromatography method (Thermo-Electron mod. 8000top, FUSED SILICA Gaschromatograph (ThermoElectron Corporation, Rodano, Milan, Italy) with OMEGAWAX 250 fused silica capillary column 30 m X 0.25 mm X 0.25 mm film thickness; analysis temperature, 125 °C; flame ionisation detector, 185 °C; carrier helium, 1.7 ml/min (Stanco et al., 2003).

Statistical Analysis

The data on laying performance were processed by a two-way ANOVA according to the model:

$$Yijk = m + Di + Wj + Dwij + eijk$$

were Y is the single observation, m the general mean, D the effect of the diet (i = BSLM or SBM), W the effect of the week of lay (j = from 26 to 45), e is the error. Comparison between means was performed by Tukey's test (SAS, 2000). Differences between weigh classes of eggs for the entire period of the trial were tested by chi-square test.

The other data were were processed by a one-way ANOVA using the PROC GLM of SAS (2000) according to the following model:

$$Yij = m + Di + eij$$

where Y is the single observation, m the general mean, D the effect of the diet (i = HILM or SBM), e is the error. Comparison between means was performed by Tukey's test (SAS, 2000).

Results

No morbidity or mortality was reordered during the experimental period. The average temperature during the trial was 21°C ± 0.5 (standard deviation) and the average humidity was 60% ± 2.5 (standard deviation). The amount of ADF-linked protein in *Hermetia illucens* larvae meal was 5.55% as fed, so the estimated amount of chitin was 5.40% as fed.

Body live weight and productive performances

The live weight of the hens was recorded at the beginning and at the end of the trial and the results are reported in the Table 7.6.

Table 7.6: Changes in body live weight of the hens during the experimental period.

	Initial body weight (Kg)	**Final body weight (Kg)**	**Weight gain (g)**
HILM	1.79	1.89[b]	102.2[b]

SBM	1.77	2.10[a]	328.9[a]
Pvalue	0.79	0.012	0.024
RMSE	0.162	0.154	181.81

Abbreviations: HILM: hermetia llucens larvae meal; SBM: soybean meal; RMSE: root mean square error; a-b Pvalue <0.05.

It is possible to observe that the initial body weight was similar between the groups, while at the end of the trial the control group showed a higher live weight than the *Hermetia illucens* larvae meal group (2.10 vs 1.77 Kg, in SBM and HILM group, respectively; P <0.05). The weight gain reordered from 26 and 45 weeks of age was higher (P < 0.05) in the SMB group (328.9 g/d) than the HILM group (102.2 g/d).
The effects of the protein sources (soybean and black soldier fly), the week of age and their interaction in laying performance are reported in the Table 7.7.

Table 7.7: Effect of main protein source and the week of production on laying performance of the hens

	Lay (%)	**Feed intake(g/d/hen)**	**Egg weight(g)**	**Egg mass**	**FCR**
HILM	91.9 b	108.0b	59.9b	55.1b	1.97b
SBM	94.5 a	125.1°	61.8a	58.3 a	2.17 a
Pvalue					
Group effect	<0.001	<0.001	<0.001	<0.001	<0.001
Week effect	<0.001	<0.001	0.0087	0.001	0.423
Interaction effect	0.45	0.20	0.38	0.005	0.20
RMSE	6.580	9.129	2.781	5.921	0.252

The lay percentage, feed intake and the average weight and the egg mass resulted lower in the hens fed insect meal (-2.6% for the lay percentage, -

17.1 for the feed intake g/d/hen, -2.1 g for the egg weight, -3.2 for the egg mass) than the ones fed soybean meal (P <0.01).
The feed conversion ratio resulted more favorable in the hens fed *Hermetia illucens* larvae meal then the birds fed soybean meal (P <0.01).
The feed conversion ratio was unaffected by week of age. The laying percentage, the feed intake, egg weight and mass are affected by the weeks of production, even if the interaction group per week resulted significant only for the egg mass (Pvalue <0.01).

Table 7.8: The effect of the diets in the weight class of the eggs during the entire period of the trial.

	S% (up to 52g)	**M% (53-63 g)**	**L% 64-73g)**	**XL% (>73g)**
HILM	7.14 a	69.1 a	21.6b	2.17 a
SMB	2.82 b	66.2b	30.9 a	0.00b
Pvalue	<0.001	<0.001	<0.001	<0.001
RMSE	0.934	2.931	0.351	0.153

Table 7.8 showed the results of egg classification according to their weight. It is possible to observe that the laying hend fed Hermetia illucens larvae meal produced a higher percentage of eggs from S, M and L classes (P < 0.01), and the SBM group had a higher percentage of eggs from the L class (P <0.01).

Blood parameters

The effect of protein source on hematologic profile ofthe laying hens is reported in the tables 9, 10, 11 and 12.

Table 7.9: Hematological traits, serum proteins, glucose, and lipids of laying hens.

Hematological traits	**HIML**	**SMB**	**P value**	**RMSE**
Hematocrit%	33.3	33.8	0.67	3.642
Haemoglobin%	11.1	10.1	0.071	1.451
RBCx10^6/mm^3	3.65	3.61	0.85	0.654
WBC x10^3/mm^3	21.1	20.9	0.86	2.982
Heterophilis%	37.1	37.3	0.35	0.841
Lymphocytes%	47.3	48.9	0.34	0.173

Monocytes%	2.94	2.69	0.97	0.471
Eosinophilis	11.4	1.01	0.22	0.090
Basophilis%	1.31	1.00	0.12	0.472
H/L	0.79	0.77	0.11	0.534
Serum protein, glucose and lipids				
Total protein g/dl	5.18	5.31	0.58	0.629
Albumin g/dl	2.72	2.58	0.44	0.501
Globulin g/dl	2.74 a	2.12b	0.030	0.763
Albumin/globulin	1.01b	1.62 a	0.033	0.771
Glucose mg/dl	274	295	0.29	55.340
Cholesterol mg/dl	134b	108 a	0.010	26.582
Triglycerides mg/dl	1296 B	1942 A	0.007	627.77

The level of globulin was lower in the SBM (2.12 g/dl) group than the HILM (2.74 g/dl), the albumin to globulin ratio is lower in the HIML (1.01) than the control group(1.62).
The cholesterol and triglycerides levels are higher (P = 0.01) in the control (+54 mg/dl for triglycerides and +26 mg/dl for cholesterol) than the insect group.

Table 7.10: The liver function of laying hens:

	HIML	SBM	Pvalue	RMSE
AST U/I	112	126	0.57	68.470
ALT U/I	139	133	0.66	97.610
GGT U/I	88.8	76.9	0.64	71.501
ALP U/I	1191	1189	0.99	906.703

There are no significant differences between the groups on the liver parameters (Table 7.10).

Table 7.11: The electrolytes of the laying hens:

	HILM	**SBM**	**P value**	**RMSE**
Ca mg/dl	10.6 A	9.46 B	0.002	0.943
P mg/dl	7.58	8.43	0.34	2.502
Mg mg/dl	5.22	6.99	0.082	2.734

Fe mcg/dl	215	224	0.18	19.091
Cl mmol/dl	133 b	137 a	0.038	7.682

The Ca amount (Table 7.11) resulted greater in the hens fed on Hermetia illucens larvae meal (+1.14 mg/dl) than the hens fed on soybean group (P <0.01). The Cl amount resulted lower in the birds fed on insect meal (+4 mmol/dl) than the ones fed on soybean meal (P <0.05).

Table 7.12: The renal and the muscle function of laying hens

	HILM	**SBM**	**Pvalue**	**RMSE**
BUN mg/dl	0.79	0.89	0.38	0.304
Crea mg/dl	0.29B	0.46 A	0.002	0.143
Uric acid mg/dl	4.15	5.02	0.24	0.060
CK U/I	663	654	0.22	478.182
LDH U/I	949	898	0.62	293.302
Lactate mg/dl	105	121	0.11	27.942

The Creatinine level of laying hens fed on Hermetia illucens larvae meal was higher (+ 17 mg/dl) than the birds fed on soybean meal diets (P <0.01).

Eggs

Chemical composition of the eggs

Table 7.13: The proximate yolk composition (%).

Yolk composition(%)	**Hermetia illucens larvae meal diet (HILM)**	**Soybean meal diet (SMB)**	**RMSE**	**Pvalue**
Water content	50.5	49.6	1.88	NS
Ash	17.8	18.8	0.25	NS
Crude protein	15.6	16.0	0.77	NS

Total lipids	30.9	32.6	5.10	NS

RMSE: root mean square error; NS: not statistically significant.

Table 7.14: the proximate albumen composition (%).

Albumen composition (%)	**Hermetia illucens larvae meal diet (HILM)**	**Soybean meal diet (SMB)**	**RMSE**	**Pvalue**
Water content	86.9	86.9	0.89	NS
Ash	7.5	7.4	0.05	NS
Crude protein	11.1	11.2	0.81	NS

RMSE: root mean square error; NS: not statistically significant.

From the data showed in the table 7.13 yolk is mainly composed by water, followed by lipid, ash, and protein both in soybean and insect meal groups. Water is instead the dominant fraction of albumen (reported in the table 7.14), followed by protein and ash. For both yolk and albumen, no differences were observed between group for chemical composition.

Physical criteria of the eggs

Table 7.15: Morphological and physical characteristics and pH of the eggs from hens fed soybean meal diet and Hermetia illucens larvae meal diet.

	Hermetia illucens larvae meal	**Soybean meal**	**RMSE**	**P value**
Weight				
Egg	59.56	62.67	5.316	NS
Shell	7.92	8.10	0.762	NS
Yolk	14.82	14.80	1.486	NS
Albumen	35.98 b	39.09 a	3.936	<0.05
Prcentage of total weight				

Shell	13.3	12.95	0.951	NS
Yolk	24.95 a	23.64b	1.901	<0.05
Albumen	60.32b	62.33a	2.411	<0.05
Yolk diameter	2.84	2.87	0.184	NS
Yolk colour				
L*	55.56	57.83	4.758	NS
A*	5.63A	1.36B	1.525	<0.01
B*	27.40	24.92	5.106	NS
Chroma	28.02	25.00	5.086	NS
Hue	78.01B	86.76A	3.425	<0.01
Ph	6.10	6.07	0.081	NS

Abbrevations: RMSE: root mean square error, NS not significant.

The weight and the percentage on egg total weight of the albumen (table 7.15) were higher in the eggs from the hens fed insect meal (+ 3.11 g and + 2.01%, respectively) than the ones fed soybean meal (P <0.05). It is possible to observe that the yolk color was more red in the eggs produced by the hens fed insect meal for the parameters A* and Hue (P <0.01). Comparing the diets for vitamins and carotenoids content emerged that HILM diet has lower level of total tocopherols than control diet, leading by a half amount of α-tocopherol, despite the high content of this vitamin in the insect. Regarding the content in retinol, H. illucens did not contained vit. A whilst its derived meal had higher level of retinol than control diet. HILM diet resulted also 4 times richer in the total carotenoid content than SBM diet, especially in lutein which level was more than three times that of soybean meal.

Effects of insect meal on egg characteristics of Lohman Brown Classic laying birds are presented in Table 7.15. Diameter of yolk, whole egg, shell, and yolk weights were not significantly affected by dietary treatments. The albumen of HIML was significantly lighter than SBM group . Red (r*)index was also affected by the treatment, specifically HIML yolks resulted redder. A neutral pH was found for both analytical groups without any significant differences.

Fatty acids profile of the eggs.

