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THESIS

"Feeding strategies to improve welfare and sustainability in fish and poultry production"

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Abbreviation List

HBP	Honey bee pollen
TNF-α	Tumour necrosis factor- α
HSP70	Heat Shock Protein 70
IMBBC	Institute of Marine Biology, Biotechnology and Aquaculture
HI	Hermetia illucens larvae meal
SBM	soybean meal
MTT	Maecenas tempus tellus
HI25	Group with 25% of Hermetia illucens meal substitution
HI50	Group with 50% of Hermetia illucens meal substitution
IAP	Intestinal alkaline phosphatase
OIE	World Organization for Animal Health
PAW	Participatory Animal Welfare
	Institute of Marine Biology, Biotechnology and Aquaculture
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OIE	World Organization for Animal Health
PAW	Participatory Animal Welfare
DM	Dry Matter
CP	Crude Protein
EE	Ether Extract
CF	Crude Fibre
AOAC	Association of Official Analytical Chemist
WG	Weight Gain
FBW	Final Body Weight
DIR	Daily Intake Rate
SGR	Specific Growth Rate
FCR	Feed Conversion Ratio
PCR	Protein Efficiency Ratio
HIS	Hepatosomatic Index
VSI	Viscerosomatic Index
AIA	Acid-insoluble ash
RPM	Revolution Per Minute
SD	Standard Deviation
PBS	Phosphate-Buffered Saline
GOT	Glutamic Oxaloacetic Transaminase

Abbreviation List

GTP	Glutamic Pyruvic Transaminase
ADC	Apparent Digestibility Coefficient
SD	Standard Deviation
PBS	Phosphate-Buffered Saline
GOT	Glutamic Oxaloacetic Transaminase
SFE	Supercritical Fluid Extraction
TMB	Tetramethylbenzidine
HBSS	Hank’s buffer
TCA	Trichloroacetic Acid
FAO	Food and Agricolture Organizzation
PAP	Processed Animal Proteins
ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
NDF	Neutral Detergent Fiber
ALP	Alkalin Phosphatase
CK	Creatine Kinase
LDH	Lactic Dehydrogenase
BUN	Blood Urea Nitrogen
NRC	Nutrient Requirements of Poultry
DPPH	Diphenyl-2-Picrylhydrazyl

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Abstract

Given the forecast of world population growth and the consequent increase in nutritional needs for both humans and animals, animal production will increase dramatically by 2050. For this reason, alternative farming methods are sought to improve sustainability, reduce the environmental impact, in order to guarantee a production consistent with the growth forecasts.
Improving welfare is one of the factors that can enhance the sustainability of a farm. Healthy animals with competent immune defences are animals from which originates a final product of better quality and on which less pharmacological aids will be used. To this purpose we decided to test honey bee pollen in two species of marine fish, to verify its antioxidant effect and the its capacity to enhance both the innate and the adaptive immune response. Honey bee pollen is gaining attention as a health-promoter in fish and polyphenols are considered the principal biomarkers of quality for commercially distributed pollen preparations.

The aim of the first trial was to evaluate the effects of the inclusion of honey bee pollen (HBP) in meagre (*Argyrosoumus regius*) juveniles diets on growth performance, diet digestibility, intestinal morphology and immunohistochemistry. Furthermore, stress-related molecular markers and biochemical blood profile of fish were assessed, together with mineral trace and toxic elements concentration in pollen and diets. Trial was conducted at the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research (Crete, Greece).
Specimens of meagre (360) of 3.34±0.14 g initial body weight, were randomly allocated in twelve 500 l circular tanks (30 fish per tank). Four diets were formulated: a control diet and three experimental diets with 1%, 2.5% and 4% of HBP inclusion as fed basis. All the growth parameters and crude protein and ether extract digestibility coefficients were negatively linearly affected by increased HBP inclusion ($p < 0.0001$). Histology of medium intestine showed slight signs of alterations in group HPB1 and HPB2.5 compared to control. Fish from HBP4 group showed severe alterations at the intestinal mucosa level. Immunohistochemical detection of tumour necrosis factor- α (TNF-α) in the medium intestine showed the presence of TNF-α+ cells in the lamina propria, that resulted in accordance with the increased level of the TNF-α protein detected by immunoblotting in the liver. This stress situation was confirmed by the increased hepatic level of Heat Shock Protein 70 (Hsp70) ($p < 0.05$) in fish fed the HBP4 diet and by the linear decrease of total serum protein levels in HBP containing diets ($p < 0.0001$). These negative effects can be related to the ultrastructure

of the bee pollen grains walls that make the bioactive substances unavailable and can irritate the intestine of a carnivorous fish such as meagre.

In view of these results, in the second study, carried out on gilthead sea bream, we wanted to verify whether these negative effects could be overcome by the inclusion in the feed of the bioactive fractions previously extracted from pollen, using advanced techniques such as SFE, which does not involve the use of solvents.This method is considered “green” and it conjugates, to the extraction power, the avoidance of solvents. The immune-stimulatory effect of two levels of honey bee pollen (5% and 10%) and its supercritical fluid extract (0.5% and 1%) included in the diet for *Sparus aurata* was tested. The preliminary evaluation of the antioxidant properties of the pollen extracts, obtained by chemical and supercritical fluids extractions, attested that the supercritical fluid extract showed the best antioxidants performances. Furthermore, the results demonstrate that the diets supplemented with pollen extract had a stimulatory effect on serum immunity, respect to the inclusion of raw pollen. More specifically, serum peroxidase, protease, antiprotease, lysozyme activities, as well as the bactericidal activity (against *Vibrio anguillarium* and *Vibrio harveyii*) were significantly increased in fish fed the diets supplemented with supercritical fluid extract, respect to the fish fed on control and on diets supplemented with 5% and 10% of raw pollen, suggesting to consider this natural resources as supplement in aquaculture.

Another aspect that we have considered is the use of innovative protein as feed. Insect meals could be an alternative protein source for livestock, and they would also be able to reduce the environmental problems related to intensive animal production systems.
European legislation currently authorizes the use of seven insect species in aquaculture, however, there is still no legislation that regulates the use in the poultry sector.

The aim of the third study was to evaluate productive performance, blood analysis, nutrient digestibility, and changes in the internal organs of laying hens fed *Hermetia illucens* larvae meal (HI) at two different substitution levels (25 or 50%) of soybean meal (SBM). A total of 162 Hy-line Brown hens (sixteen weeks old) were equally divided into three experimental groups and fed isoprotein and isoenergetic diets.

Abstract

Egg weight, feed intake, and feed conversion rate were not affected by the soybean meal substitution at both inclusion levels of insect meal. Egg mass was positively affected by the insect meal diets, as was the lay percentage, although only at the lowest inclusion level. Dry matter, organic matter, and crude protein digestibility coefficients were lower for the diet with 50% of sostitution, probably due to the negative effect of chitin. A reduction in serum cholesterol and triglycerides was observed in both insect-meal fed groups, while serum globulin level increased only at the highest level of insect meal inclusion, and, consequently, the albumin to globulin ratio decreased. Overall, a protein replacement of 25% with an insect meal from Hermetia illucens larvae in the diet of laying hens seems to be more suitable and closer to the optimal level.

The fourth and last trial was subsequent of the third one. In this case was evaluated the effects of feeding a *Hermetia illucens* larvae meal on the different intestinal traits of hens, and to determine the toxic elements' concentration in the insect meal and diets, 162 hens were randomly allotted to three groups. The control received a corn-soybean meal-based diet; the HI25 and HI50 groups received two diets in which the 25% and 50% of the dietary protein were replaced by the HI protein, respectively. The duodenal and jejunal villi height and villi/crypt were higher ($p < 0.01$) in the SBM than in the HI groups. The ileal villi height was higher ($p < 0.05$) in the SBM and HI25 groups than the HI50. The HI50 group exhibited a lower duodenal maltase activity. The intestinal alkaline phosphatase (IAP) activity linearly decreased in the duodenum and jejunum as the dietary insect meal inclusion increased. The HI50 group had a higher acetate and butyrate level than the SBM. The levels of cadmium (Cd), lead (Pb), mercury (Hg), and arsenic (As) lower than the maximum values established by the EU Commission. The 25% soybean protein replacement with Hermetia illucens larvae meal in the diet of laying hens was more suitable and closer to the optimal level than 50%.

Introduction

Animal well-being and sustainability are topics that have a magnetic attraction on public opinion in the new 20's. The debate is constantly and continuously evolving, and the amount of energy addressed to these topics, especially by the new generations, is in continuous growth. According to the latest Eurobarometer, in Italy only 3% of respondents think that animal welfare is not important, 86% believe improvements in animal conditions are needed and 80% want more information on how animals are raised (Di Mambro, 2019)
Who among us do not feel the weight of having to defend the planet? Who among us is not tormented by the talking cricket whispering from our shoulder every time we buy a product packed in a plastic wrap, every time we don't do the separate collection of waste, every time we leave a light turned on more than necessary?
In light of this, I decided to deepen the studies on these issues, evaluating feeding strategies to improve the welfare and sustainability in animal production.

The evolution of animals production is leading to a reversal of the trend seen in the last century. The increase in the average of western world population wealth and the population growth has entailed the need to increase animal production, intended both as meat and as milk and egg.
Over the years this has led to the development of an intensive type of farming, which over time has revealed all its negative aspects. It is universally recognized the enormous difference between the flavour and consistency of a cattle, or a pig raised on pasture or a chicken grown in a large area of land compared to animals born and lived indoors. Intensive breeding has led to a drastic reduction in the variety of feeds available, moreover animals housed in restricted environments have no way of developing their muscle masses. These rearing methods often can lead to a reduction of the flavour of the meat, a poorness of organoleptic qualities, a monotonous product with a stringy consistency. It is not a coincidence that high quality products such as Jamon Serrano have very strict protocols for the breeding of animals from which they are produced. The quality and type of jamon, in fact, depends on the pig feeding method and on the environment in which they are raised.

Another huge problem of intensive breeding is the bacterial contamination, only in the United States every year there are over 70 thousand cases of salmonella related to the consumption of undercooked chicken and meat

pathogens are the main cause of deaths due to the consumption of infected food, or to the antibiotic resistance. An intensive breeding strategy leads to overcrowding of the stables and to the increase of stress levels in livestock. This leads to a consequent alteration of the immune defences, the spread of stable pathology and therefore the immoderate use of antibiotics. (Available on Focus online, 2020)

Starting from this point it is easy to understand how much promoting animal welfare can be linked to the concept of one health. One Health' is an approach to designing and implementing programmes, policies, legislation and research in which multiple sectors communicate and work together to achieve better public health outcomes. Antibiotic resistance and zoonoses are two of the cardinal topics that the One Health program deals with. Improving welfare means improving immune competence and therefore preventing the spread of diseases and the use of antibiotics.

A sign of greater worldwide attention on animal welfare is the Global Animal Welfare Strategy adopted in 2017 by the World Organization for Animal Health (OIE), to which one hundred and eighty countries adhere. This program follows an iter in which animal welfare had been indicated as a priority topic. The prerequisites of this Strategy are the acknowledgment that the well-being of the animals is closely linked to their health and human health, the sustainability of socio-economic and environmental systems and that well-being is achieved with scientific knowledge, economic and production systems, cultural, religious influences and is based on ethical reflections, also considering the legislative systems. This leads to responsibilities that at various levels involve governments, academic institutions, civil societies, veterinarians, researchers, breeders and all the people who in various capacities own animals, use them and take care of them. OIE indicates that in assessing animal welfare, economic and ethical conditions are complementary to each other and must be considered united in every context, assessing that in food production systems, animal welfare contributes to improving productivity, quality and food safety, the economic performance of the breeding activity. For this reason, animal welfare contributes to ensuring the interest of consumers and ultimately the prosperity of economies that depend on animal production.
The current perspective of the OIE broadens the usual horizons, linking the sciences of well-being more closely to the scientific areas of social sciences and economics and to other sectors. The integration of these sectors is aimed

to guarantee animal welfare and respect of the environment, to obtain a better global animal - human health and economic sustainability.
The approach to welfare has radically changed even for breeders. They are trying to focus on the quality of the final product rather than on the quantity and on the higher attention of the consumer to breeding methods. An interesting example is INVERSION (agroecological innovations for the resilience and sustainability of mountain livestock), a project for some small Italian mountain areas which envisages the use of PAW (Participatory Animal Welfare) tool, capable of a systemic monitoring of animal welfare. Evaluating 24 indicators and assigning each of them a score allows you to monitor the well-being of the herd (available on Ruminantia online, dic. 2019)

An aspect that greatly stimulates consumer curiosity is the possibility of using alternatives to meat. Winston Churchill wrote in 1932: "We will avoid the absurdity of growing a whole chicken to eat only the breast or the wing, making these parts develop separately" and apparently its paradigm is becoming more and more real. In our future, will there be hamburgers prepared in the laboratory without the need to slaughter animals and allocate precious vegetable resources to feed production? Studies on the production of artificial meat are progressing, and the first products begin to be subjected to the satisfaction test of cooks and consumers. This future could come closer. “Meat production as well as we know it's at the end of the line, "says the international consulting firm A. T. Kearney. In 2040 only 40 percent of the meat sold all over the world will come from live animals. The vast majority of steaks, wurstel and meatballs will be replaced by plant imitations or by synthetic meat produced by the industry biotechnology. The turning point has already begun. Companies like Beyond Meat, for example, produce hamburger starting from a puree of peas. This meat substitute is always more common in restaurants and canteens of all the world. In the United States the chain of fast food Burger King sells the Impossible Whopper, made with proteins plant. Businesses almost every week foodstuffs like Nestlé bring in trade in new plant substitutes for meat, like the Incredible Hack. From the names of products almost looks like companies they hope for a miracle. And the next one generation of meat substitutes it will be even more futuristic. There is a refrigerator, inside they are laid, in transparent jars, tomorrow's steaks. They are still invisible to the eye, composed only bovine stem cells. But if put in a culture broth, ripen until it becomes a piece of meat. “Our technology allows us to grow a three-

dimensional fabric made of muscle cells, fats, connective tissues and blood vessel" explains biologist Neta Lavon. So as claimed by the producers of meat substitutes there are finding ways to produce animal proteins without raising nourishing and slaughtering animals, and that in this way we will be able to support food needs of the planet in the best way for the environment. Currently the main obstacle to artificial meat spread is the price, but it should drop after the start of extensive production (article published on Focus online, 2019; Lynch, 2019; Buchter, 2019).