Table 7.16: Total fatty acids composition in the eggs produced by the laying hens of the soybean meal group (SBM) and the Hermetia illucens group (HILM).

mg/Kg	Hermetia illucens larvae meal diet (HIML)	Soybean meal diet (SBM)	RMSE	Pvalue
Total fatty acids	307.58	305.44	3.114	NS
12:0	1.23	1.19	0.166	NS
14:0	20.2 A	1.70B	0.214	<0.001
14:1 n-5	0.69 a	1.64b	0.081	<0.05
15:0	0.80	0.81	0.105	NS
16:0	19.69 b	2.82 a	1.031	<0.05
16:1 n-9	1.31 a	1.17 b	0.125	< 0.05
16:1 n-7	2.87 B	3.48 a	0.371	< 0.001
Antesio 17:0	0.78	0.77	0.104	NS
17:0	0.88	0.87	0.107	NS
16:3 n-4	0.76	076	0.101	NS
17:1	0.82	0.83	0.101	NS
18:0	7.38 a	7.07b	0.255	< 0.05
18:1 n-9	30.09	80.25	1.467	NS
18:1 n-7	3.53 b	3.83 a	0.254	< 0.05
18:2 n-6	11.72 A	10.24B	1.005	< 0.001
18:3 n-6	0.83	0.84	0.133	NS
18:3 n-4	0.72	0.73	0.095	NS
18:3 n-3	1.06	1.01	0.090	NS
18:4 n-3	0.69	0.69	0.094	NS
20:0	1.14	1.15	0.165	NS
20:1 n-9	0.63	0.66	0.065	NS
20:2 n-6	0.66	0.67	0.077	NS
20:3 n-3	0.74	0.76	0.081	NS
20:4 n-6	2.62	2.70	0.021	NS
20:3 n-3	0.57	0.57	0.081	NS
20:4 n-3	0.56	0.57	0.077	NS
20:5 n-3	0.55	0.55	0.076	NS

22:0	0.61	0.61	0.097	NS
22:5 n-6	0.48B	0.74 A	0.096	< 0.001
22:6 n-3	1.40	1.40	0.159	NS
Σ SFA	35.38	35.58	0.807	NS
ΣMUFA	40.88 b	41.80 a	1.296	<0.05
ΣPUFA n-6	17.29 a	16.20 b	1.147	< 0.05
Σ PUFAn-3	4.98	4.93	0.549	NS

Data on fatty acid profile of the eggs obtained from the two tested groups are reported in Table 7.16.

Table 7.17: Tocopherols and caroteinoids content (mg/Kg) of yolk from the hens fed soybean meal and Hermetia illucens larvae meal.

	HILM	**SBM**	**RMSE**	**Pvalue**
Total tocopherols	47.8	44.3	1.83	NS
Cholesterol	10.2 b	12.6 a	0.11	<0.05
δ-tocopherol	4.3	3.9	0.20	NS
γ-tocopherol	4.0 a	2.4 b	0.20	<0.05
a-tocopherol	39.6	38.0	0.65	NS
Retinol	16.9	17.0	0.67	NS
Total caroteinoids	15.0 a	10.5 b	0.64	<0.05
Lutein	8.6	4.9	0.45	NS
Zeaxanthin	6.0	5.4	0.46	NS
β-carotene	0.33 a	0.19 b	0.01	< 0.05

Dealing with tocopherols, retinol, and other carotenoids contents of the eggs, the data present in Table 7.17. Generally, α-tocopherol was the most abundant tocopherol in both groups, amounting for 84% of the total content. The eggs from HIML group showed a higher content in total tocopherols (+7.9%), even if only γ-tocopherol was significantly higher than in SBM group being the double. Retinol was not affected by the dietary treactment, whereas total carotenoids were significantly increased in the eggs of HIML group, leading by the +70% of lutein, and β-caroten amounts.
Fatty acids composition of eggs yolk is presented in Table 7.16.

It is possible observe that yolks of both groups were extremely rich in:

- C18:1-n9,
- C16:0,
- C18:0 (stearic acid),
- C18:2-n6 (linoleic acid, LA)
- C18:1-n7
- and C16:1-n7, accounted for around 73% of total fatty acids.

Interestingly, diet significantly affected all these fatty acids except oleic acid, the main constituent of yolks lipid fraction. Particularly, significantly higher values of Linoleic acid and C18:0 were found in HIML than SBM group, whereas HIML resulted poorer of the others fatty acids. The differences in C18:2-n6 and C16:1-n7 seemed to determine the significant differences on MUFA and PUFA-n6 fractions found between HIML and SBM group. Considering the products of the activity of desaturase/elongase enzymes involved in the conversion of linoleic acid, no differences in C18:3-n6, and C20:4-n6 contents but significantly lower amount of C22:5-n6, the final product of this bioconversion, were found in HIML rather than SBM group. At the same time, none of the products of the elongation and desaturation of C18:3-n3, such as C20:5-n3 (eicosapentaenoic acid, EPA) and C22:6-n3 (docosahexaenoic acid, DHA) were significantly affected being in sum stable below the 2% of total fatty acid. Finally, cholesterol was significantly reduced in insect group yolks, of about 7% than the soybean ones.

Intestine (weight and length of single tracts)

Table 7.18: Intestinal length and weight of laying hens

	HIML	**SBM**	**RMSE**	**P value**
	Intestine weight, % LW			
Full digestive tract	10.45[a]	8.81[b]	0.68	0.0330
Empty digestive tract	6.42	5.76	0.57	0.1263

Crop	0.30	0.24	0.14	0.1116
Duodenum	1.08	1.14	0.16	0.3723
Jejunum	1.21	1.11	0.18	0.2874
Ileum	1.14	1.21	0.23	0.5758
Caeca	0.71	0.72	0.09	0.7260
Colon	0.43	0.36	0.12	0.2267
Cloaca	1.45	1.04	0.60	0.1739
	Intestine lenghts, % LW			
Entire intestinal tract	10.09	10.13	0.71	0.5698
Duodenum	1.66	1.87	0.66	0.5227
Jejunum	2.74	2.63	0.76	0.7763
Ileum	3.26	3.24	0.71	0.9395
Caeca	1.44	1.50	0.25	0.6012
Colon	0.59	0.54	0.11	0.4522
Cloaca	0.35	0.34	0.13	0.8760

It is possible observe that the full digestive tract expressed as percentage of total live weight is higher in the hens fed on Hermetia illucens larvae meal (+ 1.64%) than the control group (Pvalue <0.05).

1- Small intestine(morphometry, brush border enzyme membrane activity, and ileal digestibility)

Table 7.19: results of intestinal morphometry evaluation in the three tracts of the small intestine was measured.

	HIML	**SBM**	**RMSE**	**P value**
Duodenum				
Villi height	934.2[a]	828.5[b]	105.4	0.0321
Crypt depth	153.7	157.8	25.56	0.6240
Villi/Crypt	6.07	5.25	0.67	0.0895
Jejunum				
Villi height	809.0[b]	946.3[a]	133.5	0.0294
Crypt depth	184.6	173.4	37.39	0.5494
Villi/Crypt	4.38	5.46	0.76	0.1204
Ileum				
Villi height	916.4[b]	1055.7[a]	91.30	0.0184
Crypt depth	205.4[a]	176.6[b]	17.23	0.0329
Villi/Crypt	4.45[b]	5.98[a]	0.29	0.0236

Table 7.19 reports the intestinal morphology evaluated on the three tracts of the hens small intestine. It is possible to observe that the laying hens fed HILM diet showed a higher villi height (P<0.05), the contrary was reported for the Jejiunum tract.
In the ileum, the villi height was higher in the SBM group, and the opposite was fo the crypt depth. Thus, the villi to crypt ratio was higher in the control group than the insect one.

Table 7.20: Border brush membrane enzymes activity

	HIML	SBM	RMSE	P value
Duodenum				
Maltase	9.86^{a}	7.13^{b}	2.55	0.044
Saccarase	1.71	1.83	0.57	0.679
IAP	6.83	8.19	2.97	0.360
GGT	0.205	0.184	1.91	0.492
Jejunum				
Maltase	8.93	6.41	3.14	0.129
Saccarase	3.22	2.30	1.54	0.252
IAP	5.40^{b}	8.30^{a}	2.49	0.036
GGT	0.198	0.229	2.61	0.466
Ileum				
Maltase	11.21^{a}	5.20^{b}	3.98	0.010
Saccarase	2.35^{A}	1.40^{B}	0.57	0.005
IAP	3.57^{b}	5.20^{a}	1.29	0.026
GGT	0.107^{B}	0.188^{A}	1.21	0.001

The activity of malthase, sacarase, intestinal alkaline phosphatase and -glutamiltraspherase determinated in different digestive tracts of laying hens are reported in Table 7.20.
In the duodenum, the malthase had a higher activity (P <0.05) in insect meal than the control group.
The alkaline phosphatase in the jejunum had a higher activity (P <0.05) in the SMB group than the HILM group.
In the ileum it is possible observe that thee are differences between groups for all the tested enzyme activities: maltase and saccarase had a higher activity in hens HILM group (P < 0.01), while alkaline phosphatase and

GGT had higher activity in the SBM group (P < 0.05 and P <0.01, respectively).

Table 7.21: Ileal apparent digestibility

	HIML	SBM	RMSE	P value
Dry matter	67.63b	70.27A	2.36	0.0421
Organic matter	69.17b	72.18a	2.24	0.0177
Protein	67.67B	78.29A	1.84	<0.0001
Ether extract	73.01	77.34	5.03	0.1071

The coefficients of apparent ileal digestibility of the nutrients according to dietary treatment of hens are reported in Table 7.21. The coefficients of apparent ileal digestibility of dry and organic matter resulted lower in the hens fed Hermetia illucens larvae meal (P <0.05), mainly due to the crude protein digetibility. The coefficicients of apparent ileal digestibility of ether extract was not affected by dietary treatment.

2- Large intestine

Table 7.22: Volatile fatty acids production

	HIML	**SBM**	**RMSE**	**P value**
	Absolute values, mmol/l			
Acetate	50.68a	37.25b	11.44	0.0290
Propionate	13.83	10.25	5.24	0.1802
Butyrate	6.86°	4.22B	1.47	0.0022
Isobutyrate	0.71	0.77	0.21	0.6541
Valerianic	1.50	1.22	0.46	0.2238
Isovalerianic	1.06	0.89	0.25	0.3832
Total VFA	74.59a	54.52b	17.38	0.0312
	Relative values, % of total VFA			
Acetate	68.50	68.22	5.07	0.9128
Propionate	17.92	18.51	4.27	0.7782
Butyrate	9.26	7.98	1.63	0.1276
Isobutyrate	0.96B	1.45A	0.27	0.0033
Valerianic	1.99	2.28	0.42	0.1764

Isovalerianic	1.38	1.72	0.47	0.1500

The production of volatile fatty acids measured in caecal content of the hens submitted to different dietary treatments is reported in Table 7.22. The total volatile fatty acids production resulted higher (P <0.01) in the insect group, mainly due to higher amonut of acetate and bytyrate (P <0.05 and <0.01, respectively). When the volatile fatty acids were expressed as percentage of total VFA production, there are no differences between the experimental groups, with the exception of isobutyrate which showed a higher proportion (P < 0.05) in the control group.

Discussion

There are not many studies already published on the use of the insect meal in laying hens. In the present trial it was possible to observe that there are important differences not only for the macro nutrients, but also for the mineral and the amminoacids between the control and the insect diet (all these are very important for the poultry production).

The *Hermetia illucens* meal showed a higher content of calcium (+2.46), lysine(+1.40), methionine(+2.17), isoleucine(+1.35), valine(+2.38) and threonine(+1.35) times than the soybean meal. The sum of methionine and cysteine resulted similar in both the protein sources. At the contrary the soybean meal is richer in tryptophan (+2.33) than *Hermetia illucens* larvae meal. *Hermetia illucens* larvae meal diet is mostly distinguished for a higher content of lauric acid (C12:0) than the soybean meal diet which in turn contained more C18:1-n9 and C18:2-n6. Insect meal is overall characterized by +8% of saturated fatty acids (SFA) and -6% of polyunsaturated n6 fraction (PUFAn6), whilst PUFAn3 was slightly higher in insect meal than in soybean meal. The Hermetia illicens larvae meal and both the diets did not contained cholesterol.

The levels of nutrients for both diets are adequate for satisfied the requirements of Lohman brown classic from the 26 to 45 weeks of age in accoding to Lohman classic brown guide (2011). Productive performance of laying hens fed Hermetia illucens larvae meal based diet were lower than that of hens fed the control diet.

The results reported by Maurer et al. (2016) are not in line with our results; these authors showed that the egg production, feed intake, and feed conversion ratio (FCR) of Lohmann Leghorn laying hens fed diets in which Hermetia illucens replaced (at inclusion of 50 and 100%) the soybean cake, were unaffected by the dietary treatments. In addition, Maurer et al. (2016)

carried out a trial on the later laying period (indeed started the experimental period from 64 weeks of age of hens) and lasted for a shorter time respect to our one (10 weeks). Probably, the productive performance of laying hens fed Hermetia illucens larvae meal resulted lower because the feed intake was lower than the hens fed the control diet.