The most important discoveries of Impossible Food company, the producer of the Impossible Whopper hamburger, was that the "magic ingredient" responsible for the unique flavours and aromas of meat, "heme." Heme is an iron-containing molecule that occurs naturally in every cell of every animal and plant. The heme in the Impossible Burger is identical to the heme in meat, but it is produced with bioengineering procedures and delivers the unique taste and bioavailable iron content of beef, using only natural resources. From what the company claims Impossible Burger requires approximately 75% less water and 95% less land and generates about 87% lower greenhouse gas emissions than a conventional burger and is produced without using hormones or antibiotics and contains no cholesterol. For researchers that day has come, whereas from a single cow we can produce all the meat we want. As in all things, there are important diatribes on artificial meat. However, you turn it, it seems that the problem on food production is full of dead ends.

A British study (Lynch, 2019) published in Frontiers in Sustainable Food Systems says that laboratory-grown meat that many see as a future alternative to satisfy the world's appetite for animal protein could, in the long run, do more harm to the environment than traditional meat. The breeding of slaughter animals alone is responsible for 15% of the total of all anthropogenic greenhouse gas emissions, and the bovine one is associated with an important production of methane and nitrogen monoxide, deriving from manure and the digestive process of animals. Also weighing on the environment are the conversion of agricultural land to pasture or to the intensive production of animal feed, and the water dissipated in the entire production process. Synthetic meat is obtained by collecting stem cells from animal tissues and letting them differentiate into fibres, to obtain a sufficient amount of muscle tissue that can be united, flavoured and sold as meat. The process is laborious and still too expensive to be carried out on a large scale and to reach consumers; however, at least on paper it would have the advantage of producing less methane emissions. The long-term climatic

implications of the two different production processes, with a difference compared to previous studies: they did not convert, as often happens, all the different greenhouse gas emissions into tons of CO_2 equivalent. With the same tons of emissions, methane has in fact a much higher impact on the climate than carbon dioxide. However, it only remains in the atmosphere for about 12 years, while CO_2 persists, and accumulates for millennia. The influence of methane, therefore, is not cumulative: that's why in some circumstances, the production of synthetic meat, associated almost exclusively with CO_2 emissions, could be even heavier, in climatic terms. In order to avoid it, it will be necessary to invest in sustainable energy production methods that can power the production plants.
Apart from emissions, artificial meat would drastically reduce animal slaughtering and soil consumption and would contain water bleeding. However, if the market expands, it could lead to soil pollution problems, due to the large quantities of chemicals, hormones and growth factors used to grow the initial cells. On the other hand, the artificial meat could give greater guarantees of hygiene than the real one, avoiding the bacterial contaminations that can be caused by the slaughtering processes, but also the dangers of diseases such as avian flu or the "mad cow", and the spread of antibiotic resistance (article published on Focus n. , on).

Therefore, currently the only things that could improve the impact of the farms are the improvement of well-being, in order to improve the organoleptic and nutritional quality of the final product, to reduce stress in order to not compromise the competent immune response, to reduce pathologies due to breeding management problems and reducing pharmacological aids using natural nutraceuticals. A second aspect on which we can act to decrease the footprint of animal farming is the search for alternative protein sources both as feed and food.
The purpose of this thesis encompasses these two objectives: improve the welfare adding natural nutraceuticals to diets that reduce the oxidative stress and increase the immune competence and research new protein sources with low environmental impact.

The word “welfare” came from the Old Norse word velferth, derived from the words meaning good (vel in Old Norse) and travel (fara in Old Norse). A similar word, wohlfahrt, is used today in German language. In the Romantic languages the concept is rather different, being based on “being good” (bienestar in Spanish, bien-être in French and bemestar in

Portuguese). This variation is now supported in the United States, where the word "well-being" is predominantly used, rather than "welfare", because of potential confusion with the welfare state for people (Phillips, 2009).

Choosing a definition that best encapsulates our interest on the topic is not easy, we are essentially trying to answer the question: "How good is an animal's state in its environment?" (Phillips, 2009). This answer has four components:

- the animal's environment;
- the animal's awareness of its environment;
- the animal's internal state, based on its genetics, its past and present experiences;
- the animal's awareness of its internal state (Phillips, 2009).

Currently one of the most quoted definition of animal welfare is one of the oldest: "the state of an animal with regard to its ability to cope with its environment" (Broom, 1986). This definition is adequate at least to the first objective, and partly to the second and does not take into consideration the internal state of the animal (Phillips, 2009). To define welfare, a model that conceptualize in a fully satisfactory manner the major ethical concerns over the quality of life of animals would be preferred. Furthermore, in recent years, ethical concerns about the quality of life of animals have increasingly become the subject of public policy and controversy. Consequently, society has turned to science for guidance (Brambell, 1965; Thorpe, 1969) and animal welfare has become a focus of scientific studies (Fraser, 1997).
Scientists proposed different conceptions and definitions of animal welfare, and these led to different research methods and different ways of interpreting results (Fraser, 1998). Debating on animal welfare means talking about values that answer to the question: "what is better for an animal?", however these values cannot always be transformed into empirical evidence (Fraser, 1995). For this reason, we have to look at animal welfare as a concept that links scientific research to the ethical concerns (Fraser, 1997).
Stafleu et al. (1996) noted that when scientists dealt with a value-related concept such as animal welfare, they redefined it linking it to other scientific concepts (stress, immune competence, fitness) or to available methods of measurement (cortisol levels, disease incidence). This made the assessment of animal welfare seem like a purely empirical issue overshadowing the relevant ethical concerns. By saying this, we refer to the quality-of-life concerns given by social critics and ethicists (Duncan et al, 1997): first are

'natural-living' concerns which emphasized the naturalness of the circumstances in which animals are kept and the ability of an animal to live according to its 'nature'. A second type of concern, which is called 'feelings-based' emphasized the affective experiences ('feelings', 'emotions') of animals, so a good life for animals depended on freedom from suffering in the sense of prolonged or intense pain, fear, hunger and other negative states; some commentators also emphasized positive states such as comfort, contentment and pleasure.
Others with practical responsibility for animal care, accorded special importance to health and to the 'normal' or 'satisfactory' functioning of the animal's biological systems.
Thirdly 'functioning-based' concerns, held especially by many farmers, veterinarians and others with practical responsibility for animal care, accord special importance to health and the 'normal' or 'satisfactory' functioning of the animal's biological systems. Thus, Sainsbury (1986) wrote of protecting animal health as a duty that people owe to animals in their care, and Taylor (1972) emphasized shelter, nutrition and health care as advantages that more than compensate for the unnaturalness of keeping animals in confinement.
The best-known example of animal welfare definition is the 'five freedoms' of the Farm Animal Welfare Council of the United Kingdom referred to affective experience (fear, hunger), biological functioning (injury, disease) and performance of natural behaviour (Ewbank, 1988).
This narrower conception limited the scope of animal welfare research by ruling out or de-emphasizing certain measures in favour of others.

The Broom's definition says that: "the welfare of an animal is its state as regards its attempts to cope with its environment and refers to how much has to be done to cope, and how well or how badly coping attempts succeed" and in 1988 he stated that the welfare of an individual can be assessed in a scientific way, without the involvement of moral considerations.

This scientific definition of "animal welfare" told us very little about what animal well-being involves or how it can be assessed or expressed.
The inadequacy of scientific definitions was perhaps best explained by Tannenbaum in 1991, who argued that we cannot treat animal well-being simply as a scientific variable because it was a concept where values are inextricably involved.
Tannenbaum pointed out that well-being is value-laden because it implied something that promote a better or preferable life. Furthermore, well-being

required the “absence of suffering” and the “complete mental and physical health”. Another aspect examined by Tennenbaum is that human values are entwined with the scientific assessment of well-being because the consideration we made depend on what we felt is important for animal well-being. This does not mean that we cannot do objective scientific studies in assessing well-being.

Fraser in 1993 proposed three broad criteria to summarize the think of other workers that explain the deep meaning of animal welfare.

- Well-being implied a high level of biological functioning. The animals should be free from disease, injury, and malnutrition. They should be thriving, without significant checks on normal growth, and without reliance on pharmaceutical intervention to compensate for unsuitable environments. An unresolved issue is whether the animals should also be free from genetic traits, produced by selective breeding, which interfere with normal health and functioning.
- Well-being implies freedom from suffering in the sense of prolonged pain, fear, distress, discomfort, hunger, thirst, and other negative experiences. Short-term negative states, such as short-term pain, hunger, and anxiety, are virtually inevitable in animal life, and the border-line between acceptable and unacceptable will remain a source of debate.
- Well-being implies that the animals should have positive experiences, such as comfort and contentment, and freedom to engage in presumably pleasurable activities, such as play and exploration. This aspect is likely to cause the most controversy. Animal welfare scientists recognize the difficulty of studying positive states such as contentment (Fraser, 1998). Despite these difficulties of we cannot realistically exclude the study of positive state from our criteria for well-being.

For our research the most interesting analysis on animal welfare is understand how it could influence the productivity.

In agricultural contexts, productivity measures such as growth rate or feed intake are traditional indicators of biological functioning, and those who identify well-being closely with biological functioning may argue that high productivity is by itself adequate assurance of a high state of well-being. The link between well-being and productivity is probably very real at low levels of productivity, where depressed health, growth, reproduction, and survival indicate significant problems with normal functioning. Hurnik in 1993, for example emphasized longevity as an integrative measure that serves as an indirect indicator of quality of life. He argued that animal’s quality of life is directly related to the satisfaction of many needs (comfort, health, survival).

Therefore, farmers are not mistaken in claiming that improved survival and growth, connected to changes such as improved nutrition and protection from harsh weather, indicate positive contributions to well-being. However, productivity measures must be used with great caution.
The relationship between productivity and well-being is also obscured by many confounding factors (Sambraus, 1981), including use of antibiotics to avoid disease in sub-optimal environments and strong genetic selection for certain productivity traits which may have weakened the link between these traits and other aspects of biological functioning.
The economic performance, in fact, differs widely from the biological performance optimized by natural selection.

From the veterinary point of view, traditional veterinary studies of the incidence of disease and injury have been indicators of biological functioning (Fraser, 1993).
Unfavorable environmental conditions, incorrect nutritional plan and management errors in general, have caused pathology of different severity in farmed animals.
Pathology is the result of an alteration of the biological functioning. Examples of studies about wellbeing are those conducted by Edwards et al. (1986) or Furniss et al. (1986), that linked respiratory tract lesion to the air quality or those conducted by Anderson et al in 1968 and Muggenburg et al in 1967 that linked gastrointestinal lesion to management and nutritional factors. Furthermore, other studies have shown that the incidence of bovine mastitis was influenced by the physical environment, including stall length and width, drafts, use and type of bedding, use of cow trainers, and restriction of movement by tying or manger design (International Dairy Federation, 1987).

Another important aspect to provide for is that physiological events reflect an animal's emotional state. Selye (1950) described a lot of "stressors" that produce many different characteristic body changes. Some of these "stressors" are physical restraint, exposure to cold, injection of harmful substances. Selye demonstrated that if any of these stressors where applied, there were hypertrophy of the adrenal glands, involution of the thymus and lymph nodes, and gastric ulcers, he grouped these body modifications under the name "General Adaptation Syndrome." Selye theorized that the activation of the anterior pituitary and adrenal cortex, with increased secretion of the hormone ACTH and glucocorticoids, had (in the words of

Mason, 1968) “a unique, preeminent, and non-specific” relation to stress. Thus, it appeared that the General Adaptation Syndrome would reflect both challenges to biological functioning and unpleasant psychological states (Fraser, 1993).
However, this type of explanation was reductive, because there are a lot of controversy like the technical difficulty to identify the secretion of glucocorticoid. As Ladewig et al. (1989) showed, the cortisol secretion is strongly pulsatile and is not clear whether bodily responses to cortisol depend on the height of the peaks, the frequency of the pulses, or the average circulating levels.

Moberg in 1985 and in 1987 described the biological changes induced by “stressors” and supported that them are led to a clear pathology, such as clinical disease, failure of reproduction, or outbreaks of harmful behaviour. Nevertheless, the pathology is usually preceded by some “pre-pathological” condition, the most studied is the reduction of immune competence (Kelley, 1985; Griffin, 1989).
As Fraser stated, the knowledge about the assessment of well-being is only part to resolve animal welfare problems. Farm animal welfare is affected by the five interrelated factors of knowledge, values, technology, economics, and regulation.
Critics of agriculture often claim that animal well-being is being sacrificed in the name of efficiency, mechanization, cost-cutting, and profit. We need more research to help harmonize animal well-being and other important values like systems of raising animals should be ecologically sound and they should produce healthy food products at prices that make them accessible. Researchers need to be proactive to generate the kind of knowledge that will allow regulations to be formulated to be interpretable and of genuine benefit. Society requires significant changes in animal breeding methods and there is not enough behavioural research to answer all the questions we would like to ask about complex physiological responses and body changes to allow for safe conclusions. Hence, we need a mixed program of research whereby scientists, with the guidance of producers, consumers and animal protectionists, proceed to develop and perfect animal rearing methods which will work well for both animals and producers and be perceived as ethically positive by society and therefore sustainable (Fraser, 1993).
This is what Fraser presented to us over 20 years ago, and these statements remain more relevant than ever. His studies on wellbeing are among the cornerstones of modern science and opened the door to what is today's

concept of sustainability. With our research we aim to act as intermediaries between aquaculture production, the global need for more sustainable production in view of the progressive demographic increase, and the need to take care of animal welfare.
The key question that introduces us to the concept of sustainability is the one suggested in 1997 by Becket: How can societies shape their development in such a way to preserve the preconditions of development for future generations?