Althought both the diets contained the same amount of metabolize energy, ADF and protein, had a similar particle size, and the hens were located in the same building and, as a consequence, they were submitted to the same temperatures and humidity, other factor can affect the feed intake. It was described by Ferket and Gernat (2006) and by Tabeekh et al (2015) that in the poultry also the the feed color can affect the feed intake.

In the literature there are conflicting reports in feed color preference of poultry (Hess and Gongil, 1956; Capretta, 1969; Cooper, 1971) this was elucidated by Kennedy (1980) who concluded that all chicks showed a preference for diets of the color was fed after hatching.

Tabeekh et al. (2006) observed that the chickens showed a tendence to decrease the feed consumption of colored diet. The conventional diet used for the laying hens are based on corn and soybean, and the ingredients used for the control diet are the same thus the color of our control diet was similar to that of a standard diet for hens. Instead the Hermetia illucens larvae meal had a darker color than the soybean meal (the color of hermetia illucens meal is brown). Also the odor could be affect the laying hens feed intake, and the poultry tend to avoid the inusual ingredient than the conventional (Esmail, 2013).

The feed intake of Lohman Brown classic from 26 to 45 weeks of age was 117g/d/ hens (Lohman Classic Brown Managment Guide, 2011), this value was higher than the hermetia illucens larvae meal group, and lower than soybean meal group.

The higher feed intake of the control group affected the weight gain and, as a consequence, the final weight of hens. At 45 weeks of age (at the end of the experimental period) the average weight of the laying hens fed hermetia illucens larvae meal fell in the range reported by the Lohman classic brown Guide (2011), instead the hens fed soybean meal were of little oversized.

The feed conversion ratio (FCR) resulted more favorable for the insect meal group, indicating a greater use of feed nutrients; such hypothesis is also confirmed by the average laying percentage along the trial of hermetia illucens larvae meal that was not much lower than the conventional one given by the breeder for Lohmann Brown classic in the same period (92.36 % vs 91.94 %, for insect group and soybean group respectively). Moreover,

the average egg weight of hens fed insect meal was 59.91 g (-2.9% of the hens fed soybean meal), and are comparable to the average weight indicated from the Lohmann standard along the same lay period (59.57 g).
Also, the egg size of hens fed Hermetia illucens larvae meal showed a various range of weight variability as included all the 4 examined weight classes while this did not happen for the control group in which XL class of egg was not represented.
Leeson et al. (2011) described that the protein intake represents the most important factor affecting the egg weight, and as a consequence the size class.
More than the protein amount, methionine (Sohail et al., 2002), linoleic acid (Harms & Russell, 2004), and fat (Grobas et al., 1999b,1999c) are three important factors that can affect egg size.
Keshavarz (2003) observed that, by reducing dietary level of protein to 13% and omitting supplemental methionine from the diet, egg production, egg weight, egg mass, feed consumption, body weight gain, and extra-large plus large-sized eggs were reduced and feed conversion and the number of small plus tiny sized eggs increased. Accordingly, a reduction in the levels of these factors in the diet may decrease egg size and increase eggshell quality. Keshavarz (2003) pointed that reducing the dietary methionine level in laying hens from 54 to 72 wk of age from 0.36 to 0.23% decreased productivity, including lower egg production and smaller egg size.
Jackson *et al.* (1987) observed that egg weight was reduced and shell strength increased by reducing dietary methionine. However, these changes were obtained at the expense of lower egg production.
Nassiri Moghaddam et al. (2012) carried out an experiment to determine the effects of different levels of methionine, protein and tallow on productive performance and egg quality of laying hens in the late phase of production. A completely randomized design with a 3×2×2 factorial arrangement, with three levels (0.34, 0.31, and 0.27%) of methionine, two levels (12.8 and 14.7%) of protein and two levels (1 and 3%) of tallow with constant level of linoleic acid (1.55 ± 0.02%), was used.
Productive performance and egg quality were not affected by 12 and 20% reduction of protein and methionine, respectively. It seems that decreasing the levels of methionine and protein to lower than the recommended values can decrease egg weight without negative effects on productive performance and egg quality of laying hens in the late phase of production.
The hens fed insect meal showed a lower feed intake affected the protein intake, even if the amount of protein in the HILM diet was appropriate for

the laying hens (17.91%) to produce eggs of Lohmann classic brown hens, the daily feed consumption was from 105 and 110 g during the experimental period (Lohmann Brown Classic Management Guide, 2011). The daily crude protein intake of the laying hens was 19.33 g (0.16 g of sulphoric aminoacids) for the animals fed on Hermetia illucens larvae meal and 22.64 g (0.19g of sulphoric aminoacids) for the hens fed the conventional diet. Because the feed intake of the hens fed on insect meal based diet reported a lower feed intake than the ones fed the conventional diet, the hens of insect group ingested a lower amount of protein and as a consequence lower amount of aminoacids.

During the trial there are not mortality or clinical signs of morbidity (such as diarrhea), and the absence of weight loss, indicated that the Hermetia illucens larvae meal diet was not responsible of negative effects on laying hen health status.
The results of blood analysis showed that the most of the criteria are similar in both the experimental groups. In order to evaluate the body condition of poultry, the total protein and the albumin represents significant criteria, according to Piotrowska et al. (2011), because the plasma protein plays an important role in body homeostasis maintenance, and in according to Filipovis et al. (2007) the albumin represents the most favorable source of aminoacids for the protein synthesis. The albumin and the total proteins were similar in the two groups.
The laying hens fed Hermetia illucens larvae meal showed a higher globulin level and as a consequence the albumin to globulin ratio was lower than the control group. It is describerd (Griminger and Scanes, 1986) that the high globulin concentation in the blood and the low albumin/globulin ratio represent indicators of better disease resistance and immune response in the birds.
The insects such as Hermetia illucens and Musca domestica larvae in order to defend themselves from possible pathogens in organic waste and manure where they are rearing, may produce antimicrobical peptides (Ratcliffe et al.,2014) and these molecules also can be useful for the immune system of the poultry (Veldkamp and Bosh, 2015).
Also in our previous trial on the broiler a similar result on the albumin to globulin ratio was obtained.
This could be attributed to the chitin amount in the diet that standing our calculation was 5.55% as fed, in line to the Finke (2013) estimation. Thus,

considering the aveage feed intake of the laying hens fed HILM diet, the amount of chitin ingested per day by the birds was 1.02 g.
Also the lower level of serum cholesterol and triglycerides showed in the insect group could be attributed to che chitin content of Hermetia illucens larvae meal, in according to Hossain and Blair (2007). These authors included a commercial chitin (derived from crustacean shell waste was found to contain 373 g crude protein, 265 g ash, 23.5 g ether extract, 130 g calcium and 16.4 g phosphorus per kg, on an air-dry basis) in broiler diet from one to 21 d of age at zero, 25, 50, and 75 g/kg of diet and described a cholesterol and triglycerides reduction in the blood at all levels, but more consistent at 50 g/kg of inclusion. The reduction of cholesterol and lipid level in the blood could be explained according to Prajapati and Patel (2010) who observed that the chitin is positive charged and could be able to attract the bile sand free fatty acids (negatively charged).
The major poultry nitrogenous waste product is the uric acid (Harr, 2002), and the level of this parameter in the serum is affected by the protein catabolism. In this study the serum levels of uric acid are similar in both groups, despite the different protein intake in the HILM (19.35 g/d) and SBM (22.60 g/d) groups.
Another important indicator of the protein metabolism is the creatinine (Piotrowska et al., 2011), that is derived by the phosphocreatine metabolized in the skeletal muscle; the diet, the physical activity, the age, the muscle mass affects the blood level of this parameter as described by Wyss and Kassurah (2000) and Rajman et al. (2006). In this study, the creatinine level in the serum resulted lower in the hens fed HILM diet than the control group, and considering that the birds had the same age, physical activity and muscle mass, the main factor affecting the seric level of creatinine is the diet. The lowest level of creatinine showed in hens fed Hermetia illucens larvae meal it is probably due to the lowest protein intake in birds of this group; The creatinine is not only considered an index of protein metabolism but also of the renal function. There are no clear studies regarding the effect of chitin on renal function, but some authors (Jing et al., 1997; Davis et al., 2003; Ahmed et al., 2014) reported that chitosan (produced by chitin deacetylation) administrated to rats and dogs is responsible of the reduction of the creatinine levels in the serum.

Egg quality

While the total amount of crude protein, the essential aminoacids and the total lipids are relatively consistent in the eggs, other components such as

fatty acids composition, vitamins, cholesterol, carotenoids and antioxidant (such as tocopherols) are influenced by the dietary treatment of the hens and are more variable (Shin et al., 2013).

There is a limited information on the use of insect meal, in the diets of lying hens and especially for chemical composition of eggs. According to Makkar et al (2014) most of the experiments published to date indeed have been focused on laying performance, weights of eggs components, color, and rarely fatty acid composition.

Generally, many works remark that meal from H. illucens (Al Qazzaz et al., 2016) and Westwood (Cirina forda)(Amao et al 2010) can be good sources of protein in layers diet at different substitution levels (up to 75% obtained with Westwood larvae) without adverse effect on yolk weight (Agumbiade et al., 2007; Amao et al., 2010) while the albumen weight was shown to be significantly reduced by 50% of Westwood meal substitution whereas only a slight decreased was obtained with a total replacement. Present results confirmed that albumen appear the egg component most susceptible to the new protein sources.

Data of previous research presented no effect (Agumbiade ae al., 2007; Amao et al., 2010) or slight discoloration when 5% of H. illucens was introduced in Arabian strain, however different color evaluation methods were utilized so data cannot be comparable. In both cases, authors justify this pattern to the fact that insect are not vegetables, which would contain carotene or xanthophyllous pigments need for egg coloration development.

Results on Table 13 instead disagree with previous funding, being HILM group significantly redder. Furthermore, our data found an explanation in total carotenoid content obtained for both insect and soybean meals. The yolk of the hen fed insect meal indeed were found to be rich in β-caroten and lutein, pigments responsible for an orange-yellow color. Both pigments, as retinol and zeaxanthin, as whole are transferred to the egg against a concentration gradient so they can accumulate at higher levels in the egg than the availability in the feed would predict.

In according to Moreno et al.,(2016) both meals and yolks carotenoids composition the discrepancy from the first and the latter clearly emerged, especially for zeaxanthin which doubled in the insect group and increases 7 times in the hens fed conventional diet . Egg yolks are known to contain predominantly lutein and are poor sources of pro-vitamin A molecule, such as β-caroten, presumably because it is utilized by the hen (Surai et al 2001) or more efficiently stored in liver (Moreno et al., 2016) and carotenoid profiles here presented are in agreement. Looking at the accumulation

patterns, both lutein and zeaxanthin are transported against a concentration gradient in both groups, notwithstanding HILM seems to accumulate in a shallower way that SBM suggesting that these carotenoids are less efficiently assimilated from H. illucens than soybean meal. Different mechanism and context (eg. competition, matrix effects or bioavailability) have been demonstrated to affect carotenoids transfer and absorption (Moreno et al.,2016) and the presence of α-tocopherol in feed is one of these (Islam et al.,2016). The presence of a double α-tocopherol quantity in soybean meal compared to the insect meal could be responsible for a higher accumulation pattern in SBM than HILM eggs as much the dietary α-tocopherol have been proved to enhance bioavailability of lutein. Nevertheless, in the context of human health (Islam et al 2016), eggs from birds feeding with H. illucens meal resulted a better source of carotenoids, especially lutein, which play a role in the prevention of cataract and age-related macular degeneration, as well as heart disease, stroke (Ribaya-Mercado et al., 2012) and in improving cognitive function in elderly (Jonson,2012).

Grobas et al. (2001) reviewed whether crude protein and metabolized energy levels in laying hens affected proximate eggs composition in relation on the species, however limited information was available on the effect of protein sources on egg composition.

Our results showed that dietary treatments had no effects on the main constituents (such as crude protein, essential aminoacids and fat) of eggs, so the total replacement of soybean meal with Hermetia illucens larvae meal could be suitable. An in-deep view on composition was considered necessary for confirm this assertion, so fatty acid composition of both meals and yolks were analyzed. The lipid content of insects is largely dependent on their diets and stage of development.(Oonincx et al 2015)(Mustonim et al 2015) The lipids of H. illucens larvae fed on cow manure contained 21% of lauric acid, 16% of palmitic acid, 32% of oleic acid and 0.2% of n-3 fatty acids, while these proportions were respectively 43%, 11%, 12% and 3% for larvae fed 50% fish offal and 50% cow manure.(St-Hilaire et al 2007) C12:0, C16:0 and C18:1-n9 were however reported to be the most abundant fatty acid of black soldier flies(St- Hilaire et al 2007;Oonincx et al 2015) and present results are in agreement. Fatty acid composition of the two experimental diets have been influenced by their ingredients, in particular soybean is responsible for the high content of linoleic acid of SBM diet whereas the presence of a higher quantity of maize grain might explain the difference between the fatty acid composition of H. illucens larvae meal diet.