In contrast to the international discussion concerning the concept of "global change", which is structured by a series of ecological crisis phenomena, like climate change, land waste, water and air pollution, and described in natural science terms, the concept of sustainable development is based on a society-oriented definition of problem, for this reason, putting sustainability concretely into practice requires knowledge about the interactions among society, economy, politics and environment.
In our field of research, the primary thing to take into consideration to make animal rearing more sustainable, in addition to the already widely mentioned animal welfare, is the impelling need for new protein sources.
The benefits of agriculture have been immense, modern agriculture now feeds 6,000 million people. As Tielman says, the global cereal production has doubled in the past 40 years, mainly from the increased yields resulting from greater inputs of fertilizer, water and pesticides, new crop strains, and other technologies of the 'Green Revolution' (FAO, 2001; WHO,1990; Tilman 2002). This has increased the global per capita food supply, reducing hunger, improving nutrition and sparing natural ecosystems from conversion to agriculture (Waggoner, 1995).
By 2050, global population is projected to be 50% larger than at present and global grain demand is projected to double (Cassman, 1999; Alexandratos, 1999). This doubling will result from a doubling food production and sustaining food production. Those are the major challenges and doing so in ways that do not compromise environmental integrity and public health is a greater challenge still (Ruttan, 1999).
The production of 1 kg of meat can require between 3 and 10 kg of grain. During the past 40 years, global per capita meat production has increased more than 60%. Livestock production is becoming an industrial-scale process in which several thousand cattle or pigs, or 100,000 or more chickens, are fed grains and produced in a single facility (Tilman, 2002).

In this regard, there is the need to search for new and more sustainable protein sources to meet the needs of human demographic increase. Currently we are focusing our research on alternative protein sources for feeds production. To study more in dept the impact of feed and the need of new protein sources we must analyse the role of the soybean. Soybean meal (SBM) is the main protein source used for feed composition and is the only protein source used in chicken breeding.

It represented 61% of the proteins used to feed livestock about the two-thirds of the total world output of protein feedstuffs, including all other major oil meals (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). Soybean meal is the by-product of the extraction of soybean oil and it is usually classified for marketing by its crude protein content. There are two main categories, the “high-protein” SBM, with 47-49% protein content and 3% crude fibre, obtained from dehulled seeds, and the “conventional" SBM, with 43-44% protein content, that contain the hulls (Cromwell, 2012). But why is SBM so widely used?

Because in comparison to other plant protein sources SBM has a very high digestibility amino acids, with high content of lysine. This composition allows the formulation of diets with less total protein content, consequently, less excess nitrogen in the feed and for this reason less nitrogen excretion into the biosphere (Pettigrew et al., 2008).

As illustrated above, the demand for meat products is expected to increase from current levels by more than 75% in 2050. It is somewhat disproportional that meat represents 15% of the total energy in the global human diet, while approximately 80% of agricultural land (3,400 million ha as pastures and 500 million ha as crop land) is used for animal grazing or the production of livestock feed and fodder (Herrero et al, 2015; Herrero et al, 2016). Furthermore, about a third of the world’s cereal production is fed to animals (Mottet et al, 2017). The increase in global demand for meat and the restricted availability of land prompt the search for alternative protein sources. Van Huis in 2017 indicate that half of the mitigation potential for agriculture, forestry and land use sectors lies within livestock production. The production of 1 kg of beef requires about 50 times more land than the production of 1 kg of vegetables (Nijdam et al, 2012). Another mitigation measure is using feed with a low environmental impact to produce fish, pigs or poultry. One suggested alternative protein source are insects.

The question we need to answer is: Is the production of insects as an alternative protein source environmentally more sustainable than the faming of other animals (Abbasi et al, 2015; Gahukar, 2016)?
The production of feed is a major driver of environmental impact in conventional livestock systems and insect production systems are no exception. As widely demonstrated by van Huis (2017), insect mini-livestock is a valid method of production of alternative protein sources. It involves a considerable reduction of the land use to breed them, the potential reuse of waste substances comes as their nourishment, a considerable reduction of water consumption, a high protein conversion capacity.
Sustainability in feed production is now closely linked to the concept of waste minimization thas has become a priority. Nowadays, according to the circular economy approach based on the "reduce, reuse, repair, and recycle" theory, wastes from food can be valorised leading to several possibilities to produce proteins (EU 2018; Stevens et al. 2018). This situation leads the possibility to use wastes as substrate for insects' farming.
An article published on the Guardian reports that is estimated for both food, and feed market a 24.4% growth per year over the next decade with a global production of more than 730,000 tonnes in 2030 (EAAP 2019). Recent data indicated an EU insect yearly production of 6,000 tonnes, corresponding to an average of 2,000-3,000 tonnes of insect-derived PAPs (IPIFF 2019; Mancuso et al. 2019). For 2025 the insect proteins production will surpass 1.2 million tonnes reaching about 10% of the EU share of total protein supply (IPIFF 2019; Mancuso et al. 2019).
When compared to chicken, 1 g of edible protein requires two to three times as much land and 50% more water compared to mealworms (Oonincx et al, 2012; Miglietta et al, 2015). A gram of edible protein from beef requires 8–14 times as much land and approximately 5 times as much water com-pared to mealworms.
One of the main reasons why insects are considered as potentially sustainable sources of animal protein is because of their high feed conversion efficiency (Nakagaki et al, 1991).Whereas poultry provided with optimized diets converts 33% of dietary protein to edible body mass, yellow mealworms utilize 22–45% of dietary protein, black soldier fly larvae about half (43–55 %).
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 larvae fly (*Hermetia illucens* (HI)) belongs to the order of Diptera. The life cycle in ideal condition last about 2 months, but if the organic matter is not enough can last up to 4 months (Harduin et al, 2003).
Black soldier fly larvae can be fed with a huge variety of substrates like: fruits, vegetables, coffee bean pulp, distillate grains, fish offal, animal manure, human excreta. Adult animals do not need to be fed, they use the fat reserves accumulated in larval life. 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.
The HI larvae contain 40-44% of crude protein, the amount of fat is extremely variable because it depends of the diet. For example, if the substrate is oil rich food waste, the reared larvae can contain 42-49% of crude lipids (Barry et al 2004). Larvae fed on poultry, swine and cattle manure contain respectively from 15 to 25% (Arango Gutierrez et al, 2004) of crude protein and from 28% to 35% of fat. For the high level in Ca (5-8%) and P (0.6-.0.8%) (Newton et al, 1977; St. Hilarie et al., 2007) Hi meal is suitable for the animals at rapid growth such as broilers. As said above, the fatty acids composition of larvae depends on the fatty acid composition of the diet.
The use of *Hermetia illucens* in animal feed should be taken seriously into consideration, not least because of their reduced environmental impact and to be harmless to human health as they are not pathogen carriers (Newton et al, 1977; Sheppard et al, 1994)

The first trial on the inclusion of *Hermetia illucens* larvae meal on chicken was conducted by Hale in 1973. He replaced soybean meal with insect meal and observed a better weight gain in the broilers fed insect meal than the control group a higher feed intake and so a better feed conversion ratio.
EU legislation authorises the use of proteins from seven insect species for fish feed. Insects have obvious advantages in nutritional value and the amino acid composition of their proteins generally meet animal requirements for good growth and health. Although, in the normal feeding of poultry insects are present, there is still no legislation for the use of insect meal in feed for these species. In this regard, we set out to expand the studies available on poultry production, in order to encourage the use of this new protein source.

Among the various studied insect meals it can be seen how HI meal is the one that most easily adapts to the application in our field of study, for its

protein content, for its calcium content, for the amino acid balance and for its digestibility.
In light of the premises just illustrated, the purpose of my research work is to evaluate feeding strategies to improve the welfare and the sustainability in animal production, and it is based on 4 completed trials. Two feeding trials facing to two different species of marine fish with the aim of testing nutraceuticals in order to improve wellbeing and two feeding trials facing to laying hens with the aim of testing the effect of alternative protein sources with the aim to enhance animal rearing sustainability:

- The first trial "Honey bee pollen in meagre (*Argyrosomus regius*) juveniles diets: effects on growth, diet digestibility, intestinal traits and biochemical markers related to health and stress" was conducted at the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research (Crete, Greece).

- The second "The inclusion of a supercritical fluid extract, obtained from honey bee pollen, in the diet of sea bream (*Sparus aurata*), improves fish immune response by enhancing anti-oxidant and anti-bacterial activities" was carried out in the aquaculture facility of the Department of Veterinary Medicine and Animal Production, Napoli Federico II University (Naples, Italy).

- The third research project "Laying performance, blood profiles, nutrient digestibility and inner organs traits of hens fed an insect meal from *Hermetia illucens* larvae" was carried out for in a private laying hens company located in Sardinia (Italy).

- The fourth trial "Intestinal Morphometry, Enzymatic and Microbial Activity in Laying Hens Fed Different Levels of a *Hermetia illucens* Larvae Meal and Toxic Elements Content of the Insect Meal and Diets" represents the continuation and completion of the study described in the previous paragraph and was carried out in the same private laying hens farm located in Sardinia (Italy) as the previous one.

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Chapter 1

Honey bee pollen in meagre (*Argyrosomus regius*) juveniles diets: effects on growth, diet digestibility, intestinal traits and biochemical markers related to health and stress

Panettieri V., Chatzifotis S., Messina C.M., Olivotto I., Manuguerra S., Randazzo B., Ariano A., Bovera F., Santulli A., Severino L., Piccolo G. (2020) Honey bee pollen in meagre (*Argyrosomus regius*) juveniles diets: effects on growth, diet digestibility, intestinal traits and biochemical markers related to health and stress. Animals 2020, 10, 231; doi:10.3390/ani10020231

1.1 Simple Summary

Recently, several studies have focused on the use of nutraceuticals and honey bee products to improve the welfare and sustainability of animal husbandry. Honey bee pollen is rich in bioactive substances, presenting a strong antioxidant activity with possible positive effects on growth performance and non-specific immune responses in reared fish. Despite its favorable characteristics, the addition of honey bee pollen to a meagre (Argyrosomus regius) diet in our trial resulted in a reduction of growth performances and diet digestibility, histological alterations of intestinal morphology, and high levels of biomolecular stress markers, probably due to its complex ultrastructure, which is indigestible for monogastric animals. These negative effects could be overcome by using bioactive component extraction methods and thus eliminating the indigestible fractions. Our results confirmed the general assumption that it should always be considered necessary to test nutraceutical additives of natural origin in a given species in order to verify the effective positive action and exclude any negative repercussions on animal health.

1.2 Abstract

This research aimed to evaluate the e ects of the inclusion of honey bee pollen (HBP) in meagre (Argyrosoumus regius) juveniles' diets on growth performance, diet digestibility, intestinal morphology, and immunohistochemistry. Furthermore, stress-related molecular markers and biochemical blood profile of fish were evaluated, together with mineral trace and toxic elementconcentration in pollen and diets. Specimens of meagre (360) of 3.34 ± 0.14 g initial body weight, were randomly allocated to twelve 500 L circular tanks (30 fish per tank). Four diets were formulated: a control diet and three experimental diets with 1%, 2.5%, and 4% of HBP inclusion. All the growth parameters and crude protein and ether extract digestibility coeffcients were negatively linearly affected by increased HBP inclusion ($p < 0.0001$). Histology of medium intestine showed slight signs of alterations in group HPB1 and HPB2.5 compared to control. Fish from HBP4 group showed severe alterations at the intestinal mucosa level.

Immunohistochemical detection of TNF-α in the medium intestine showed the presence of TNF-α+ cells in the lamina propria, which resulted in accordance with the increased level of the TNF-α protein detected by immunoblotting in the liver. This stress situation was confirmed by the increased hepatic level of HSP70 ($p < 0.05$) in fish fed the HBP4 diet and by the linear decrease of total serum protein levels in HBP-containing diets ($p < 0.0001$).

These negative effects can be related to the ultrastructure of the bee pollen grain walls, which make the

bioactive substances unavailable and can irritate the intestine of a carnivorous fish such as meagre.

1.3 Introduction

In the past 20 years, researchers have focused their attention on several natural molecules to be used as possible antioxidant therapeutic and preventive agents (Ferreira et al. 2006).
A growing amount of scientific evidence is demonstrating that supplementing diets with natural compounds that act as protective factors or immunostimulants can improve growth performance, product quality, fish health, and physiological response to stress situations and diseases (García Beltrán et al., 2019; Guardiola et al., 2017; Bahi et al., 2017; Bulfon et al. 2015; Messina et al., 2015).
Bee-derived products have been widely investigated for their positive characteristics, such as their strong antioxidant activity, their positive e ects on non-specific immune responses, and their capacity to increase growth performance in various animal species (Babaei et al., 2016; El-Asely et al., 2014; Attia et al., 2010). Research has been carried out to test the inclusion of these nutraceutical products in aquafeeds because of their antioxidant potential to prevent or treat aquatic animal diseases and to increase their performance (Choobkar et al., 2017; LeBlanc et al., 2009; Thorp et al., 2000).
Honey bee pollen (HBP) consists of the male generative cells gathered by honeybees from flower stamens and the anthers of flowers, which are collected by foraging bees and carried to the hives, where pollen agglutinates with bee secretions and the addition of nectar (LeBlanc et al., 2009; Thorp et al., 2000). The consumption of HBP has increased in the recent years because it has been considered a healthy and therapeutic product because of its nutritional properties, source of proteins, lipids, vitamins, minerals, amino acids, carotenoids, flavonoids, phenolic compounds, antioxidants, carotenes, and xanthophylls (Campos et al., 2008; Jean-Prost et al., 2007). Phenolic acid, flavonoids, and tannins act as potent antioxidants and protective agents and their free radical scavenging activity encourages their application in the biomedical field (Attia et al., 2014; Mărghitaş et al., 2009).
The introduction of HBP in freshwater fish diets has led to improved growth and immune status (Abbass et al., 2012), increase in the number of phagocytic cells, (i.e., neutrophils and monocytes), and reduction of the mortality caused by Aeromonas hydrophila in Nile tilapia (Oreochromis niloticus) (El-Asely et al., 2014).

Despite these favourable characteristics, no research has been carried out to evaluate the effects of the inclusion of HBP in diets for marine fish species. The present research intended to fill this gap, evaluating growth performance, diet digestibility, intestinal morphology and immunohistochemistry, stress-related molecular markers, and biochemical blood profile in meagre (Argyrosoumus regius) juveniles fed diets containing increasing levels of commercial HBP. In addition, the research aimed to contribute to the knowledge of the mineral trace element concentration in honey bee pollen and in fish diets.

1.4 Materials and methods

A chestnut honey bee pollen (HBP) purchased from a local organic farm located in the city of Naples (Napoli, Italy) was chosen for the research. Both growth and digestibility trials were conducted at the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research (Crete, Greece). The experimental protocol was designed according to the guidelines of European Directive (2010/63/EU) on the protection of animals used for scientific purposes. The digestibility trial was approved by the experts of ASSEMBLE Plus project (Contract number: SR020220189047). The growth trial procedures and the blood sampling were approved by Ethics experts of the TNA selection panel of the AQUAEXCEL 2020 (project number: AE040069).

1.4.1 Growth trial

1.4.1.1 Fish and Experimental conditions

Meagre juveniles were obtained from the IMBBC hatchery. After a 2-week period of acclimation to the experimental conditions, three hundred and sixty fish of 3.34 ± 0.14 g initial body weight were individually weighted under moderate anaesthesia conditions (used anaesthetic: MS222-Tricain Methanesulphonate at 50 mg/L dosage) and randomly allocated in twelve 500 l circular tanks (30 fish per tank) supplied by flow-through borehole aerated seawater (renewal 200% per hour).
The trial was conducted from July to October 2018 under constant temperature (19.0 ± 0.5 °C), salinity (36‰), and dissolved oxygen (DO 8 ± 0.5 ppm) and a dark:light cycle of 12 : 12 hours.

1.4.1.2 Fish diets

To determine the effects of the inclusion of HBP, four isonitrogenous (53% crude protein (CP) on as fed basis),isolipidic (15% ether extract (EE) on as fed basis) and isocaloric diets were formulated to meet the meagre nutrients and energy requirements according to Chatzifotis et al. (2010): a control diet with no addition of HBP(HBP0) and three experimental diets in which HBP was included at 1% (HBP1), 2.5% (HBP2.5) and 4% (HBP4) as fed basis.
The experimental feeds were prepared at the IMBBC laboratory. All dietary ingredients were finely ground and thoroughly mixed; water was then

blended into the mixture to obtain an appropriate consistency for pelleting using a 2.5 mm die meat grinder. After pelleting, the diets were dried in a ventilated oven at 40°C for 24 h and stored in plastic bags until used.
Chemical composition of the experimental diets was determined as follows: Dry Matter (DM), Ash, Crude Protein (CP), Ether Extract (EE) and Crude Fibre (CF) (procedure numbers 934.01, 942.05, 954.01, 920.39, and 962.09, respectively) according to AOAC [19]. Before starting the analysis, the pellets were finely ground using a cutting mill. The ingredients and proximate composition of the diets are reported in Table 1.1 and the proximate composition of the bee pollen is reported in Table 1.2.

Table 1.1 Ingredients and chemical composition of experimental diets for meagre

Ingredients, g/kg	**HBP0**	**HBP1**	**HBP2.5**	**HBP4**
Fish Meal	440	438	435	430
Soy Bean Meal	240	238	235	230
Corn Gluten Meal	100	98	96	95
Wheat Flour	100	98	93	90
Fish oil	100	98	96	95
Honey Bee Pollen	0	10	25	40
Mineral mix	10	10	10	10
Vitamin mix	10	10	10	10
Chemical Composition				
Dry matter %	90.0	92.7	90.6	93.0
Ash %	9.4	9.8	9.0	9.4
Crude Protein %	53.0	53.6	52.5	52.5
Ether Extract %	15.0	14.9	15.0	14.8
Crude Fibre %	7.7	7.7	7.7	7.5
Na %	0.086	0.075	0.058	0.075
Mg %	0.130	0.117	0.131	0.125
K ppb	381.65	351.92	381.93	377.90
Ca ppb	870.39	696.49	456.83	562.36

The experimental diets were randomly assigned to triplicate groups of 30 fish.
Each diet was administered three times per day (09:00 h; 12:00h and 16:00 h) to apparent satiety (until the first pellet was refused), 7 days per week. Any not-ingested pellet was recovered, dried and weighed. The exact quantity of feed distributed in each tank was recorded daily. The trial lasted 88 days.

Table 1.2 Chemical composition of honey bee pollen

	DM(%)	Ash(%)	CP(%)	EE(%)	CF(%)	Na(%)	Mg(%)	K(%)	Ca(%)
HBP	85.02	3.79	23.18	7.82	19.23	0.067	0.062	0.019	0.061

Abbreviations: HBP: honey bee pollen; DM: dry matter; CP: crude protein; EE: ether extract; CF: crude fiber

1.4.1.3 Trace Elements

The trace elements contained in the honey bee pollen and in the diets were also determined. Before analysis, the samples (0.5 ± 0.02 g) were placed in a Teflon vessel with 5.0 ml of 65% HNO3 and 2.0 ml of 30% H2O2 (Romil UpA). The vessel was sealed and placed in a microwave digestion system (Milestone, Bergamo, Italy). Microwave-assisted digestion was performed with a mineralization program for 15 min at 200°C. The vessel was then cooled at 30°C, the digestion mixture was transferred into a 50.0 ml flask and the final volume was obtained by adding Milli-Q water (Ariano et al., 2019). Trace elements concentrations were determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) technique using a Perkin Elmer Optima 2100 DV instrument coupled with a CETAC U5000AT. The calibration curve and two blanks were run during each set of analyses, to check the purity of the chemicals. A reference material (CRM DORM-4, National Research Council of Canada (NRC-CNRC), Ottawa (Ontario), Canada) was also included for quality control. All the values of the reference materials were within the certified limits. The instrumental detection limits are expressed as wet weight (w.w.) and were determined following the protocol described by Perkin Elmer ICP application study number 57 (Bernard et al., 1993).

1.4.1.4 Growth performance

At the end of the trial, fish were starved for 1 day, lightly anesthetised (MS222-Tricain Methanesulphonate at 50 mg/L dosage) and group weighed.
The following growth performance indexes and protein efficiency ratio (PER) were calculated according to the following formulas (Piccolo et al., 2017):

- Weight gain (WG%) =100 × [(FBW, final body weight (g) − IBW, initial body weight (g))/initial live weight (g)]

- Daily intake rate (DIR, %/day) = 100× [(feed intake (g)/mean weight (g))/days]
- Specific growth rate (SGR, %/day) = [(lnFBW − lnIBW)/number of feeding days] × 100
- Feed conversion ratio (FCR) = [total feed supplied (g)/weight gain (g)]
- Protein efficiency ratio (PER) = [weight gain (g)/total protein fed (g)]

1.4.2 Somatic traits

At the end of the feeding trial, 45 fish per treatment (15 fish per replicate) were randomly chosen, weighed and sacrificed by over anaesthesia (MS222-Tricain Methanesulphonate at 250 mg/L dosage) and dissected. Liver and gut were weighed to determine Hepatosomatic (HSI) and Viscerosomatic (VSI) indexes as described in Piccolo *et al.* (2013). Liver, muscle and intestine of 4 fish for each tank were sampled.

1.4.3 Digestibility trial

Dry matter, protein and lipid digestibility of the tested diets was evaluated in a separate trial. One hundred and eighty meagre of 23.53 ± 2.16 g initial weight obtained from the IMBBC hatchery were distributed in 12 circular fiberglass tanks (3 tanks per treatment; 15 fish per tank) of 270 l equipped with a settling column. The water and environmental conditions were the same as described for the growth trial.
Diets were prepared at the IMBBC laboratory following the same procedure described above and using the same inclusion levels of HBP (1%, 2.5%, 4%). The apparent digestibility coefficients were measured using the indirect acid-insoluble ash (AIA) method, for this reason, 1% celite® (Fluka, St. Gallen, Switzerland) was added to the diets used in the growth trial as an inert marker.
Fish were fed the experimental diets ad libitum 3 times a day for 4 weeks. Faeces accumulated in the settling column were collected daily before the morning meal, centrifuged at 7000 rpm for 10 min and stored at -20°C until analysis.
30 minutes after the last feeding in the afternoon, tanks were cleaned to remove excess of faeces, and then the settling column was placed.

Feeds and faeces were analysed according to AOAC (2004). The AIA contents of feeds and faeces were determined according to Vogtmann *et al*. (1975).
The apparent digestibility coefficients of dry matter (ADC DM), crude protein (ADC CP), ether extract (ADC EE) were calculated following Palmegiano et al. (2006)

1.4.4 Histology

For the histological analysis, segments of medium intestine were isolated from growth trial fish and immediately fixed in modified buffered Karnovsk fixative for at least 24 hours. After fixation, samples were dehydrated in graded alcohol solutions, cleared in xylene and embedded in solid paraffin. Cross sections of 5 µm (cut with a LEICA microtome) were stained with Mayer's haematoxylin and eosin Y and examined under a Zeiss Axio Imager.A2 microscope; images were acquired by mean of a combined colour digital camera Axiocam 503 (Zeiss). For the morphometric evaluation of intestinal folds height, and mucous cells abundance, 9 fish for each treatment (3 for each tank) were considered. Three sections for each fish were observed at an interval of about 200 µm in order to avoid repetitions in morphometric measurements of folds and quantification of mucous cells. Intestinal folds were measured from the apex to the base excluding the underlying connective layer and values were expressed by mean and SD.

1.4.5 Immunohistochemistry

For the immunodetection of TNF-α in the medium intestine, 9 fish for each treatment (3 for each tank) were used and sections (5 µm) were placed on gelatinized slides (0.5% fish gelatin, 0.05% chromopotassium sulfate) in order to prevent sections detachment during the reaction. Sections were deparaffinised and rehydrated through serial graded ethanol solutions. Endogenous peroxidase activity was blocked by treating sections with 0.3% hydrogen peroxide for 10 min at room temperature. To prevent aspecific antibody binding, slides were rinsed in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 15 min and blocked using 5% BSA in Tris for 20 min. After rinsing in PBS for 15 min, sections were incubated overnight at 4°C with anti-TNF-α rabbit polyclonal primary antibody specific for zebrafish (ANASPEC). After rinsing the slides with PBS, the sections were incubated

with the secondary fluorescent antibody (Goat Anti-Rabbit IgG H&L, Alexa Fluor 488 No. ab150077; Abcam, dilution 1:400) at room temperature for 1 h and 30 min. Following incubation, the slides were mounted with Fluoreshield Mounding Medium with DAPI (ab104139) for nuclei staining. The sections were observed under a Zeiss Axio Imager. M2 microscope and images were captured with a high-resolution camera Zeiss Axiocam 105 color. Negative controls were obtained by the incubation without primary antibody. For the relative quantification of TNF-α+ cells three sections from three different fish for each treatment were analyzed. Observations were performed counting TNF-α+ labeled cells in three undamaged folds for each section. An arbitrary unit was adopted on the mean of the count: 0 - 20 = +, 20 - 50 = ++, 50 - > 100 =+++.

1.4.6 Extraction of total proteins and detection of hepatic biomolecular markers by immunoblotting

For total proteins extraction, aliquots of lyophilized livers were homogenized on ice (1:3 w/v) with 100 mM HEPES pH 7.4, 10 mM EDTA, 10 mM EGTA and 4 M NaCl 23.37 g / 100 ml, in presence of 10 µl proteases inhibitor mix (NaF 42 mg/ml, Aprotinine, Na3PO4 183.9 mg/ml, Leupeptine 4X). The homogenate was centrifuged at 6000 rpm at 4°C for 15 minutes and the supernatant was stored in ice for protein quantification according to Lowry et al. (1951). Biomolecular markers in liver were analysed by immunoblotting.
Equivalent amounts of proteins (30 µg) diluted with Laemmli buffer (1970) (Sigma-Aldrich), were loaded, after protein denaturation for 5 min at 90 °C, on pre-cast gel for SDS–polyacrylamide electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA) and blotted using a Trans Blot Turbo Transfer System (Bio-Rad). The correct amount of protein loaded was confirmed by red Ponceau staining. Filters were used for protein detection by primary antibodies (AbI) specifics for TNF-α (Ab polyclonal from rabbit) and HSP70 (Ab monoclonal from rabbit). (Sigma-Aldrich, Dorset, UK; Santa Cruz, CA, USA). The primary antibodies were diluted in buffer at the concentrations suggested by the manufactures for each AbI. In relation to the origin of the AbI, the appropriate secondary antibodies were used (anti mouse or anti-rabbit secondary) as mean of detection the secondary antibodies were conjugated with horseradish peroxidase (GAR/M-HRP Bio-Rad, Hercules, CA, USA) or alkaline phosphatase. The signals originated by immunoreaction were detected using enhanced chemo-luminescent

(ECL) reagents (Clarity Western ECL Blotting Substrate, Bio-Rad, CA) and a BCIP/NBT substrate system (Bio-Rad). Images were obtained, photographed and digitalized with Chemi Doc XRS (Bio-Rad, Hercules, CA, USA), and further analysed with Image Lab software (Bio-Rad, Hercules, CA, USA). The results were expressed as fold increase of each treatment in relation to the respective control; the images shown are representative of almost three separate experiments, for which the mean quantification is reported in each figure, together with the significance of the differences ($p < 0.05$).