Modifications of the fatty acid composition of egg yolks can be obtained by feeding different level and type of lipid sources. (Grobas et al, 2001; Pal et al., 2002; Naiet et al 2016). After feeding cod liver oil in comparison with pumpkin seed oils diets, a significant higher content of n3 fatty acids and lower n6 values were observed in yolks (Neijet et al., 2016). Moreover, dietary intervention with olive oil increases MUFA content of the yolk, especially of oleic acid whereas linseed oil increases the level of n3 fatty acid, especially C18:3n3. However, not all the differences between fatty acid profile in yolk can be attributable to dietary components and present results are in agreement to Grobas et al. (2001). First of all, C12 drastically decrease in hens fed hermetia illucens larvae meal diets (HILM) yolks even if it was abundantly contained in the HILM diet.

In turn, C16 and C18 in yolks almost doubled compared with meal contents, regardless the diet utilized. In addition, it is mostly known that laying hens have remarkable desaturase and elongase activities which transform C18:2-n6 and C18:3-n3 in their derivatives (Grobas et al.,2001). Specifically, it seemed that the most of the increase in n6 fatty acids, especially C20:4-n6 and C22:5-n6, as well as increases in C20:5-n3, C22:5-n3 and C22:6-n3 can be due to small increases in the dietary concentration of C18:2-n6 and C18:3-n3, respectively, which enhance enzymatic activities(Grobas et al 2001) Hence, considering the lower C18:2-n6 content in HILM diet together to the higher values of the same fatty acid found in HILM than in SBM eggs and significant lower values of its derived fatty acids, C20:3-n6 and C22:5-n6, it seems possible to speculate that enzymatic activity of SBM group was slightly reduced. Again, as found for carotenoids, the overall fatty acid composition of HILM yolks was broadly comparable with the control group)

In the present study, despite the absence of cholesterol in the two experimental meals, cholesterol was found in a concentration fitting the proposed value (15 g kg-1),(Han et al., 1993) thus confirming that cholesterol levels in the hen result from a balance of dietary intake, biosynthesis, and excretion of cholesterol and cholesterol by-products (Sutton et al., 1984). Interestingly, HILM presented a cholesterol depletion which need further investigations in order to understand the possible metabolic role of insect meal.

Baumgartner et al. (2008) described that chicken eggs supply about 30% of dietary cholesterol in the American diet the public's perception of the role of eggs in the diet has changed over the past 50 years. Eggs are considered to be an important contributor to high serum cholesterol levels and an increased risk of cardiovascular disease in according to Simcic et al. (2009).

In addition, egg consumption per capital has been declining in many countries because of concerns associated with cholesterol (Suk and Park, 2001).

In according to Some research has indicated that the cholesterol content of eggs is influenced some factors such as genetic factors (Shafey et al., 1998), dietary composition (McNaughton, 1978; Liu et al., 2010), laying intensity (Mahmoud et al., 2010), layer age (Hall and Mckay, 1993), and medical treatment (Simcic et al., 2009). McNaughton et al. (1978) carried out two experiments to determine the effect of dietary fiber source and level on egg yolk, liver, and plasma cholesterol concentrations of White Leghorn laying hens. In the first experiment, dietary fiber levels of 2.05, 4.41, 6.68, and 8.79% furnished mainly by sunflower meal were fed to laying hens for 140 days.

In the second experiment, alfalfa meal, ground whole oats, sunflower meal, rice mill feed, or wood shavings was added to a corn-soybean meal basal diet to furnish 2.00% added crude fiber and fed to laying hens for 84 days. The authors described that yolk cholesterol decreased in the hens fed crude dietary fiber levels of 4.41, 6.68, and 8.79%, the hens as compared to a corn-soybean meal basal diet containing 2.05% crude fiber.

Egg yolk cholesterol was significantly decreased by feeding alfalfa meal, oats, sunflower meal, rice mill feed, or wood shavings to laying hens when compared to yolk cholesterol of hens fed the basal diet.

The greatest reduction in egg yolk cholesterol was found by feeding either oats or wood shavings. No significant differences were found in plasma cholesterol due to dietary fiber level.

Plasma triglycerides decreased and liver cholesterol increased as dietary fiber level increased in diets fed to laying hens. When laying hens were fed alfalfa meal, oats, rice mill feed, or wood shavings, plasma cholesterol significantly decreased.

The chemical structure of the chitin can be comparable to the cellulose, and consider for this reason as “fiber”; in our trial the chitin content of the insect meal diet was 5.40 % (as feed), our “fiber” content was in according to the ones showed by McNaughton et al. (1978). The chitin content could be affect the seric and yolk cholesterol content.

Our results showed that the seric cholesterol content is lower in the hens fed insect meal than the ones fed soybean meal (Pvalue <0.05), also the cholesterol amount in the eggs showed that the eggs produced by the hens of the insect group il lower than the control group.

Intestine

In the present trial, the weight and the lenght of the different tracts of the small intestine are not affected by the dietary tractments. In literature there are no many studies on this regard and the object of the studes are birds in growing.
Ballitoc and Sun (2013) observed that in the broilers fed Tenebrio molitor larvae meal up to 10% of inclusion level the small intestine increased in weight and in length compare to the control group while no differences are described in the other intestinal tracts. In this thesis previously was described the use of Tenebrio molitor larvae meal in the broiler in total replacement of soybean meal, and are described a greater full intestine length and weight and a greater length and weight of the ileum and ceca (Bovera et al.,2016). The absence of the effects on the intestine length observed in the present research can be attributed to the age of the hens. In according to Uni et al. (1995, 1996) the development of the digestive system in the birds (including villi size and area) is very quick in the first two days after hatch and reach a plateau at 5-10 days of age. After this period the intestinal tracts as a growth similar to the increase of the live body weight until somatic maturity (Uni et al.,1999). The gastrointestinal tract of the hens, in that trial, has been measured at 45 weeks of age when the animal growth and as a consequence, the development of the intestine are completed.
The production of the caecal volatile fatty acids was affected by the dietary treatment, indeed the hens fed HILM diet showed an increase of 36.8% of total production of VFa compared to the soybean meal group. The acid acetic increased by the 36.1% and the butyric acid resulted increased oh 62.6% than the SBM group. In the broiler fed Tenebro molitor larvae meal, described previously, the total amount of volatile fatty acids increased of 45.6%, the acid acetic of 45.6% and the butyrate of the 40.3% than the broiler fed soybean meal (Loponte et al.,2016).
In according to Barnes et al. (1979) the high concentration of volatile fatty acids are ascribed to the fermentation produced by the obligate anaerobic bacteria.
The butyric acid is necessary for the development of the Gut Associated Lymphoid Tissue (Mroz, 2005), and it is also reported by Bovera et al (2010) that is the prime enterocytes energy source; and is considered the major intestinal energy source even when other fuel sources, such as glucose and glutamine, are available, and is described that could stimulate the development of the colon-rectal and ileal mucosal cells (Montagne et al.,2003; Topping and Clifton, 2001). The butyrate is important not only the

mantaining of the caeco-colon function (as described to Montagne et al.,2003) but for the full gastrointestinal tracts.
The higher amonut of butyric acid is responsible of the increase of nutrients for the eneterocytes intensify the blood flows throught the gastrointestinal tract and as a consequence increase the tissue oxygenation and the nutrient transport and absorption (Mahdavi and Toki, 2009). In according to Mroz et al (2005) the mechanism of action may enhances local neural networks as well as chemo-receptors together with direct effect on smooth muscle cells. Saemann et al. (2000) described that the butyric acid has anti- inflammatory property, and it can play a key role in the regulation of the immune response; there are not so much studies available in literature that demonstrate the association of butyrate with immune and anti-inflammatory response (Khempaka et al., 2011).
The increase of volatile fatty acids has a bacteriostatic effect on some enteric pathogens bacteria (such as *Salmonella thyphimurium*), and they not inhibit the development of Lactobaccillus (beneficial gastrointestinal bacteria) in the chickens (Van der Wielen., 2000). Moreover, Van der Wielen et al (2002) described that there is a negative correlation between acetate levels and the Enterobacteriacee colonization (such as Salmonella spp.).
Also Nisbet et al (1996) reported a negative correlation between the proprionate levels and CFU of Salmonella in young chickens. Van der Wielen described also that the increase of butyrate was correlated to the decrease of Salmonella colonization.
The higher concentration of butiric acid in the laying hens fed Hermetia illucens larvae meal than the soybean group, could be related to the mechanism that inhibit the Enterobacteriacee colonization.
In literature are not available previously studies that demonstrate that the hens fed insect meal diet has a positive effect on intestinal microbiota and on the production of volatile fatty acids, but the results of the present trial could be due to the chitin amount fed by the laying hens of the HILM group, and this results can be ascribed to the probiotic activity of the chitin, such as described also in the previous trial of this thesis (Bovera et al 2015 a and b). At the moment are in course studies at the Department of Veterinary Medicine and Animal Production of the University of Napoli “Federico II” on the possible changes of microbiota on the laying hens used in this trial.
Other authors (Khempaka et al, 2011) showed that the inclusion of shrimp head (another chitin source) at 15 an 20% as fed, as well as the inclusion of 1.9% of purified chitin in broiler diet is responsible of the increase of the butyrate production in the caeca.

In addition, the highest butyric acids amount in the caecal content of the laying hens fed hermetia illucens larvae meal diet had contradictory effect on the different tract of the small intestine. In the duodenum it is described that has a positive effect on highest villi weigh and can explain the highest maltase activity. In the ileum (the longest tract of the mass intestine) it is reported a negative effect on villi height and decreased, as a consequence, the villi to crypt ratio and the activity of alkaline phosphatase indicating a reduction of absorption surface in according to Han et al (2012), but is reported also a positive effect on the activities of maltase and sucrose. Brush border disacharase (maltase and sucrose) represents the small intestine digestion of the starch (Nichols et al.,2003), and the increased of their activity is ascribed to an improvement of utilization of the nutrients (Han et al.,2012). In addition, some author (Svihus et al.,2004; Zimoja and Svihus, 2009) showed that the ileum plays a key role in starch digestion and absorption in fast growing broiler.
The activity of alkaline phosphatase resulted higher in the Jejiunum and ileum of laying hens fed the soybean meal diet. Vaishnava and Hooper (2009) demonstrated that the alkaline phosphatase suppress the inflammatory responses from the host that may induced by the LPS (lipopolysacharide) from the beneficial bacteria and promote several beneficial effect to improve the intestinal health, the regulation of calcium absorption, modulation of intestinal bacteria growth(Alam et al., 2014; Brun et al.,2014; Malo et al .,2014);in addition, in according to Sabatakou et al (2007) the alkaline phosphatase is considered a marker for crypt and villus differentiation in the poultry.

The studies previously published on intestinal changes in function of the diet are concentrated on broiler post-hatch chickens, and for this reason it is difficult cooperate the results reported in this trial with the others (Iji et a.,2001).
In the ileum is also reported a reduction of gamma glutamil transpherase of hens fed Hermetia illucens larvae meal, this results can be ascribed at a significant decrease of ileum villi height. Ferraris et al (1992) reported that Gamma glutamil- transpherase in the mouse has a lower activity in the crypt and the activity increased up to the villus, the gammaglutamil transpherase activity is stimulated by dietary protein.This enzyme, in addition, improve the aminoacids transport in the intestine(Smith et al.,1991; Cotgrave and Scuppe-Koinstinen, 1994). The lowest amount of gamma- glutamil transpherase showed in the ileum of the hens fed Hrmetia illucens larvae

meal can be ascribed to the lower protein availability for the digestion observed in this tract of the small intestine.

Conclusions

Defatted *Hermetia illucens* larvae meal could be an interesting protein source for laying hens, able to sustain the egg production without negative effects on animal health and enhancing immune status of birds. On the other hand, when used as complete replacement of soybean meal, it negatively affected feed intake and thus production performance of hens even if feed conversion ratio of insect diet was more favourable than that of soybean diet. This can be ascribed to the darker colour of the insect meal as compared to soybean. Possible solution to this problem could be to use lower percentage of *Hermetia illucens* larvae meal inclusion in hen diet or to feed chick with insect meal starting from the first day after hatching.

The yolk composition was positively affected by the Hermetia illucens larvae meal and all the analytical parameters resulted similar or better performance than the hens fed soybean meal based diet. The yolk of the hens fed insect meal resulted rich in in PUFA, n-6, n-3, and showed lower cholesterol content that the group fed soybean meal diet. The specific role of the protein sources on the metabolic pattern of laying hens need more studies.

The total replacement of the soybean meal of with Hermetia illucens larvae meal showed a negative effect on nutrient digestibility and in particular on the protein. The enzymatic activity in the small intestine and probably the microbiota in the caeca were modified due to dietary inclusion of insect meal and these changes were positive in terms of enhanced production of butyric acid in the caeca , or negative as the reduction of some enzymatic activities in the ileum.

Further studies are needed to find out the optimal inclusion level of Hermetia illucens larvae meal to balance the negative effects of feed intake and nutrient digestibility, and the positive effects on the digestive system of the laying hens, and on this purpose is in course another trial to investigate the partial inclusion of Hermetia illucens and Tenebrio molitor (at 25 and 50% of inclusion) in laying hens.

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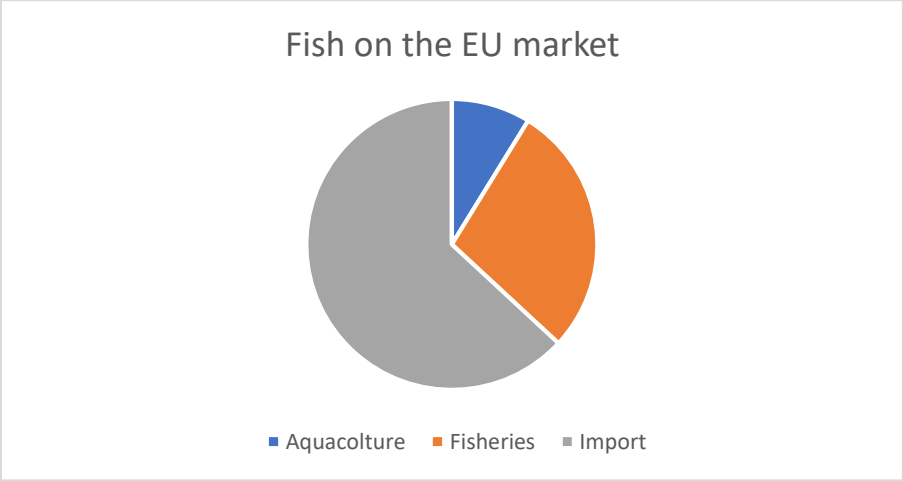
VIII
The use of feather meal, offal meal and blood meal as possible alternative source to fish meal in European sea bass

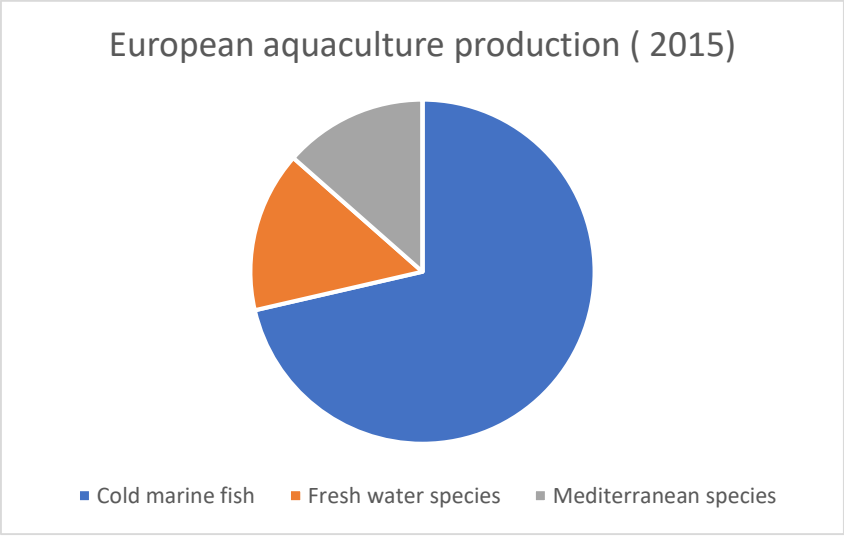
The fish present on the market may come from aquaculture or from capture. The capture fishery activity impoverishes natural resources and affect marine biodiversity, for these reasons the aquaculture in the next years will play a key role in sustainable fish production both from the environmental and economic point of view. Considering that world human population will continue to increase in the next future and in about 30 years it will reach 9 billion, the demand for protein sources of animal origin is expected to increase, and it is estimated that the demand for fish will increase by about 75% by 2050 (FAO, 2016). On the other hand, a significative amount of the fish caught annually is used for fishmeal and fish oil production and most of them are used as ingredients destined to aqua feeds formulations. Fishmeal and fish oil in fact are still considered the most nutritious and digestible ingredients for farmed fish feeds. To offset their high prices, as feed demand increases, the amount of fishmeal and fish oil used in compound feeds for aquaculture has shown a clear downward trend, with their being more selectively used as strategic ingredients at lower concentrations and for specific stages of production, particularly hatchery, bloodstock and finishing diets (FAO, 2016).
The FEAP annual report (2016) described that in the 2014 were present 12.26 million tons of fish product (capture and aquaculture) on the European Union market. It is observed that 2.16 million tons of fish produced were exported; the aquaculture produced the 8.8% of fish on the EU market. The fish provided by capture fishery represented the 28.1%(4.5 million tons) of the fish present on the European market. The 63.1% of the fish were imported (9.1 million of tons).

Graph 8.1: Data on the fish product present on the European market (2014) as previously described are resumed in the following graph (%).



In 2014, the consumption of fish in the European Union was increased by about 80,000 tons, compared to 2013 (+0.7%) (FEAP, 2016). Based on these data, it is possible to deduce that in the European Union 24.4 Kg fish per capita are consumed (FEAP,2016). The major external suppliers of European Union are the Norway (the EU imports from the Norway have increased by 70% for the last 8 years) and the China (EU Fish Market, 2015). During 2015 the European aquaculture production increased by 0.4% (for a total of 2.35 million tons) compared to 2014. In the same year, 1.67 million tons of cold water marine fish were produced, fresh water species represented the 15.1% (0.35 million tons) and Mediterranean species were 0.31 million tons (gilthead seabream and European seabass in particular, but also other species as meagre, sole and turbot (FEAP, 2016)).
The data about the European aquaculture are resumed in the Graph 8.2.

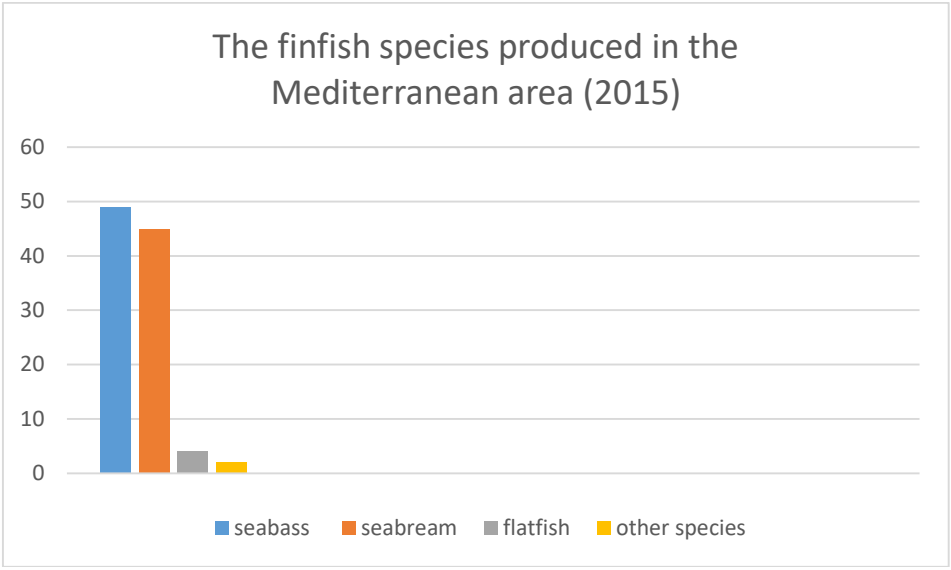
Graph 8.2: European aquaculture production (2015).



The most important producer country in Europe is Norway (58% of total production). United Kingdom, Greece and Turkey produced about 0.1 million of tons annually each.

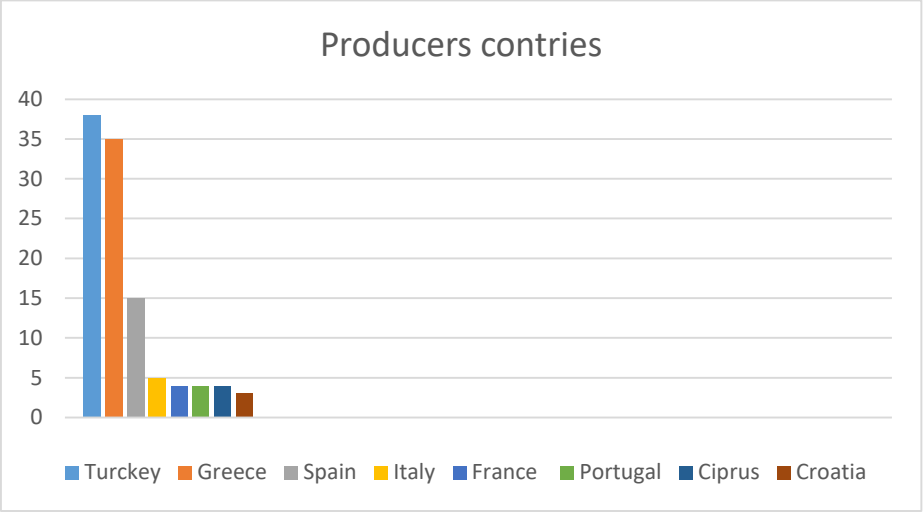
The main species produced are represented by the European sea bass (*D. labrax*, Linnaeus, 1758) and gilthead seabream (*Sparus aurata*; Linnaeus, 1758), indeed the production of these two species was 0.3 million tons during the 2015 (FEAP, 2016).

Graph 8.3: the main species produced during the 2015 in the Mediterranean area.



The most important producers of southern Europe are Turkey (38%) and Greece (35%), followed by Spain (15%) and Italy (5%). The four countries previously cited provided the 94% of the production (FEAP, 2016). France, Cyprus and Portugal during the 2015 produced the 4% of fish and Croatia the 3% of the fish produced in the Mediterranean area (total tons produced were 317,029, with an increase of more than 1.6% compared to 2014).

Graph 8.4: Producers countries.



It is well known that fish has a high nutritional value, in fact it is an excellent protein source, provides omega 3 polyunsaturated fatty acids, it is a good source of macronutrients and micronutrients (iron, zinc, iodine, magnesium, calcium) and vitamins; in fact, it is rich in vitamins of group B and D (vitamin / hormone essential for calcium metabolism).
To produce good quality final products, fish must be fed with good quality feeds, the protein sources employed must have a good quality, an optimal amino acid profile for the bred species, good palatability (In this regard, free amino acids seem to play a crucial role in fish) and must not contain antinutritional factors that may adversely affect digestibility (Barrows et al, 2008).
The major protein sources used to product fish feed are fish meal and soybean meal.

Aim of the trial

Aim of this trial was to evaluate the effects of fish meal substitution with feather meal, alone or in combination with two other PAPs (blood meal and offal meal), on growth performances, diet digestibility, somatic indexes and fish chemical composition in European sea bass (*Dicentrarchus labrax*) juveniles.

Material and methods

Three experimental trials were carried out in the facilities of Hellenic Centre for Marine Research (HCMR)-Aqualabs of Heraklion, Crete (Gr).