1.4.7 Assessment of biochemical parameters in blood samples

Biochemical measurements were performed on blood samples from fish 2 months old of the species *Argyrosomus regius*, within a weight 20-25 g. The purpose of the test was to evaluate the effect of bee pollen enrichment in accordance with the requirements of the AquaExcel2020 program.
On the day of the sampling, fish were euthanized, and their weight and length were measured. Immediately, blood was collected from the caudal vessel via heparinized syringes. Hematocrit was measured, using capillary tubes and a specialized centrifuge to determine the percentage of red blood cells by volume. Additionally, hemoglobin was determined using a colorimetric assay kit (Spinreact, Spain).
Following these procedures, blood samples were centrifuged at 2,000 x g for 10 minutes and the resulting plasma was stored at - 20 °C until further analyzed. Plasma samples were used to assess the following biochemical parameters: cholesterol, triglycerides, albumin, total proteins, glucose, creatinine, sodium, potassium, calcium, phosphorus, lactate, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GTP).
More specifically, plasma cholesterol (CO/PAP, Biosis, Greece), triglycerides (GPO/PAP, Biosis, Greece), albumin (BCG, Biosis, Greece), total serum proteins (Biuret, Biosis, Greece), glucose (GOD/PAP, Biosis, Greece), calcium (Arsenazo III, Biosis, Greece), phosphorus (UV, Biosis, Greece), lactate (LO-POD, Spinreact, Spain), creatinine (Cayman Chemical Company, USA) concentrations were assessed using commercial enzymatic colorimetric kits. Moreover, GOT-AST (IFCC/LIQUID, Biosis, Greece), GPT-ALT (IFCC/LIQUID, Biosis, Greece) concentrations were estimated using enzymatic kinetic assays. Finally, a flame-photometer was used to determine plasma sodium and potassium concentrations.

2.8 Statistical Analysis

The data were tested for normal distribution and equal variances before analysis (Steel and Torrie, 1980; SAS, 2000). All the data were analysed by one-way ANOVA, using the GLM procedure of SAS (2000), according to the model:

$$Yij= m +Di + eij,$$

For growth and digestibility trials the experimental unit was the tank and each value was obtained as average of the 30 fishes of each tank. For somatic indexes experimental unit was the individual fish (15 fish for each tank). Finally, for the blood parameters, the unit derives from the average of 4 fish per tank.
In addition, the mean comparison has been performed using orthogonal contrast analysis. The examined component were linear and quadratic (Steel and Torrie, 1980; SAS, 2000). $P < 0.05$ was considered the threshold for statistical significance.

1.5 Results

1.5.1 Trace elements

The Table 1.3 shows the content of toxic and essential trace elements in experimental feeds and honey bee pollen

Table 1.3 Toxic and essential trace elements in experimental feeds and honey bee pollen.

Trace elements (ppb)	HBP0	HBP1	HBP2.5	HBP4	Honey bee pollen
Fe	499.27	490.53	511.96	512.95	134.63
Cu	40.17	38.13	44.73	38.86	6.16
Zn	186.06	178.50	193.95	191.28	29.77
Co	0.15	0.15	0.15	0.15	0.02
Al	62.98	46.01	56.82	66.21	58.13
Se	0.55	0.53	0.54	0.57	1.85
Mn	259.04	262.06	257.79	268.78	31,73
Pb	0.22	0.23	0.23	0.24	0.16
Cd	0.12	0.10	0.11	0.11	0.03
Cr	0.81	0.79	0.83	0.82	0.30
Ni	1.92	1.81	1.90	1.88	0.34
As	2.45	2.23	2.41	2.35	0.59

1.5.2 Growth performance

In Table 1.4, the growth parameters measured during the trial are reported.

Table 1.4 Growth Performance of meagre fed experimental diets

						P values		
						Contrast analysis		
	HBP0	HBP1	HBP2.5	HBP4	RMSE	Linear[1]	Quadratic[1]	Cubic[1]
Initial BW (g)	3.35	3.34	3.34	3.34				
Final BW (g)	26.78	23.29	22.69	21.36	0.66	<0.0001	<0.0001	0.0659
FCR	0.96	1.04	1.18	1.23	0.06	0.0007	0.0003	0.3186
SGR	2.37	2.21	2.18	2.11	0.08	0.0044	0.0062	0.4158

Intake‰ ABW/d	16.48	17.23	19.08	19.43	0.98	0.0061	0.0025	0.336
WG%	705.3	606.9	579.7	539.7	52.9	0.0050	0.0059	0.5564
PER	1.96	1.80	1.61	1.55	0.10	0.0008	0.0004	0.5619

Abbreviations: Initial BW: initial body weight; Final BW: final body weight; FCR: feed conversion ratio; SGR: specific growth rate; Intake‰ ABW/d: intake per kg adjusted body weight; WG%: weight gain percentage per initial body weight; PER: protein efficiency ratio. [1] Contrast Analysis.

No significant differences in somatic indexes emerged among the fish fed the experimental diets, except for the gut length that showed a linear increase with the increasing of honey bee pollen inclusion in the diet.
All the growth parameters were significantly influenced by the diet. Daily feed intake increased as bee pollen inclusion in the diet increased ($p < 0.01$), while final weight ($p < 0.0001$), FCR ($p < 0.001$), SGR ($p < 0.01$), PER ($p < 0.001$) and WG %ABW ($p < 0.01$) were negatively linearly affected by bee pollen inclusion in the diet. The growth parameters reached the worst values in the HBP4 diet.

1.5.3 Somatic indexes, slaughter traits

In Table 1.5 the viscerosomatic indexes and the slaughter traits are shown.

Table 1.5 Somatic indexes of meagre fed experimental diets

					P values			
						Contrast analysis		
	HBP0	**HBP1**	**HBP2.5**	**HBP4**	**RMSE**	**Linear[1]**	**Quadratic [1]**	**Cubic[1]**
F. Weight	37.4	33.9	36.8	33.9	4.41	0.0584	0.316	0.037
VSI%	4.37	4.27	4.20	4.11	0.70	0.3299	0.333	0.9185
HIS%	1.62	1.69	1.67	1.72	0.36	0.4245	0.517	0.6901
K,	1.17	1.05	1.06	1.06	0.13	0.0397	0.078	0.3916
Gut length %BW	37.50	45.64	40.09	44.05	7.46	0.0413	0.282	0.0206

F. weight: final body weight; HIS: Hepatosomatic index; VSI: Viscerosomatic index. K: condiction factor; [1] Contrast analysis.

No significant differences in somatic indexes emerged among the fish fed the experimental diets, except for the gut length that showed a linear increase with the increasing of honey bee pollen inclusion in the diet.

1.5.4 Digestibility trial

The estimated ADC of DM, CP, and EE of the four diets are reported in Table 1.6.

Table 1.6 Digestibility trial results

					P values			
						Contrast analysis		
	HBP0	**HBP1**	**HBP2.5**	**HBP4**	**RMSE**	**Linear[1]**	**Quadratic[1]**	**Cubic[1]**
ADC DM	64.24	74.28	36.03	45.63	0.82	<0.0001	<0.0001	0.0001
ADC CP	90.26	91.36	78.71	78.56	0.76	<0.0001	<0.0001	0,0003
ADC EE	93.45	92.15	85.725	85.91	0.91	<0.0001	<0.0001	<0.0001

ADC DM: dry matter apparent digestibility coefficient; ADC CP: crude protein apparent digestibility coefficient; ADCEE: ether extract apparent digestibility coefficient. [1] Contrast analysis.

ADC DM linearly decreased as the HBP content in the diets increased. Furthermore, linear and quadratic components indicated a gradual reduction of crude protein and ether extract digestibility coefficients going from the control to HBP4 diet. Nevertheless, the ADCs of HBP1 diet resulted higher than those shown by HBP2.5 and HBP4 groups as indicated by the quadratic component of the variance ($P < 0.001$).

1.5.5 Histology

Histology of medium intestine showed slight signs of alterations in group HPB1 and HPB2.5 if compared with HBP0 group. No significant di erences were shown by the morphometric evaluation of intestinal folds among experimental groups (Table 1.7).

Table 1.7. Morphometric evaluation of mucosal folds.

	MF Height (_m)
HBP0	462.3±18,7
HBP1	487,8±23.5
HBP2,5	453.1±24.8
HBP4	476.6±46.4

MF height: mucosal fold height. Data are expressed as mean and SD.

A significant linear increase in mucous cells in response to pollen inclusion was observed (Figure 1.1).

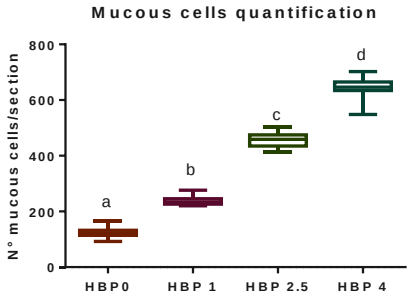

Figure 1.1. Abundance of mucous cell in medium intestine. The number of mucous cells is reported as mean of the observations performed on three trasversal intestinal sections from individual fish (n=9) from the different experimental groups. Different letters indicate ignificant differences among groups ($p<0.05$).

The most frequently encountered alterations consisted in leucocyte infiltration at the level of the lamina propria and thickening of the submucosa (Fig. 1.2g). Intestine samples from HBP4 group showed severe alteration at the level of the mucosa even preserving epithelial integrity. In this group, a substantial increase in the number of mucous cells was observed (Figure 1.1 and Figure 1.2). Moreover, melano macrophage-like, melanin-containing cells, were often observed at the base of the epithelial layer and infiltrating lamina propria and submucosa (fig. 1.2h).

Figure 1.2 Intestinal histology of meagre fed experimental diets after 88 days of trial

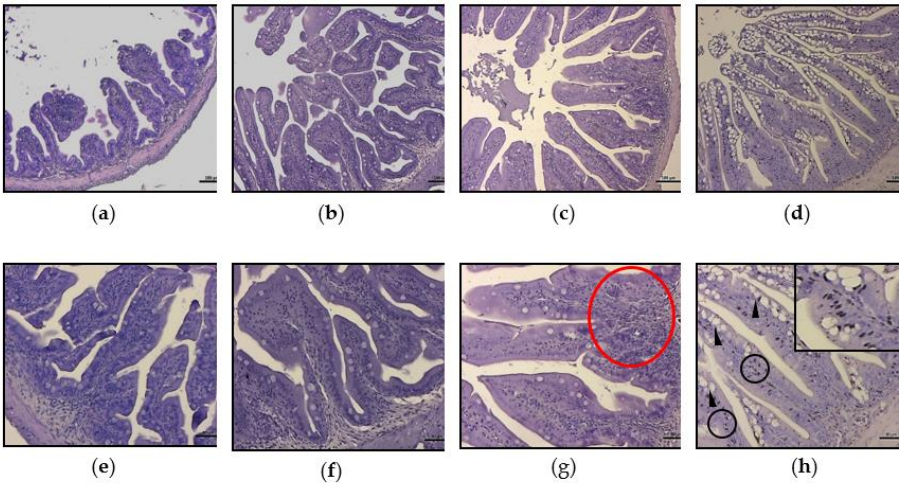

Figure 1.2 Histology of *A. regius* medium intestine from the different experimental groups: a, e) normal histology from HBP0 group; b, f) HBP1 group did not shown signs of pathological

alteration; c, g)in HBP2.5 group was possible to detect inflammatory influx in some portions of the intestinal submucosa (red circle); d, h) HBP4 group showed a severe degree of mucipar hyperplasia of the mucosa (arrowhead) with an abundant presence of melano macrophages at the base of the enterocytes (black circle and box). Scale: a, b, c, d = 200 µm; e, f, g, h = 50 µm.

1.5.6 Immunohistochemistry

Immunohistochemical detection of TNF-α in medium intestine showed the presence of TNF-α+ cells in the lamina propria and submucosa of bee pollen treated fish. While a weak reaction was recorded in the epithelial layer of intestinal folds in all treated groups, a moderate influx of TNF-α+ cells was recorded in particular in groups HBP2.5 and HBP4 (Figure 1.3). In particular round macrophage/neutrophil-like TNF-α+ cells were observed in the submucosa and lamina propria of HBP1, HBP2.5 and HBP4 groups. The relative abundance evaluation of TNF-α + cells infiltrating submucosa and lamina propria showed low values in HBP1, while HBP2.5 and HBP4 showed high values with no significant differences between the two groups (Table 1.8) at base of the epithelial layer and infiltrating lamina propria and submucosa.

Figure 1.3 Intestinal immunohistochemistry

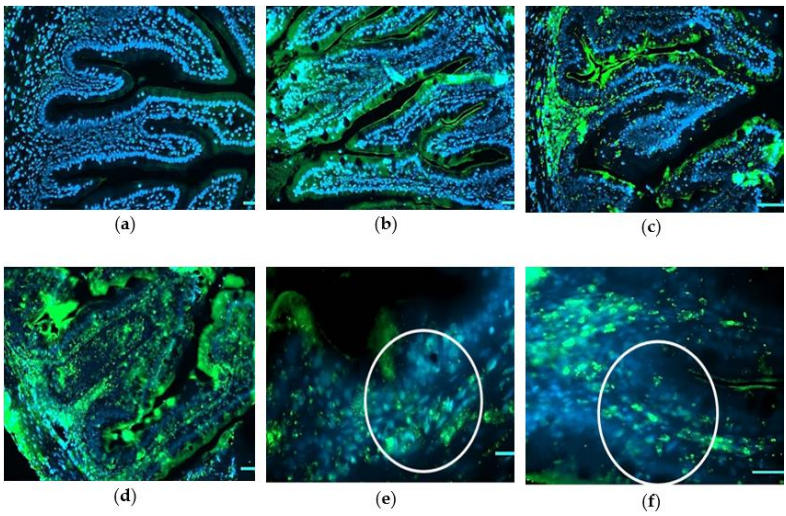

Figure 1.3. Immuno-histochemical detection of TNF-α in medium intestine of the different experimental groups (a,b,c,d). High magnification images showing TNF-α+ cells (white circle) in the submucosa and in the lamina propria of intestine from HBP2.5 (e) and HBP4 (f) groups. Positive

reaction appears in green. Nuclei were stained with DAPI (blue). Scale bar: a,b,c,d= 50µm; e,f= 20µm.

Table 1.8 Relative density of TNF-α + cells in medium intestine

CTRL	HBP1	HBP 2.5	HBP 4
+	+	+++	+++

1.5.7 Hepatic biomolecular markers

The evaluation of the molecular markers related to immunostimulation and stress resistance showed that the levels of the protein TNF-α presented a significant increase only in fish fed the diet with the highest level of HBP inclusion (HBP4) ($p < 0.05$), while HSP70 resulted increased both in HBP2.5 and HBP4 groups compared to the control group (Figure 1.4).