Trial n. 1 digestibility trial

In a first separate trial, 105 European sea bass juveniles (average BW 150 g) were randomly allocated in 21 conic fiberglass 250 L tanks. The tanks were equipped with a faeces collecting tube and were inserted in a flow-through sea water tanks system. Seven experimental diets were formulated using a diet formulator. The diets were isoproteic (45% of crude protein) and isoenergetic (20.6 MJ/Kg). The control diet (0%) contained fish meal as the sole protein source. The diets 15% FEM, 25% FEM, 50% FEM and 75% FEM presented a progressive increase of feather meal in fish meal substitution. In the diets 50% BOF and 75% BOF, 50% and 75% of fish meal respectively was replaced by a mix of feather meal, blood meal and offal meal in equal parts. The 21 tanks were randomly assigned in triplicate to each experimental diet. The experimental diets were formulated and constituted in the aquaculture laboratory of the HCMR using a meat maker and dried in an oven at 40°C for two days. In the diets was included Celite® 245 (Sigma Aldrich) at 1% as external indigestible marker.

In table 1, the nutritional values of the alternative ingredients used to formulate the experimental diets are reported. The values are the average values provided by the producer (Soleval, Janeve, France).

Table 8.1: Chemical composition and aminoacidic profile of blood meal, feather meal and offal meal.

Chemical composition	blood meal	offal meal	feather meal
DM%	4	4	6
Ash%	2	16	3
CP%	91	65	85
Lipid%	0	12	6
Aminoacidic profile (% protein)			
Alanine%	6.68	4.36	3.89
Arginine%	4.57	4.35	5.84
aspartic acid %	8.57	4.89	5.66
Cysteine %	1.26	0.65	4.09
glutamic acids%	9.02	7.72	9.32

Histidine%	4.96	1.25	0.7
glycine%	3.5	7.31	6.41
Isoleucine%	3.03	2.02	3.98
Leucine%	9.71	4.02	6.75
Lysine%	7.51	3.68	1.85
Methionine%	1.09	1.18	0.52
Phenylalanine%	5.61	2.25	4.09
Proline%	3.16	4.52	7.63
Serine%	3.73	2.66	9.56
Threonine%	4	2.34	3.98
Tryptophan%	1.8	0.47	0.57
Tyrosine %	2.76	1.84	2.09
Valine%	0	2.51	5.98

In table 8.2 are reported the ingredients, chemical composition and amino acid profile of the experimental diets.

Table 8.2: Ingredients, chemical composition and aminoacidic profile of the experimental diets.

Ingredients (g/Kg)	**0%**	**15% FEM**	**25% FEM**	**50% FEM**	**75% FEM**	**50% BOF**	**75% BOF**
blood meal	0	126	150	295	435	101	152
offal meal	0	0	0	0		102	152
feather meal	0	0	0	0		102	152
corn starch	280	278	270	278	260	242	235
mineral premix	15	15	15	15	15	15	15
vitamin premix	25	25	25	25	25	25	25
Methionine	0	0	0	2	5		5
Chemical composition							
DM%	90.24	90.20	90.29	89.93	88.86	88.72	88.81
Ash%	8.38	7.40	7.20	6.06	5.95	6.20	7.27
GE MJ/kg	19.85	20.03	20.26	20.17	20.38	20.90	20.52
DE MJ/kg	14.39	13.94	14.10	13.22	12.86	14.48	14.46
CP%	45.02	45.07	45.04	45.04	44.93	44.96	45.07
Dig CP%	39.40	38.64	38.47	37.56	36.59	37.48	38.19

Lipid%	15.23	14.98	15.73	14.28	14.85	15.26	15.43
Fibre%	1.79	1.56	1.51	1.27	0.99	1.26	1.43
Aminoacidic profile (% of protein)							
Arginine%	3.73	3.6	3.57	3.38	3.16	2.98	3.25
Histidine%	1.14	1.01	0.99	0.84	0.7	1.26	1.23
Isoleucine%	2.15	1.72	1.63	1.14	0.64	1.03	1.41
Leucine%	3.58	3.71	3.73	3.88	3.98	4.16	3.98
Lysine%	3.89	3.43	3.34	2.81	2.28	3.12	3.38
Methionine%	1.35	1.15	1.11	1.06	1.1	1.17	1.38
Phenylalanine %	1.94	2.04	2.05	2.16	2.23	2.33	2.21
Threonine%	2.29	2.21	2.19	2.09	1.97	2.08	2.15
Tryptophan%	0.53	0.48	0.47	0.42	0.36	0.48	0.5
Valine%	2.42	2.88	2.96	3.49	3.96	3.24	2.97
M+C%	1.88	1.99	2	2.12	2.22	1.7	1.76
P+T%	3.41	3.53	3.55	3.68	3.77	3.69	3.6
M+C%	1.88	1.99	2	2.12	2.22	1.7	1.76
P+T%	3.41	3.53	3.55	3.68	3.77	3.69	3.6

The aminoacidic profile of the diets were calculated using the diet formulator taking into account the aminoacidic profile of the used ingredients as provided by the producers, for the alternative protein source, and that of an average fish meal for the used fish meal (only the essential amino acids are reported).
Methionine was added to the diets 50% FEM, 75% FEM and 75% BOF to meet the European sea bass amino acids requirement as reported by Kousolaki et al., 2015.
The trial lasted 6 weeks. Water temperature was 20 °C ± 0.5. Fish went through a first acclimation period of 1 week, during which were fed with the control diet, a second acclimation period of 2 weeks in which were fed the experimental diets without faecal collection. The faecal collection started at the end of the third week of the trial and lasted 3 weeks. The animals were fed two times a day at 9:00 and 14:00; at 15:00 the tanks were cleaned to avoid the feed contamination of the faeces. Faeces were collected at 8:00 every morning, centrifugated at 4000 RPM*x 10 minutes and stoked at -20°C. At the end of the collection period, faces were freeze-dried and analysed to determinate the chemical composition and the Acid Insoluble Ashes (AIA), in order to evaluate the in vivo digestibility coefficients.

Diets, ingredients and faeces samples were analysed for moisture and ash and crude protein in according to AOAC 2004(procedure numbers; 934.01, 942.05 954.01) , total lipids according to the method of Folch modified (Folch, 1957) .The marker (Celite 245) was determined using the acid-insoluble ash AIA method (Attkinson et al 1984).
The apparent digestibility coefficients ADC of the nutrients of the test and reference diets were calculated as follow (Cho et al., 1982) :

Dry matter ADC= (1-(AIA in Diet/AIA in Faeces)) x100

Crude protein ADC (%)= {[(% crude protein in the diet /% AIA in the diet) − (% crude protein in faeces /% AIA in faeces)] / (% crude protein in tested diet/% AIA in diet)} ×100

Crude lipids ADC (%)= {[(% crude lipids in the diet /% AIA in the diet) − (% crude lipids in faeces /% AIA in faeces)] / (% crude lipids in tested diet/% AIA in diet)} ×100

Trial 2: Feather meal growth trial

In this experiment a total of 360 sea bass juveniles (average initial body weight 10.21 g ± 2.26 g) were group weighted and randomly allocated in 12 fiberglass circular tanks (water capacity 500 L), 30 fish per tank, and they were divided into four diets groups (each group constituted of 3 tanks). The four diets used in this trial were the same used in the digestibility one (without Celite®). Due to a limited number of tanks at our disposal, and considered the results obtained in the digestibility trial, the 75% FEM diet was excluded from this experiment. The ingredients and chemical composition of the diets are shown in table 2. The experimental period lasted 122 days. The animals were fed three times a day (i.e. 9:00, 12.00 and 16:00) at satiety (the feed administration stopped at the first pellet refused). The feed consumption was recorded and morbility and mortality were checked daily. Temperature along the trial was 19.5 ° ± 0.5 C. On a monthly basis, fish were group weighted and growth performance parameters were calculated as follow:

FCR – Feed Conversion Rate (As fed) = [total feed supplied as fed (g)/Weight Gain (g)]

FCR – Feed Conversion Rate (Dry matter) = [total feed supplied as dry matter (g)/Weight Gain (g)]

SGR - Specific growth rate (% day−1) = [(lnFBW − lnIBW)/number of feeding days] × 100
DIR - Daily intake rate (g dry matter/kg Average Body Weight/days) = 1000* [(feed intake (g)/mean weight (g))/days]
PER - Protein efficiency ratio = [Weight gain (g)/total protein fed (g)].

At the end of the experimental period, all the fish were group weighted and 5 fish per tank were collected randomly and killed by anaesthetic overdose (phenoxyethanol). The sacrificed fish were individually weighed and total length was measured in order to evaluate the condition factor as follows:

CF = 100 * [body weight (g)/total length (cm)^3]

After that, an abdominal cut was made through a surgical scissors and the liver and the visceral fat were collected to calculate the hepatosomatic index:

HSI = 100 * [liver weight (g)/body weight (g)]

and the viscero-somatic index:

VSI = 100 * [visceral weight (g)/body weight (g)]

After this, the 5 fish per tank were pooled, freeze-dried and destined to the whole body chemical analyses. The dry matter, ash and crude protein were analysed in according to AOAC 2004 (the procedure numbers were respectively:934.01, 942.05 954.01. The total lipids were determined in according to Folch modified (1957).

Trial n 3 BOF (Blood – Offal – Feather) growth trial

To test if the negative effects obtained in the previous trial with the use of feather meal alone could be reduced by adding other PAPs to the diet in combination with it, a further trial was carried out. For this trial, a total of 225 European sea bass juveniles (average initial body weight 13.2 ± 0.1 g) were group weighed and randomly distributed in 9 fiberglass tanks (500 L volume), 25 fish per tank. Three diets were formulated. In the 0% diet, fish meal was the main protein source. In the diets 50% BOF and 75% BOF, 50% and 75% of fish meal respectively was replaced by a mix of feather meal, blood meal and offal meal in equal parts. These three diets differed from those tested in the digestibility trial for the presence of wheat gluten and for a higher crude protein content (55% CP). This was made to take into account the minor protein digestibility registered for BOF diets in the digestibility trial and to try if by better covering the protein requirements of the fish

species it would have led to better results in terms of growth performance. Methionine was added to the diets 50% BOF and 75% BOF to meet the European sea bass amino acids requirement as reported by Kousolaki et al., 2015. The experimental diets were isoproteic (55%) and isolipidic (15% total lipid). Each experimental diet was administered to the tanks in triplicate. The trial lasted 90 days. The ingredients and chemical composition of the three diets are reported in table 8.3.

Table 8.3: ingredients, chemical composition and aminoacidic profile of the diets.

Ingredients (g/Kg)	**0%**	**50% BOF**	**75% BOF**
fish meal	592	297	149
blood meal	0	124	186
offal meal	0	124	186
feather meal	0	124	186
wheat gluten	148	75	37
corn starch	150	129	116
fish oil	70	85	94
mineral premix	15	15	15
vitamin premix	25	25	25
Methionine	0	2	6
Chemical composition			
DM%	91.01	90.33	89.74
Ash%	8.22	6.56	5.71
GE MJ/kg	20.77	21.41	21.70
DE MJ/kg	15.96	15.41	15.14
CP%	54.89	55.03	54.95
Dig CP%	49.31	47.29	46.14
Lipid%	15.09	14.99	15.09
Fibre%	1.66	1.39	1.26
Amonnoacidic profile (% of protein)			
Arginine%	3.88	3.59	3.44
Histidine%	1.25	1.42	1.51
Isoleucine%	2.44	1.62	1.2
Leucine%	4.93	5.12	5.2

Lysine%	3.9	3.7	3.59
Methionine%	1.46	1.22	1.37
Phenylalanine%	2.4	2.7	2.84
Threonine%	2.49	2.47	2.45
Tryptophan%	0.55	0.55	0.56
Valine%	2.74	3.51	3.89
M+C%	2.11	2.05	2.02
P+T%	1.26	4.43	4.51

The animals were fed three times a day (9:00, 12:00 and 16:00) at satiety (the feed administration stopped at the first pellet refused). The feed consumption was recorded and morbility and mortality were checked daily. Temperature along the trial was 19.5 ° ± 0.5 C. On a monthly basis, fish were group weighted and growth performance parameters were calculated as follow:

FCR – Feed Conversion Rate (As fed) = [total feed supplied as fed (g)/Weight Gain (g)]

FCR – Feed Conversion Rate (Dry matter) = [total feed supplied as dry matter (g)/Weight Gain (g)]

SGR - Specific growth rate (% day−1) = [(lnFBW − lnIBW)/number of feeding days] × 100

DIR - Daily intake rate (g dry matter/kg Average Body Weight/days) = 1000 x [(feed intake (g)/mean weight (g))/days]

PER - Protein efficiency ratio = [Weight gain (g)/total protein fed (g)].