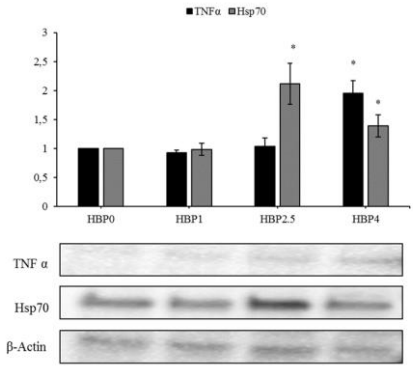

Figure 1.4. Immunoblot of TNFα, HSP70, evaluated on samples of the liver of *A. regius* fed on three different diets containing increasing levels of commercial HBP (HBP1, HHBP2.5, HBP4) and in standard condition (HBP0). β-Actin was used as the internal control. The images are representative of at least three separate experiments. The relative protein quantification is represented in the graphic (* $p < 0.05$ vs HBP0).

1.5.8 Assessment of blood samples

The results obtained from the blood analysis are shown in Table 1.9.

Table 1.9. Biochemical assessment of blood samples

						p value		
						Contrast analysis		
	HBP0	**HBP1**	**HBP2.5**	**HBP4**	**RMSE**	**Linear**	**Quadratic**	**Cubic**
HCT,%	28.67	26.00	27.00	26.67	3.19	0.46	0.62	0.56
HGB, g/dl	3.83	3.80	3.76	3.56	0.40	0.43	0.45	0.88
ıcose, mmol/L	4.71	5.21	4.92	5.96	1.20	0.24	0.34	0.51
TSP, g/dl	10.20	8.15	6.53	3.73	0.98	<0.0001	<0.0001	0.58
Albumin, mg/dl	1.61	1.91	1.38	1.64	0.60	0.94	0.60	0.33
Globulin, mg/dl	8.56	5.72	5.14	2.07	1.27	0.0004	0.0007	0.2331
A/G	0.19	0.43	0.36	0.83	0.24	0.01	0.03	0.27
Creatinine, µmol/L	80.00	132.50	94.67	104.33	16.51	0.16	0.76	0.03
GOT, UI/L	360.00	345.00	355.67	243.33	67.7	0.11	0.16	0.48
GPT, U/L	335.50	298.50	273.33	386.67	49.45	0.30	0.48	0.41
Triglyceride, mmol/L	13.83	16.43	15.10	17.10	2.45	0.14	0.28	0.28
Cholesterol, mg/dl	6.63	7.63	9.55	6.90	0.91	0.73	0.19	0.07
P, mg/dl	7.57	9.50	9.77	11.90	4.63	0.29	0.32	0.78
Ca, mg/dl	27.07	30.87	22.53	33.47	7.31	0.32	0.75	0.14
Na, mmol/L	210.33	179.67	206.67	187.00	24.35	0.27	0.67	0.14
K, mmol/L	2.70	2.17	2.07	2.53	0.73	0.79	0.75	0.95
Lactate, mmol/L	3.17	4.15	2.75	3.3	0.73	0.83	0.45	0.11

HCT: hematocrit; HGB: hemoglobin; TSP: Total Serum Protein; A/G: albumin globulin ratio; GOT: glutamic oxaloacetic transaminase; GTP: glutamic pyruvic transaminase. statistically different.

This table highlights the differences among the groups. Total serum protein, globulin and albumin globulin ratio, linearly and quadraticly decreased as the HBP content in the diets increased.

1.6 Discussion

The trial results showed that honey bee pollen induced negative effects on meagre growth performance and diets digestibility. These data are not in line with the current, although limited bibliography, in which bee pollen had notable beneficial effects in other fish species as described by El-Asely et al., (2012) for Nile tilapia and Choobkar et al., (2017) for rainbow trout, even if it must be remarked that these latter authors used a bee pollen alcoholic extract.

The bad results obtained with the in vivo results can be explained by looking at the other performed investigations. The intestinal histology evidenced pathological alteration of the mucosa morphology showing a severe mucipar hyperplasia and an abundant presence of melano macrofage-like cells at the base of the enterocytes. These alterations became more evident as the inclusion of bee pollen in the diets increased where, besides the inflammatory influx, the migration and infiltration of melanin-containing cells indicate a further, extreme index of an immune response. The presence of TNF-α cells, resembling in shape neutrophil cells or macrophages, in fish fed different HBP inclusion corroborated the histological results. Tumor necrosis factor-alpha is an important pro-inflammatory cytokine playing an important role in cell survival through the activation, proliferation, differentiation of macrophages and released by several immune cells during infection and tissue damage and its inhibition increases the susceptibility to disease and a reduced capability to resolve infection (Hong et al., 2013; Roca et al., 2008; Johnston et al., 2006). TNF-α isoforms have been identified in various fish species (Hong et al., 2013; Roca et al., 2008; García-Castillo et al., 2002), showing high levels of conservative regions with mammals and a constitutive expression in healthy fish tissues (Roca et al., 2008). Inflammation is a high energy-demanding process and it has been widely shown that intestinal inflammation correlated to anti-nutritional factors in fish diet is able to affect growth in fish (Wang et al., 2017; Zhong, et al., 2019).

Regarding the variation of the liver biomolecular markers related to general stress response and immunostimulation, also in this case the increase of TNF-α could be ascribed to a significant production of mediators of inflammation, acting a strategy of protection (Chang et al., 1992). The positive modulation of TNF-α was observed in specimens of Cyprinus carpio treated, during vaccination, with Aloe vera and also on immunity response in poultries, mice and humans (Abdy et al., 2016). In that case, the

effects were attributed to some, not well defined, compounds of the plant (Abdy et al., 2016).

Members of the HSP70 family are widely used as biomarkers of environmental stress in ecological and toxicological studies in fish (Girilal et al., 2015; Metzger et al., 2016; Radons et al., 2016).

The functions of the different HSP70 family members depend on their cellular localization, acting both as chaperons and immunomodulators (Radons et al., 2016), with the general aims to maintain the protein homeostasis (Zuo et al., 2016), in normal and stressful conditions. HSP70 can be induced in response to thermal stress, hypoxia, oxidative stress, ultraviolet radiation, nutrient deprivation, osmotic pressure, heavy metals, chemical agents, microbial infections and inflammation (Radons et al., 2016; Zuo et al., 2016; Han et al., 2016; Teigen et al., 2015).

The levels of HSP70 protein increased in fish fed with the highest doses of bee pollen inclusion (HBP2.5 and HBP4) (Figure 1.4) indicating a stress condition that could be related to metabolic or oxidative stress. In fact, this protein is normally present at a low level in organism with a good balance of antioxidants and over-expressed in a situation of oxidative stress (Messina et al., 2016; Messina et al., 2014; Chopra et al., 2013). Our previous study on the inclusion of dehydrated lemon peel in the diet for seabream showed a reduction of the HSP70 levels in fish fed on increased levels of bioactive compounds, due to the well antioxidant's composition of this resources, that is able to guarantee protection against oxidative stress (García Beltrán et al., 2019) . Accordingly, Di Giancamillo et al. (2015) described a decrease of HSP70 in pigs fed a diet supplemented with a natural verbascoside extract, compared with pig fed a high-fat diet. These authors suggested the role of HSP70 in modulating hepatic oxidative stress (Giancamillo et al. 2015).

The main evidence that emerged from the blood analyses regard the linear reduction of the total serum protein levels, concomitant with the increase of HBP inclusion in diets. TSP is a non-destructive parameter that is robust, easy to measure everywhere and cheap, representing a suitable way of monitoring the overall welfare of fish by its regular increase (Coeurdacier et al., 2011). It has been reported that this parameter tends to decrease in fish in response to various stress conditions such as hyperoxia, hypercapnia, stocking density, transfer to another tank or nodavirus injection (Coeurdacier et al., 2011). Many explanations have been suggested to explain TSP decrease like changes in blood volume and plasma hydration, alterations in permeability of environmental barriers, tissue destruction,

malabsorption, and tissue injury by parasites or other pathogens (Steinhagen et al.,1997). These results confirm what emerged in the intestinal histology and immunohistochemistry and in hepatic stress-related biomolecular markers.

Given that the fish of the different groups were subjected to the same environmental conditions, received isoproteic and isoenergetic diets that varied only according to the level of pollen inclusion, our results can be explained through a negative action carried out by the pollen in terms of intestinal inflammation

For what concerns the mineral content, the macro elements and the trace elements contents in our HBP was in line with the values reported by Taha (Taha et al., 2015). Compared to the maximum levels (MLs) of heavy metals set by the EU Commission [Directive (EC) No 2002/32 49], Cd, Pb and As levels in honey bee pollen were lower than the maximum values established for the feeding stuff and feed materials, indicating that the risk of exposure to heavy metals deriving from consumption of the honey bee pollen used in this trial is relatively low and in compliance with EU regulations. However, in all the diets used the trial the levels of potentially toxic elements resulted lower than the MLs stated by EU regulation that establishes the following MLs of heavy metals content in mg/kg (ppm) relative to a feed with a moisture content of 12%: the Cd MLs in the feed materials and complete feed are 2.0 and 0.5 mg/kg, respectively; the Pb MLs in the feed materials and complete feed are 10.0 and 5.0 mg/kg, respectively; the As MLs in the feed materials and complete feed are both 2 mg/kg. These elements, therefore, may not have been important in determining the observed deterioration of the growth performance and health status of the intestine in such fish species.

To deeply comprehend the reason for such negative results it is necessary to dwell on the microscopical structure of bee pollen. Honey bee pollen is composed by a multitude of microscopic particles (6-200 μm in diameter) with variable shape, usually spherical or oval. The pollen cell walls consist of a series of stratified concentric layers (Zuluagad et al., 2014). As Roulstone has described the outermost layer of the pollen wall is the pollenkitt (Roulstone et al., 1999), a semi-solid coating comprised primarily of neutral lipids, hydrocarbons, terpenoids, and carotenoid pigments (Dobson, 1988). Inside the pollenkitt is the exine, an often intricately ridged matrix of the complex carbohydrate, sporopollenin. Sporopollenin is a compound that provides chemical resistance to bee pollen and preserves the compounds which are within it (Atkin et al., 2011). The chemical analysis

of sporopollenin has been limited due to the difficulty of obtaining large quantities of exine without affecting or modifying the structure of this molecule. Atkin et al. (2011) recently found that this molecule has an empirical formula C90H144O27. Nowadays, it is suggested that the sporopollenin consists of a series of biopolymers, homologous to the compounds cutin, suberin and lignin.

The exine greatly resists decay and digestion but is commonly perforated by one-to-several pores or slits (germination pores) that lead to the inner wall layer, known as the intine. The intine, composed primarily of cellulose and pectin, also very resistant, and forms the final barrier to the nutrient-rich cytoplasm. From reports based on literature, it is known that the exine is rich in antioxidant compounds, while the intine has the rest of the nutritional and bioactive compounds (Mackenzie et al., 2014).Thus, any animal consuming pollen contacts pollenkitt nutrients through external probing of pollen grains or ingestion of pollen grains but must penetrate or dismantle two resistant pollen wall layers in order to access cytoplasmic nutrients (Roulstone et al., 1999). Whereas the pollen has such complex ultrastructure, it is not certain that the beneficial substances contained in it are available for monogastric animals. Previous studies showed that exine and intine are indigestible by the human digestive tract (Franchi et al., 1997). Furthermore, administering pollen suspended in water to mice by means of gastric intubation resulted in grains being in the faces (Franchi 1987). The artificial digestion at various pH values and after various incubation times, appeared unaffected after the acid treatment (corresponding to gastric digestion) with the exception of the digestion of substances located outside the walls. Partial digestion of grains occurred only during the alkaline treatment, in presence of pancreatic enzymes (Franchi 1985); grains may be partially emptied if the enzymes are able to penetrate the exine pores and it depends also by the thickness intin layer.

Taking into account the difficulties of monogastric animals to digest bee pollen grains and the similarity of the pollen walls with indigestible fibrous substances such as suberin and lignin, it can be assumed that bee pollen had a pro-inflammatory action on the intestinal mucosa of meagre, a fish with carnivorous feeding habits (Chatzifotis et al., 2010; Chatzifotis et al., 2012). On the contrary, the bee pollen structure may not represent a problem in herbivorous fish species such as the Nile tilapia, with an enzymatic profile more suitable for the digestion of fibrous substances and in which positive effects of honey bee pollen addition to the diet has been reported (El-Asely et al., 2014).

1.5 Conclusions

In conclusion, the results of the present trial showed that the addition of honey bee pollen to meagre juveniles diets not only did not have the positive effects described in the literature for other fish species, but it even had negative effects on both growth performance and diet digestibility. Moreover, the inflammatory state of the intestine progressively worsened as the level of HBP inclusion increased. These effects could be ascribed to the ultrastructure of the bee pollen grains walls (exine and intine) that makes the bioactive substances unavailable for the fish, while the intestinal inflammation could be due to the bee pollen grains chemical composition rich of lignin and suberin-like substances that can irritate the intestine of monogastric animals such as carnivorous fish. These HBP negative effects could be overcome using an extraction method able to concentrate the bioactive substances and eliminate the indigestible fractions. As a general conclusion, it is worth highlighting how it should be always necessary to test nutraceuticals additives of natural origin in each species in order to verify the effective positive action and exclude any negative repercussions on animal health.