Furthermore, 5 fish per tank were collected randomly and killed by anaesthetic overdose (phenoxyethanol). The sacrificed fish were pooled, freeze-dried and destined to the whole body chemical analyses.
The dry matter, ash and crude protein were analized in according to AOAC 2004 (the procedure numbers were respectively:934.01, 942.05 954.01). The total lipids were determined in according to Folch modified (1957).

Statistical analyses

All the data were analysed by one-way ANOVA, using the GLM procedure of SAS (2000), according to the model:

$$Yij= m +Di + eij$$

where Y is the single observation, m the general mean, D the effect of the diet (i= 0%, 15% FEM, 25% FEM or 50% FEM diet), and e the error.
For growth and digestibility trials the experimental unit was the tank, while for somatic indexes the experimental unit was the individual fish and for the whole body chemical composition the experimental unit was represented by a pool constituted by 5 fish per tank.
In addition, to assess the probability of the linear and quadratic component and to compare treatments, the means were compared using orthogonal single degree of freedom contrast (Steel and Torrie, 1980; SAS, 2000). This latter analysis was applied only to growth trials.

Results

Trial 1: digestibility trial

The ADC (Apparent Digestibility Coefficient) of dry matter, crude protein and crude lipids are reported in table 8.4.
In general, dry matter digestibility resulted higher in the 0% diet than in the feather meal containing diets. The digestibility in fact decreased as the level of feather meal inclusion in the diets increased with a clear descending trend. In particular, control diet differed significantly ($P < 0.001$) from 25% FEM, 50% FEM and 75% FEM (72.39 vs 61.64, 60.87 and 56.80 respectively).
For what regards the BOF meals, 50% BOF dry matter ADC did not differ significantly ($P > 0.001$) from the control diet (69.98 vs 72.39), while 75% BOF resulted to show the worst value (53.88), significantly different from control diet and comparable with the 75% FEM ADC value).
The crude protein apparent digestibility coefficient showed the same trend of the dry matter one. The control diet showed the highest value (88.58). The rest of the diets showed progressively decreased values compared to control group. 50% BOF diet resulted in an ADC value lower but comparable with the control diet CP ADC (80.58 vs 88.58; P value > 0.001). The apparent digestibility coefficients of crude protein appeared at the lowest levels in 75% FEM and 75% BOF diets (69.79 and 66.99 % respectively).
The apparent digestibility coefficients of the crude lipid followed the same trend of the previous ones. The control diet showed higher values ($P<0.001$) compared to the FeM containing diets with 50% FeM and 75% FeM

presenting the lowest values (62.94 and 62.73). In this case 50% BOF diet resulted in higher but not significantly different values compared to control diet, while 75% BOF diet showed an ADC value slightly lower than the one of the control diet but not statistically different from it.

Table 8.4 Apparent Digestibility Coefficients of dry matter, crude protein and total lipids of experimental diets containing increasing levels of Feather meal and BOF meal.

	ADC Dry Matter %	ADC Crude protein %	ADC Lipids %
Control	72.39A	88.58A	82.97AB
15% FeM	67.38AB	81.14AB	67.46DE
25% FeM	61.64BC	78.06ABC	71.70DC
50% FeM	60.87BC	74.38BC	62.94E
75% FeM	56.80C	69.79BC	62.73E
50% BOF	69.98 A	80.58AB	87.16A
75% BOF	53.88C	66.99C	76.91BC
P value	<0.001	<0.001	<0.001
RMSE	2.294	3.748	2.413

Row not sharing the same letters differ for P < 0.001 (ABC)

Trial 2: Feather meal growth trial

The results of growth trial are reassumed in table 8.5.

The initial body weights resulted not homogeneous among the groups. In particular, there was an increasing trend passing from the control to 50% FEM diet as shown by the P value of the linear component of the variance (average initial body weight 10.21 g ± 2.26 g; P value <0.001). Nevertheless, we decided not to discharge the results because it is still possible to appreciate the effects of feather meal inclusion on some performance indicators such as specific growth rate, FCR, DIR and these parameters seem to show clearly and undoubtedly a statistically significant negative trend in their values as the feather meal inclusion rate increases in the diets.

SGR (specific growth rate) value resulted significantly higher (P 0.0003) in the control group compared to the feather meal containing diets as evidenced by the contrast analysis and there was a descending trend of its value passing from the control diet to the 50% FEM diet (1.42, 1.29, 1.16, 0.95 respectively for 0% FEM, 12% FEM, 25% FEM and 50% FEM) as demonstrated by the significant value of the linear component of the variance (P < 0.001).
The same trend was reported for DIR (daily intake rate): this value was higher in the control, and started to decrease in the 15% FEM diet to reach the lowest level in 50% FEM diet (P = 0.0038). The contrast analysis shows that there was a significant difference between the control diet and the feather meal containing diets (P = 0.0122).

Final weight, FCR and PER did not show significant differences among the groups.

Table 8.5: Growth performance of European sea bass at the end of feather meal growth trial

	0% FeM	15% FeM	25% FeM	50% FeM	Ctrl vs FeM	Linear	Quadratic	RMSE
Initial weight (g)	8.05	8.59	11.39	12.79	<0.0001	<0.0001	0.1511	0.4673
Final weight (g)	45.37	41.34	46.79	40.68	0.2641	0.3048	0.5695	3.0322
FCR (As Fed)	1.41	1.41	1.40	1.65	0.4519	0.0981	0.1860	0.1496
FCR (Dry Matter)	1.27	1.27	1.26	1.48	0.4824	0.1082	0.1855	0.1335
SGR	1.42	1.29	1.16	0.95	0.0003	<0.0001	0.3663	0.0723
DIR (As fed)	16.12	15.33	13.84	13.62	0.0122	0.0038	0.5837	0.8646
DIR (Dry	14.55	13.83	12.50	12.25	0.0118	0.0035	0.6165	0.7813

matter)								
PER As Fed	1.58	1.58	1.59	1.37	0.4553	0.0933	0.1733	0.1274

The somatic indexes and the whole body chemical composition of the European sea bass individuals at the end of the growth trial are reported in table 8.6.
No statistically relevant differences were found for CF, VSI and HSI. The quadratic component of variance resulted significant for DM, CP and lipid content indicating that these parameters increased in 15% and 25% FeM diets while in in 50% FeM group returned at levels comparable or lower than those of control diet.

Table 8.6: Carcass traits and fish composition at the end of the feather meal growth trial

	0% FEM	**15% FEM**	**25% FEM**	**50% FEM**	**linear**	**Quadratic**	**RMSE**
CF	1.74	1.65	2.00	1.86	0.0628	0.7425	0.1273
VSI	6.67	6.84	6.62	6.86	0.7196	0.8769	0.3610
HSI	1.21	1.52	1.14	1.40	0.6863	0.8207	0.1726
DM	30.52	32.76	32.56	29.02	0.2852	0.0135	1.5894
CP	18.33	19.36	20.12	17.37	0.5268	0.0298	1.2411
LIP	8.04	8.36	9.07	6.61	0.1837	0.0359	0.9572

ASH	10.31	10.60	11.08	11.93	0.0461	0.6022	0.8779

Abbreviations:
CF: composition factor; VSI: viscerosomatic index; HSI: hepatosomatic index; DM: dry matter; CP: crude protein; LIP: lipids; RMSE: root mean square error

Trial n. 3 BOF (Blood – Offal – Feather meal) growth trial

Table 8.7: Growth performance of European sea bass individuals fed on BOF (blood, offal, feather meal) trial.

	0%	**50% BOF**	**75% BOF**	**Ctrl vs BOF**	**linear**	**Quadratic**	**RMSE**
				P Value	P Value	P Value	
Initial weight (g)	13.05	13.20	13.25	0.6223	0.6173	0.8691	0.4047
Final weight (g)	45.78	45.16	39.45	0.0195	0.0027	0.4050	1.2588
FCR (As Fed)	1.09	1.20	1.39	0.0476	0.0187	0.5874	0.0938
FCR (Dry Matter)	1.04	1.27	1.30	0.0592	0.0222	0.5220	0.0880
SGR	1.79	1.76	1.56	0.0495	0.0105	0.1507	0.0644
DIR (As fed)	17.37	18.67	19.48	0.0809	0.0603	0.7455	0.9553
DIR (Dry matter)	16.52	17.53	18.31	0.1150	0.0813	0.8671	0.8979
PER	1.67	1.52	1.32	0.0312	0.0135	0.7271	0.1021

The results of BOF growth trial are reassumed in table 8.8.
For Final weight, FCR, SGR and PER the linear component of the variance resulted statistically significative indicating that these parameters worsened as the BOF levels increased in the diet. This was particularly true for FCR and PER, while for final weight and SGR the diet 50% BOF showed values comparable to those of control diet. These results seem to confirm the ones obtained in the digestibility trial for 50% BOF diet.

In table 8 are reported the effect of BOF diets on whole body chemical composition of fish. No significant differences have been found except for the lipid component that resulted higher in BOF groups compared to control (6.94 vs 7.51 and 7.44 for 0% BOF, 50% and 75% BOF respectively).

Table 8.8: Fish whole body chemical composition at the end of BOF trial.

	0% BOF	50% BOF	75% BOF	Ctrl vs BOF	linear	quadratic	RMSE
DM	30.42	30.19	30.07	0.6298	0.6195	0.9269	0.8075
CP	18.71	18.40	19.83	0.5934	0.2205	0.2610	1.0002
LIP	6.94	7.51	7.44	0.0237	0.0512	0.1198	0.2517
ASH	9.74	8.86	9.24	0.3667	0.5607	0.4099	1.0064

Abbreviations:
DM: dry matter; CP: crude protein; LIP: lipids; RMSE: root mean square error

Discussion

The results of the digestibility trial carried out at the facilities and laboratories of the Hellenic Centre for Marine Research of Heraklion (Crete, GR) showed that there was a decreasing trend of the apparent digestibility coefficients of the dry matter, crude protein and total lipids as the processed animal proteins in the diets increased. This was particularly evident in the diets containing the only feather meal. The results of the apparent digestibility coefficients of the 50% BOF diet that resulted comparable to the control diet, are encouraging, because they suggest that a mix of alternative animal protein sources could make it feasible a higher fish meal substitution without detrimental effects on digestibility. Digestibility results have been confirmed by the results obtained in the two growth trials, with the FEM containing diets that led to a worsening of the growth performance parameters starting from the 15% fish meal substitution rate and this was particularly true for daily intake rate and for specific growth rate, while 50%

BOF diet led to obtain specific growth rates and final weights comparable to the ones obtained with a control diet.
The results obtained in the studies previously published are various and often contradictory.
The use of processed animal protein in fish nutrition have been largely studied in the past before their prohibition of 2001 by the European Union regulation for the control of the BSE transmission (EU 999/2001).
The use of animal by product was largely investigated in the fish nutrition, in particular in salmonids and in Tilapia (Buerau et al 1999; Tacon et al., 1999).
Tacon et al (1983) observed that a meat and bone meal hexane-extracted or a meat and bone meal:blood meal included in a diet for tilapia (*Oreochromis mossambicus*) at 4:1 ratio with the addiction of methionine , histidine and lysine could replace fish meal protein up to 50% (Tacon et al , 1983). Hydrolysed feather meal supplemented with methionine, histidine and lysine could replace only 30% of FM protein (Tacon et al., 1983). Our diets were added only of methionine according to Kousulaki et al, (2015).
Davies et al (1989) demonstrated that the optimum meat and bone meal:blood meal ratio was 3:1 or 2:3 ratio, and this ratios could replace the fish meal completely in the *O. mossambicus* diets. When the blood meal where used as a total replacement of fish meal the growth rate was comparable to the control diet.
Other two authors (Mansour, 1998; al Sayed 1998) described that the Nile tilapia fed on meat and bone meal and poultry by product meal as a single dietary protein source produced a significant retard on growth rate and on feed efficiency. The results on the use of the hydrolysed feather meal are contraddictory.
Tacon et al (1983) on Nile tilapia, Viola and Zaher (1984) on Monzambique Tilapia and Davies et al. (1989) on tilapia hybrids found that diets containing hydrolysed feather meal showed poor growth performances, presumably due to poor digestibility and unbalances of essential amino acids.

Our results seem to be in agreement with the researches previously cited.