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Chapter 2

The Inclusion of a Supercritical Fluid Extract, Obtained From Honey Bee Pollen, in the Diet of Gilthead Sea Bream (*Sparus aurata*), Improves Fish Immune Response by Enhancing Anti-oxidant, and Anti-bacterial Activities

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2.1 Abstract

In the present study, the immune-stimulatory effect of two levels of honey bee pollen (5 and 10%, P5 and P10 treatment, respectively) and its supercritical fluid extract (0. 5 and 1%, E0.5 and E1, respectively) included in the diet, was tested in gilthead seabream (*Sparus aurata*). The in vivo trial was preceded by the evaluation of antioxidant properties of three different bee pollen extracts obtained by water, ethanol 80%, and Supercritic Fluids Extraction (SFE). The preliminary evaluation attested that the SFE showed the lowest extraction yield (10.47%) compared to ethanol 80% (48.61%) and water (45.99%). SFE extract showed good antioxidant properties with high polyphenol content (13.06mg GAE/g), radical scavenging activity (3.12 mg/ml), reducing power (38.68 mg/mL EC50). On the contrary, the water extract showed the significantly lowest polyphenol content (2mg GAE/g; $P < 0.05$). The results of in vivo trial demonstrate that the diets supplemented with SFE bee pollen extract had a stimulatory effect on fish serum immunity, respect to the inclusion of raw pollen, this latter revealing some inhibitory effects in the immune response, such a decrease of serum peroxidase and lysozyme activities, particularly in P10 group significantly different ($P < 0.05$) from the control group. On the contrary, serum peroxidase, protease, antiprotease, were significantly increased in fish fed the diets supplemented with supercritical fluid extract, respect to the fish fed on control and on diets supplemented with 5 and 10% of raw pollen. For what concerns the bactericidal activity against *Vibrio harveyii*, all the treatments containing bee pollen regardless of the type showed their serum bactericidal activity significantly increased with respect to the control groups ($p < 0.05$). Given its high antioxidant properties, the absence of toxic solvents and the positive action carried out on improving the humoral response in gilthead seam bream, honey bee pollen SFE extract can be taken into account in the formulation of fish feeds.

2.2 Introduction

Recently, there has been an increased interest on using plant, medicinal herbs and others compounds as natural immunostimulants, able to enhance the disease resistance in cultured fish (García Beltrán et al., 2017). Products of vegetable origin seem to represent a very promising source of bioactive molecules, being both easily available, cheap, and biocompatible (Bulfon et al., 2015). Some plants, certain parts of them or the extracts, can improve fish health enhancing both the innate and the adaptive immune response, against bacteria, viruses, or parasites (Reverter et al., 2014; García Beltrán et al., 2017). This action is represented by the modulation of some enzymes or complexes such as lysozyme,complement, anti-protease, peroxidase, or by the ability to improve some activities as respiratory burst, phagocytosis, antibody production, antioxidant, anti-stress, anti-helminthic, anti-protozoa, anti-fungal, anti-bacterial, and anti-viral (Harikrishnan et al., 2011; Reverter et al., 2014; Bulfon et al., 2015; García Beltrán et al., 2017). The use of natural compounds due to their biocide activities represents a promising substitute to the use of the antibiotics and vaccines which are commonly applied in aquaculture to control fish and molluscs diseases (Bulfon et al., 2015; García Beltrán et al., 2017). In addition, many of these vegetable-derived products have other positive benefits for fish, including enhanced growth, increased weight, appetite stimulation, and they also have the ability to facilitate the sexual maturation of farmed species, while acting as anti-stress and anti-infection agents, including many other health benefits (Harikrishnan et al., 2011).

Honey bee pollen (HBP), in particular, is becoming increasingly popular as functional food for human consumption as a potential source of energy and for its high content of compounds with positive health effects, such as essential amino acids, antioxidants, vitamins, and lipids (Xu et al., 2009; Ares et al., 2018). Pollen preparations are distributed worldwide for dietary scope and as diet integrators. In the USA bee pollen is described by the Dietary Supplement Health and Education Act of 1994 as Dietary Supplement employed to supplement the diet by increasing the total dietary intake (Kroyer and Hegedus, 2001). The pollen collected by bees contains nutritionally essential components such as carbohydrates, proteins, amino acids, vitamins, mineral substances and trace elements, but also lipids, such as fatty acids, sterols, and several other bioactive compounds (Ares et al., 2018). Polyphenol substances, mainly flavonoids, are considered, among the

ingredients, the principal biomarkers of quality, and may be used for the establishment quality standards for health control of commercially distributed pollen preparations and in relation to their nutritional-physiological properties (Kroyer and Hegedus, 2001). The bioactive quality of bee pollen decreases over time, and that conditioning carried out on fresh pollen before storage affects its nutritional and functional value (Conte et al., 2017; Ares et al., 2018). In order to extend the shelf life of bee pollen and avoid rapid fermentation and deterioration, a dehydration process (artificial drying) is necessary, since its composition has a high level of moisture. In recent years, the growing interest in the extraction and determination of these beneficial bee pollen compounds, has been confirmed by the number of published researches on this topic (Ares et al., 2018).

For the extraction of natural compounds, solvents with different polarity, from water, to hydro-alcoholic solution are used (Trabelsi et al., 2010; Messina et al., 2019b). The antioxidant activity of the extracts and their extraction yield are influenced by the polarity of the solvent.

Therefore, the use of different solvents is related to the nature of the polyphenols in the samples (Franco et al., 2008). For the extraction of many compounds, among these also phenolic ones, the supercritical fluid extraction (SFE) technology is applied. This alternative and green method is often put in comparison to traditional extraction methods with different solvents (ethanol and water), in terms of yield and product quality evaluated by the antioxidant activity of the extracts (Messina et al., 2019b). Today, bee pollen has been used for improving chicken, mammal, and fish growth (Wang et al., 2007; Attia et al., 2011; El-Asely et al., 2014). However, there are few studies on the use of bee pollen and its extract to improve welfare and immunity of fish against fish pathogens.

Our recent study on meager (Argyrosomus regius) demonstrated a negative effect of the addition of raw pollen in the diet on growth, diets digestibility, intestinal traits, and biochemical markers related to health and stress (Panettieri et al 2020), probably due to the structure of bee pollen grains. In view of these results, in the present study on gilthead sea bream, we wanted to verify whether these negative effects could be overcome by the inclusion in the feed of the bioactive fractions previously extracted from pollen, using advanced techniques such as SFE, which does not involve the use of solvents.

Therefore, the aim of the present study was to evaluate the effect of the inclusion of raw and SFE extracted pollen on the immunity response of Sparus aurata, one of the most important fish species for Mediterranean

aquaculture, so that the results emerging from the trial could potentially found practical applications. A preliminary evaluation of the antioxidants properties of HBP extracts, obtained by chemical and SF extractions, was done. Among immune-related parameters we focused on the activity of different enzymes present in the serum of sea bream (peroxidase, protease, anti- protease, and lysozyme), which are considered as good descriptors for health status in marine organisms (Esteban, 2012; Mansour et al., 2018). In addition, bactericidal of *S. aurata* serum against two pathogenic *Vibrio* species were evaluated

2.3 Materials and Methods

2.3.1 Extraction with Solvents

About 2 kg of HBP from chestnut, purchased from a local organic farm located in the city of Naples (Napoli, Italy), and collected in about 1 week, were utilized for the trials. For the extractions ethanol 80% and water (1:10 w/v) were used (Messina et al., 2019b, 2019c). The materials were then homogenized according to a consolidated protocol (Fish et al., 2002; Musa et al., 2011; Gharbi et al., 2017; Messina et al., 2019a). The matrices extracted were centrifuged and then filtered (Whatman® qualitative filter paper, Grade 93–10 µm, Merck KGaA Darmstadt, Germany) and freeze-dried (Messina et al., 2019b, 2019c).

2.3.2 Supercritical Fluid Extraction (SFE)

A supercritical extraction unit (SFE System model HELIX, Applied Separations Allentown, PA, USA) was used.
Before the dynamic extraction, a static treatment with SC-CO_2 was carried out to break the cell walls of bee pollen (Xu et al., 2009). Dynamic extraction was conducted on dried bee pollen following the method applied by Xu et al. (2009) with some modifications. For each extraction the dried powder and hydroscopic dispersing agent (Applied Separations, Allentown, PA, USA) were mixed and placed in the extraction vessel, the unit was pressurized, and dynamic extraction was carried out at pre-established conditions of temperature and pressure with a CO_2 and a co-solvent flow for 2h. An additional extraction hour was applied changing the CO_2 and co-solvent flow.
The obtained extract was stored at −20 °C and subsequently extracts were dried.

2.3.3 Characterization of the antioxidant power of HBP extracted by the three different extraction methods

2.3.2.1 Total Polyphenols Contents

Total phenolics were analysed using Folin–Ciocalteu's assay. Gallic acid was used as standard and results were expressed as mg of gallic acid equivalents (GAE) per g of extract of bee pollen (Oki et al., 2002; Manuguerra et al., 2018; Messina et al., 2019b, 2019a). Each sample was analysed in triplicate.

2.3.3.2 DPPH Radical Scavenging Activity

The DPPH (1,1-diphenyl-2-picryhydrazyl) radical scavenging activity was assessed using the method described by Bernatoniene et al. (2011) slightly modified by Messina et al. (2019a): 400 µL of various concentrations of the extracts was replenished up to 2.0 mL with 0.1 mM DPPH radical solution in ethanol.
After 30 min of incubation the absorbance was read against the blank at 517 nm. Gallic acid was employed as the reference. Inhibition of DPPH free radical in percent (I%) was calculated as given below (Manuguerra et al., 2018):

$$\boldsymbol{I\% = 1 - \left(\frac{Asample}{Ablank}\right) * 100}$$

where Ablank is the absorbance of the control reaction, and Asample is the absorbance of the test sample. The results are expressed as IC 50, mg/mL (Yeddes et al., 2013).

2.3.3.3 Reducing power

The power of the extracts to reduce iron (III) was determined according to the method of Oyaizu (1986). 300 µL OF sample solutions at different concentrations were mixed with phosphate buffer and potassium ferricyanide 1%; the mixture was incubated at 50 °C for 20 min. 300 µL of trichloroacetic acid (10%) was added to the mixture, prior to centrifugation. The upper layer of solution (300 µL) was mixed with distilled water (300 µL) and FeC13 (600 µL, 0.1%), and the absorbance was measured at 700 nm against gallic acid as standard. The results are expressed as EC50, mg/ mL (Falleh et al., 2011; Manuguerra et al., 2018).

2.3.3 Fish and experimental conditions

The trial was performed in the experimental aquaculture facility of the Department of Veterinary Medicine and Animal Production of Federico II University (Naples, Italy) in respect of the Directive 2010/63/EU and was approved by the University Federico II Ethical Committee and authorized by the Italian Ministry of Health, authorization n. 651/2017-PR; it lasted 30 days and was carried out in an indoor marine water recirculating system (Italian Ministry of Health authorizations n. 78/2013-A and 25/2019-UT) using 90 gilthead sea breams (294.7 ± 12.8 g average initial body weight – IBW) supplied by a local fish farm. The system assured control of water temperature and was equipped with mechanical sand filter, biological filter and UVA sterilization lamp apparatus.
The trial started after a fifteen days adaptation period of the fish to the experimental conditions. The water quality parameters were as follows: daily water renewal < 1%, artificial day length 12 h, temperature 22 ± 1.5 °C, salinity: 33.0 ± 2.0 g/l, dissolved oxygen 6.5± 1.1 mg/l, pH 7.9 ± 0.5, total ammonia nitrogen <0.3 mg/l, nitrite <0.01 mg/l, nitrate <38 mg/l).
On a daily basis water temperature with a mercury thermometer, pH with an Orion digital pH meter and dissolved oxygen with an oxygen meter (WTW, OXI 330, Weilheim, Germany) were measured daily.
Bi-weekly, total ammonia nitrogen (N-NH3), nitrite-nitrogen ($N\text{-}NO_2$) and nitrate nitrogen ($N\text{-}NO_3$) were determined by colorimetric methods, using commercial kits and a spectrophotometer (Hanna Instruments, C-203, Leighton Buzzard, UK).

2.3.3.1 Fish diets

Fish were randomly distributed in 15 fiberglass 220 l tanks (6 fish per tank) and were fed 5 isoenergetic and isoproteic diets. Each diet was randomly assigned to 3 tanks: a control diet; two diets in which HBP was included at 5% (P5) and at 10% (P10) and two diets in which HBP extract, obtained by SFE, was included at 0,5% (E 0.5) and at 1% (E1). The two pollen inclusion levels were chosen in order to have the same content of total polyphenols both in diets containing raw pollen (P5 and P10) and in diets containing the SFE extracts (E0.5 and E1). The diets were formulated to meet nutrient requirements of gilthead sea bream (Gómez-Requeni et al., 2004; Peres and Oliva-Teles, 2009).

The ingredients and chemical composition of the experimental diets are reported in Tables 2.1 and 2.2 respectively.

Table 2.1. Ingredients of control diets provided to Sparus aurata (g/Kg)

Ingredients	Control
Soy bean meal	240.0
Fish meal	210.0
Corn Gluten	190.0
Fish oil	160.0
Gelatinized starch	100.0
Wheat gluten	80.0
Mineral	10.0
Vitamins	10.0

The experimental diets (P5, P10, E0.5 and E1) were obtained subtracting from the premixed control diet an equivalent quantity to that of the substance to be added (50g and 100 g of honey bee pollen HBP were added to the diets P5 and P10; 5g and 10g of SFE from HBP were added to the diets E0.5 and E1)

Table 2.2. Chemical composition (% as feed) of experimental diets

	Control	P5	P10	E0.5	E1
Dry matter	88.94	88.19	87.63	88.43	88.62
Ash	5.5	4.99	4.35	4.71	4.51
Crude Protein	39.78	39.92	38.26	38.91	39.46
Ether Extract	17.85	17.25	17.15	17.75	17.15
Crude Fibers	7.17	7.54	8.63	7.37	7.62

P5 and P10: inclusion of honey bee pollen HBP at 5% and 10%; diets E0.5 and E1: inclusion of SFE from HBP at 0.5% and 1%)

The diets were physically constituted at the laboratories of the Department of Veterinary Medicine and Animal Production, Napoli Federico II University (Naples, Italy). The HBP was finely chopped, mixed with 10ml of water to create a paste, then mixed in fish oil and incorporated into the mixture. For E 0.5 and E1 diets, the extract was dissolved initially in 10ml of fish oil, then incorporated into the mixture. Before the final mixing, all ingredients were ground through a 0.5mm sieve, then water was added, and the mixture was pelleted through a 3mm dye using a meat-grinder (Bosh mod. MFW68660, Germany). The diets were then dried in a ventilated oven at a temperature of 40◦C for 24 h. This temperature was chosen as it is very similar to the temperature maintained in the hive (34.6◦C on average) in

order to preserve the pollen quality. The feeds were stored at 4◦C until use. Each diet was administered twice a day (09:00 and 16:00 h), 7 days per week. Feeds were administered at 1% of the average body weight. At the end of the trial, all the fish were individually weighted and final weight and FCR were group/tank determinedorder to preserve the pollen quality. The feeds were stored at 4 °C until use. Each diet was administered twice a day (09:00 h and 16:00 h), 7 days per week. Feeds were administered at 1% of the average body weight. At the end of the trial, all the fish were individually weighted and final weight and FCR were group/tank determined.