Bureau et al. (2000) evaluated the potential of feather meal and meat and bone meal as protein sources in rainbow trout diets. The feather meal was used alone or in combination with corn gluten meal and blood meal to replace herring meal in diets. The authors found that the incorporation of up to 15% feather meal in the diet was possible without affecting growth, feed

efficiency, nitrogen or energy gains of the fish. The incorporation of up to 24% meat and bone meals in the diet was possible without affecting growth but resulted in a small yet significant reduction in feed efficiency compared to control diet. The authors concluded that feather meal and meat and bone meal have good potential for use in rainbow trout diets. In our trial, 15% fish meal/feather meal substitution rate, led to comparable final weights and FCR compared to control diet, although SGR and feed intake were penalized.

Bureau et al (1999) carried out an experiment testing the digestibility of 20 types of processed animal proteins in rainbow trout. The authors concluded that the processing cycle could affect the quality and as a consequence the digestibility and the fish growth performance and reported a significant difference of digestibility in the feather meals, blood meals and meat and bone meals used for their trial. The different results could be linked to the dried equipment used in the processing. In the case of the blood meal the authors observed that the ring- dried blood meal had a higher digestibility than the rotoplate dried blood meal.
The hydrolysis conditions have been shown to affect the digestibility and the nutritive value of feather meal (Papadopulus et al., 1985; Latshaw et al., 1994; Wang and Parson, 1997). The results obtained by these studies suggest that drying conditions, such as the utilised equipment, may also have an effect on the apparent digestibility coefficients of poultry feather meal and could affect the fish growth performance.
Moritz and Latshaw (2001) investigated different time and pressure during the processing of the hydrolized feather meal, in order to investigate if different time and pressure of exposure could affect the nutritional value of this animal by product. A constant time series (36 min) was completed to evaluate the effect of increasing pressure (207 to 517 kPa) on nutritional value. Feather meal processed at the lowest pressure had the highest nutritional value, and vice versa. True amino acid availability determined with force-fed White Leghorn cockerels demonstrated that increasing pressure decreased true available (TA) cystine more than any other amino acid. Increased steam pressure also resulted in decreased, undegraded intake protein. Various combinations of time (106 to 4.5 min) and pressure (207 to 724 kPa) were used to prepare a constant density series (483 kg/m3). In this series, feather meals were similar in nutritional value. The authors concluded that there was no indication that high hydrolysis pressure was detrimental to feather meal quality, if the appropriate time is used.

The major compound of the feathers is the keratin, that is disrupt during the hidrolisis processing. One of the most sensitive amino acid in the keratin composition is the cysteine; more extentive hydrolysis is responsible of the decreases of the cysteine and the increase of lanthionine, according to Davies et al., (1961).The chemical conversion of the cysteine in lanthionine is linked to the production of additional sulfur compounds (Friedman, 1977), and these products because are volatile are lost during the hydrolisis process. The hydrolysis process is responsible of the trasformation of the cysteine; the raw feathers presents a high content of this aminoacid in lanthionine and also increases the digestibility.
The lanthionine, can replace the cysteine in seveal isometric form in animal diets, but is not as effective as the cysteine, on weight basis (Jones et al.,1948; Robbins et al.,1980).

The apparent digestibility coefficients of dry matter reported by Bureau et al (1999) for different feather meals ranged between 79% and 84%. The comparison with our results is difficult because our values refer to diet containing different inclusion rates of feather meal. However, our values were between 67.38% for 15% FEM and 53.88% for 75% BOF diet; the apparent digestibility coefficient of crude protein in Bureau at al. (1999) ranged between 81-87%. Our results ranged between 81.14% for the 15% FEM and 66.99% for the 75% BOF diet. Finally, the apparent digestibility coefficient of lipids showed a higher variability: from 40% up to 83%. Our results ranged from 87.16% of 50% BOF to 62.73% of 75% BOF diet.
The apparent digestibility coefficients of crude protein reported by Bureau et al (1999) for poultry by product meal were higher than our results and the results previously obtained by Cho and Slinger (1979), NCR (1981), and Cho et al (1982).
Pfeffer et al (1995) tested the addition of three by-products of poultry slaughtering to replace wheat gluten at levels of 250 and 500 g/kg in a basal diet of wheat gluten + lysine for the rainbow trout and reported that the digestibility of energy and crude protein was hardly affected by dietary proportion in poultry blood meal (84 and 85%) and poultry offal meal (80 and 81%). Furthermore, increasing dietary proportions from 250 to 500 g·kg−1 reduced the respective values of feather meal (88–81 and 86–83%).
A recent study (Esmaelli et al, 2016) evaluated the effects of the replacement of the fish meal with meat and the bone meal with or without the addition to the diets of garlic powder on the apparent digestibilty, growth performance, digestive enzymes, body composition and fatty acid profile of juvenile

rainbow trout (O. mykiss). The authors reported that increased dietary content of meat and bone meal impaired growth and production performance, body composition, digestive enzyme activity, tissue fatty acid profile and overall digestibility. Addition of garlic powder instead could correct fish performance, body composition, enzyme activity and digestibility to some extent. Dietary supply of garlic resulted in increased digestive enzyme activity improving fish performance. The authors attributed the registered impaired growth to a reduction of digestibility of protein, fat, energy and dry matter.

It is known that the terrestrial animal by products are characterized by a high protein content and generally they present a good aminoacidic profile according to Tacon et al. (1993). However, they may be deficient in one or more of the EAA. Some may contain a low Lysine content (such as poultry feather meal), low methionine (meat and bone meal, blood meal and poultry hydrolysed feather meal), and in general the blood meal is poor in essential amino acids (NCR, 1983; Tacon and Jackson, 1985).

In our trial the content of lysine and threonine was acceptable, but the methionine content during the formulation of the experimental diets appeared to be low ; in addition it was possible to observe that the methioninne content decreased at increasing level of the experimental ingredients. According to Kousulaki et al (2015), it was necessary to add cristalline methionine to the diets presenting a high PAP's inclusion rate.

However, Kousulaki et al (2015) reported that when a cistalline aminoacid is added to the diet, there is the risk that the essential aminoacid adsorbed by the fish will not reflect the aminoacid present in the diets, thereby reducing the protein utilization efficiency.

Salama et al (2013) evaluated the effect of the addition of cristallin essential aminoacids in the sea bass and observed that the diets deficient in essential aminoacids, such as lysine and methionine, are linked to lower feed intake than the control diet, and a reduction of the growth rate (De la Higueira, 2001).

In our trial PER resulted decreased in feather meal content diet only at the highest fish meal substitution level (50%), while a significant reduction of PER index was registered for BOF diets.

Furthermore, histidine, leucine, phenilalanine and valine dietary requirements, as well as the optimal levels of dietary taurine in sea bass fed law fish meal diets, are not yet studied (Kousulaki et al, 2015).

The reduction of essential aminoacidic content in the diet is responsible of the reduced growth performance and feed conversion ratio (Wilson and

Halver, 1986). In Salmonids a diet poor in methionine affects not only the growth rate but is responsible also of catarat (Walton et al, 1982 ; Rumsey et al, 1983 ; Cowey et al., 1992).
In our trial, the decreased digestibility of dry matter and nutrients could be the major factor that caused a decreased growth performance in fish fed on PAP's containing diets. To this result could have contributed the suboptimal aminoacidic profile of the experimental diets.

HSI is an index normally utilized to investigate the effects of feeding on the liver functionality which is a key organ for metabolism (Dernekbaşı, 2012). Values of the HSI higher than the standard values (between 1 and 2%) show that feeding or the feed cause some troubles in fish, especially in the carbohydrate and fat metabolism, the existence of oxidized feed in the diet, and extra carbohydrate and vitamin deficiency (Munshi and Dutta, 1996). In our growth trial there (trial n-2 Feather meal) are no difference statistically different for HSI.
CF and VSI are also normally used to state the general condition of the fish. In our trial no differences were found beetwen fish fed on fish meal based diet and fish fed PAP's containing diets.
The chemical composition of the fish showed a decrease in the percentage of dry matter, protein and lipids in the fish fed on only feather meal (quadratic contrast <0.05), and this results indicate a descrease of fish quality.
Are reported same variation also in the chemical composition of the fish fed on the mix of ingredients, the lipids increased at increasing level of experimental ingredients in the diets, also this results could indicate a worsening of fish quality.

Conclusion

The experimental diets containing the only feather meal in replacement of fish meal are showed a decreasing of digestibility, a worsening of growth performances and, in addition are reported also a decrease of fish quality, based on our results the only suitable diet is 15 % FEM.
On contrary, the experimental diets showed promising results because the digestibiliy coefficient of 50 % BOF diet is comparable to the control diet and also the growth performance of the fish fed on the ingredients mix is encouraging, the blood, offal and feather meal colud replace the fish meal at inclusion level of 50%. The higher inclusion of BOF is responsible of a

decreasing of digestibility, growth performances and fish quality (probably because the amount of feather meal in the diet is higher than 50 % BOF).

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The results obtained in the trials described along the different chapters of the present thesis clearly showed that the use of environment-friendly protein sources in poultry and fish nutrition is possible and, in my opinion, necessary to guarantee the sustainability of these animal production systems in the future.

At the beginning of this PhD course and thus of my studies on these topics, not much researches were available on the use of the insect meals in the poultry, and, in particular, in the laying hens under an intensive production system. It seems clear that *T. molitor* larvae meal can completely replace the soybean meal in the broiler diets without negative effects on growth performance, meat quality and with an improved immunity status of the birds. Different was the situation in the layers. Probably, the inclusion level of the *Hermetia illucens* larvae meal should be reduced as the full replacement of the soybean meal had negative effects on the feed intake and thus on the hens performance, even if the eggs obtained from hens fed insects showed some improved nutritional characteristics.

More in particular, the use of meal from Tenebrio molitor as main protein source in broiler in total replacement of soybean meal along a period from 30 to 62 days of age had no significant effect on most of growth performance, carcass traits (such as dressing out) and chemical and physical properties of meat, the latter important for marketing purposes. The intestinal length and weight as well as the absolute and relative spleen weight were higher in broilers fed TML in comparison to SBM and this can be attributed to the effect of chitin which reduces nutrient digestibility and act as a prebiotic. The prebiotic effect of *Tenebrio molitor* larvae meal could be a suitable alternative protein source for chicken broilers also when used as principal protein source in the diet.

Further studies are needed to investigate possible changes in the intestinal microbiota of chickens fed with insect meal based diet and it would be interesting to evaluate the suitability of insects in other bird species farmed for the meat production.

Hermetia illucens larvae meal could be an interesting protein source for laying hens, able to sustain the egg production without negative effects on animal health and enhancing immune status of birds. However, it negatively affected feed intake and thus production performance of hens even if feed conversion ratio of insect diet was more favourable than that of soybean diet. The yolk of the hens fed insect meal resulted richer in PUFA, n-6, n-3, and showed lower cholesterol content than the group fed soybean meal diet. The

specific role of the insect meal as alternative protein source on the metabolic pattern of laying hens need more studies.
The total replacement of the soybean meal of with *Hermetia illucens* larvae meal showed a negative effect on nutrient digestibility. The enzymatic activity in the small intestine was modified due to dietary inclusion of insect meal and these changes were positive in terms of enhanced production of butyric acid in the caeca, or negative as the reduction of some enzymatic activities in the ileum.
Futher studies are needed to find out the optimal inclusion level of *Hermetia illucens* larvae meal to balance the negative effects of feed intake and nutrient digestibility, and the positive effects on the digestive system of the laying hens.
In European sea bass, the experimental diets containing the only feather meal in replacement of fish meal showed a decreasing of digestibility, a worsening of growth performances and, in addition, also a decrease of fish quality. Based on our results the only suitable diet was the one presenting the 15 % of fish meal substitution with FeM.
On the contrary, when a mix of ingredients (blood, offal and feather meal) was used, the experimental diets showed promising results because the digestibility coefficients of the 50 % BOF diet was comparable to those obtained with the control diet. These results were confirmed by the growth trial in which the fish fed on the ingredients mix included in the diet at the 50% fish meal substitution rate gained growth performance comparable the control group. The higher inclusion (75%) of BOF was responsible instead of a decreasing of digestibility, growth performances and fish quality, probably due to the higher amount of feather meal in the diet).
Additional studies are required on the possible presence of antinutritional factors in processed animal proteins, and other studies are needed to find the optimal inclusion levels of these ingredients in fish diets.