2.3.3.2 Sampling

After 30 days, on the day of the sampling, three fish per tank (336.2 ± 11.4 g average final body weight – FBW) were euthanized (over anesthesia (MS222-Tricain Metansulphonate at 250 mg/L dosage) and their weight and length were measured. Immediately, blood was collected from the caudal vessel via 5 ml sterile syringes. Blood samples were collected in tubes with separator gel and are left to clot at 4 °C for 4 h. Serum were collected after centrifugation (10,000 rpm, 10 min, 4 °C) and stored at -80 °C.

2.3.4 Serum immune parameters

2.3.4.1 Peroxidase activity

The peroxidase activity in serum of sea bream fed the different diets, was measured, according to Quade and Roth, (1997) by oxidation of 3,3',5,5'-Tetramethylbenzidine (TMB). Briefly, 5µL of serum were diluted with Hanks's buffer (HBSS) without Ca^{+2} or Mg^{+2} to a final volume of 50µL in a flat-bottomed 96-well plate. Were added 100µL of 10mM TMB with 0.025% of 30% H_2O_2, as substrate, and the colour change reaction was stopped by adding 50µL 2M H_2SO_4. The OD was read at 450 nm in a plate reader. Sample without serum were used as blank, and the OD values were subtracted for each sample value. One unit was defined as the amount producing an absorbance change of 1, and the activity expressed as U mL-1 for the serum samples. All samples were analyzed in triplicate.

2.3.4.2 Protease activities

Protease activity was quantified, according to the method described by Ross et al. (2000), using the azocasein hydrolysis assay. 10µL of serum were incubated with 100µL of ammonium bicarbonate buffer and 125µL of 2%

azocasein (Sigma Aldrich) in sterile eppendorfs overnight (at RT and in agitation). The reaction was stopped by adding 250µL of 10% trichloroacetic acid (TCA). The mixtures were centrifuged (6,000 g, 5 min), 100µL of the supernatants transferred to a flat-bottomed 96-well plate, and 100µL of 1N NaOH added. The OD was read at 450nm using a plate reader. For the positive controls (100% of protease activity), the serum, was replaced by trypsin (5mg mL-1, Sigma) whereas by ammonium bicarbonate buffer for the negative controls (0% of protease activity). The activity for each sample was expressed as % protease activity in relation to the controls. All samples were analyzed in triplicate.

2.3.4.3 Antiprotease activities

Antiprotease activity was determined by the capacity of serum to inhibit trypsin activity (Hanif et al., 2004). Briefly, 10µL of serum were incubated (10 min, RT) with 10µL of trypsin solution (5mg ml^{-1}, Sigma) in sterile eppendorfs. After were added 100µL of ammonium bicarbonate buffer and 125µL of 2% azocasein (Sigma Aldrich) and the mixtures incubated for 2h at RT. Following were added 250µL of 10% trichloroacetic acid (TCA) and the samples incubated for an additional 30 min at RT. The mixtures were then centrifuged (6,000g, 5 min), 100µL of the supernatants transferred to a flat-bottomed 96-well plate, and 100µL of 1N NaOH added. The OD was read at 450 nm using a plate reader. (100% trypsin inhibition) was used buffer with no sample or trypsin, and for the negative controls (0% trypsin inhibition) was used a combination of buffer and trypsin solution. The activity for each sample was expressed as % trypsin inhibition in relation to the controls. All samples were analyzed in triplicate.

2.3.4.4 Lysozyme activity

Lysozyme activity was measured according to the turbidimetric method described by Espinosa Ruiz et al. (2019). 25 µL of serum were placed in a flat-bottomed 96-well plate. To each well, 175 µL of freeze-dried Micrococcus lysodeikticus in 10mM PBS, pH 6.2 (0.3 mg mL^{-1}, Sigma, UK) was added as lysozyme substrate. The reduction in absorbance at 450 nm was measured over 15 min at 3 min intervals at RT in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of $0.001min^{-1}$. The units of lysozyme present in serum were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma, UK),

and the results were expressed as U mg protein^{-1}. All samples were analyzed in triplicate.

2.3.5.5 Bactericidal activity

Bactericidal activity was determined following the method describe by Espinosa Ruiz et al. (2019). Samples of 20 µL of serum were added (in three replicates) to the wells of a flat-bottomed 96-well plate. PBS solution was added to some wells instead of the serum as positive control. Aliquots of 20 Ml of the previously cultured bacteria were added and the plates were incubated for 5 h at 25 °C. Then, 25 µL of MTT (1 g L^{-1}) were added to each well and the plates were newly incubated again for 10 min at 25 °C to allow the formation of formazan. Plates were then centrifuged (4500 rpm, 10 min), and the precipitates dissolved in 200 µL of DMSO. Then, 100 µL from each well were transferred to another flat-bottom 96-well plate. The absorbance of the dissolved formazan was measured at 570 nm. Bactericidal activity was expressed as percentage of nonviable bacteria, calculated as the difference between absorbance of surviving bacteria compared to the absorbance of bacteria in positive controls (100%).

2.3.5 Statistical analysis

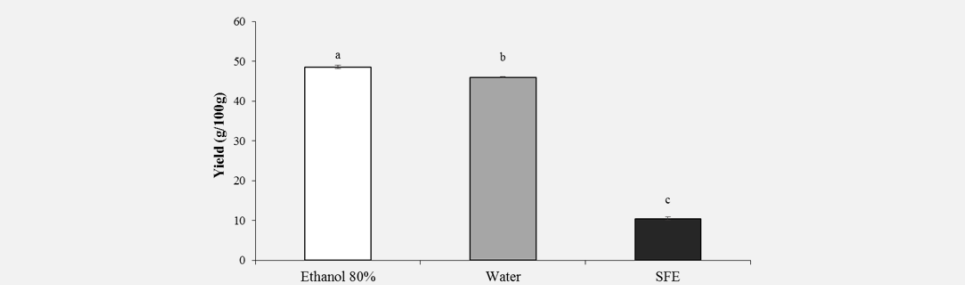
Statistical differences among the groups were assessed by one-way ANOVA analyses, followed by the Tukey or Games Howell test depending on the homogeneity of the variables. Normality of the variables was confirmed by the Shapiro–Wilk test and homogeneity of variance by the Levene test. The significance level was 95% in all cases ($P < 0.05$). All the data were analysed by the computer application SPSS for Windows® (version 20.0, SPSS Inc., Chicago, USA).

2.4 Results and discussions

2.4.1Extraction yield of HBP

The extraction yield obtained from HBP with different solvents (Ethanol 80%, water and SFE) is shown in Figure 2.1. It was observed that the solvent with the highest extraction efficiency is ethanol 80% (48.61%) followed by water (45.99%). Significantly lower yields were obtained with SFE (10.47%).

Figure 2.1 Yield (g/100g) of honey bee pollen (HBP) extracts obtained with different solvents.



Lowercase letters indicate significant differences between different solvents (a, b, c: $P<0.05$).

The extraction yield and antioxidant activity of plant and other natural extracts strongly depend, both qualitatively and quantitatively, on the polarity of the solvent used during extraction. (Franco et al., 2008; Messina et al., 2019b).

Previous studies have shown that usually the highest yields are obtained with ethanol, methanol and their mixtures with water (Franco et al., 2008). Kroyer and Hegedus (2001) in their study on bee pollen obtained a yield on aqueous extracts comparable to our results, instead their obtained a lower yield for ethanol extracts.

Total yield obtained from bee pollen using SC-CO_2 and co-solvent (ethanol 96%) was higher than the one reported by Xu et al. (2009), by applying the same pressure and temperature parameters. These authors in fact reach a maximum yield of 5,66% but without using ethanol as co-solvent. Other authors reported that SFE with CO_2/EtOH could give the best performance,

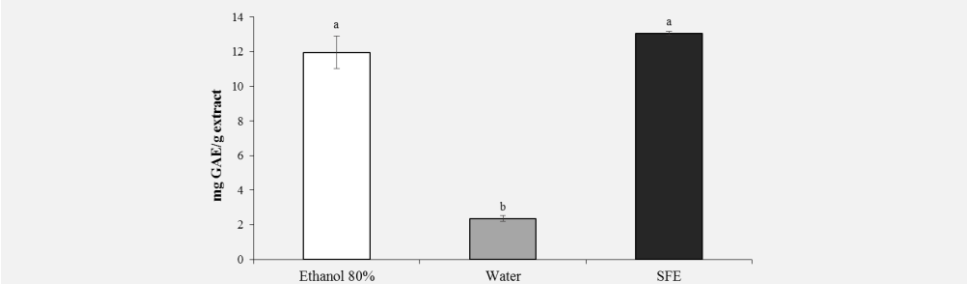
combining extraction yield and product quality (antioxidant activity and total phenolic compounds) (Castro-Vargas et al., 2010).

2.4.2 Characterization of the Antioxidant Power of HBP

2.4.2.1 Total Polyphenols Contents

Figure 2.2 shows the results of the polyphenol content. It was observed that the highest content of phenolic compounds was recorded in the pollen extract obtained with ethanol 80% (11.96mg GAE/g) and in the extract obtained with the SFE (13.06mg GAE/g). With regard to the ethanol extract, this result was expected, being ethanol a very efficient solvent for antioxidant extraction (Messina et al., 2019c).

Figure 2.2 Total phenolic content (mg GAE/g extract) of honey bee pollen (HBP) extracts obtained with different solvents.



Lowercase letters indicate significant differences between different solvents (a, b, c: $P < 0.05$).

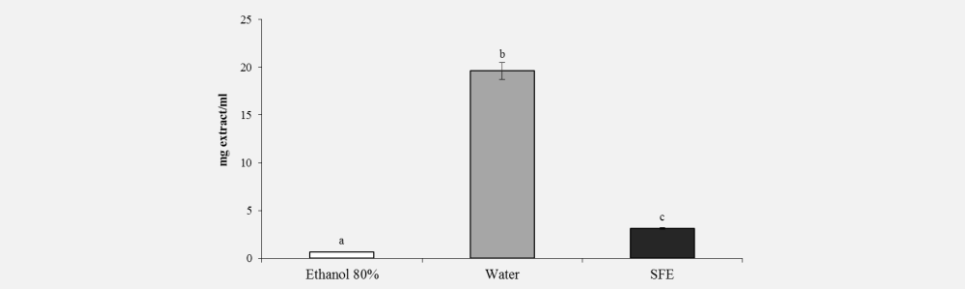
For extracts obtained with SFE, on the other hand, a polyphenol content comparable to the ethanol extract is observed, confirming that extraction with SFE offers greater selectivity, shorter extraction times and does not use toxic organic solvents. It is important to stress how the water extract showed the significantly lowest polyphenol content (2mg GAE/g; $P<0.05$).
Given its eco-friendly characteristics and the absence of solvents, extraction with SFE is a suitable technique to produce extracts that can be employed for fish feed.

2.4.2.2 1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

The DPPH test was used to evaluate the scavenging activity of bee pollen against free radicals.

Different values can be found in the literature regarding the activity of scavenging of bee pollen; values between 0 and 97% can be observed in relation to different species of flowers, their chemical composition and extraction with different solvents (Silva et al., 2006; LeBlanc et al., 2009; Basuny et al., 2013; Gabriele et al., 2015).
As shown in Figure 2.3, ethanol extracts of bee pollen showed high scavenging activity of free radicals with IC50 values of 0.66mg/ml, similar to those observed by Gabriele et al. (2015). Extracts obtained with SFE also showed high scavenging activity (3.12mg/ml).

Figure 2.3 DPPH radical scavenging activity (IC 50, mg/mL) of honey bee pollen (HBP) extracts obtained with different solvents.

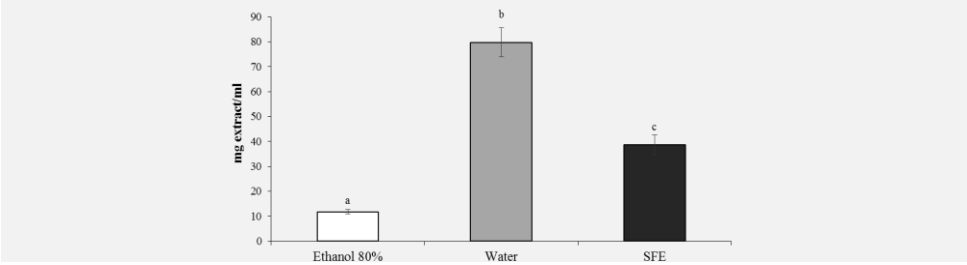


Lowercase letters indicate significant differences between different solvents (a, b, c: $P < 0.05$).

2.4.2.3 Reducing Power

The antioxidant activity is improved by the reducing power, which is linked to the presence of reducing agents that, through the donation of a hydrogen atom to free radicals, convert them into stable compounds, breaking the oxidizing chain reaction (Bayar et al., 2016; Messina et al., 2019b). Through the power reduction assay, the reducing components of a sample can be evaluated directly by measuring the reduction of Fe^{3+} in Fe^{2+}.
In the reducing power assay, the results are expressed as EC50/mg extract, where EC50 value (mg mL^{-1}) is the effective concentration of the extract at which the absorbance was 0.5. The results presented in Figure 2.4 confirm the previous results of the DPPH (Figure 2.3) and polyphenols (Figure 2.2); in fact, a greater reducing activity is observed for the samples extracted with ethanol 80% (11.76) and SFE (38.68).

Figure 2.4 Reducing power (EC50, mg/ml) of honey bee pollen (HBP) extracts obtained with different solvents.

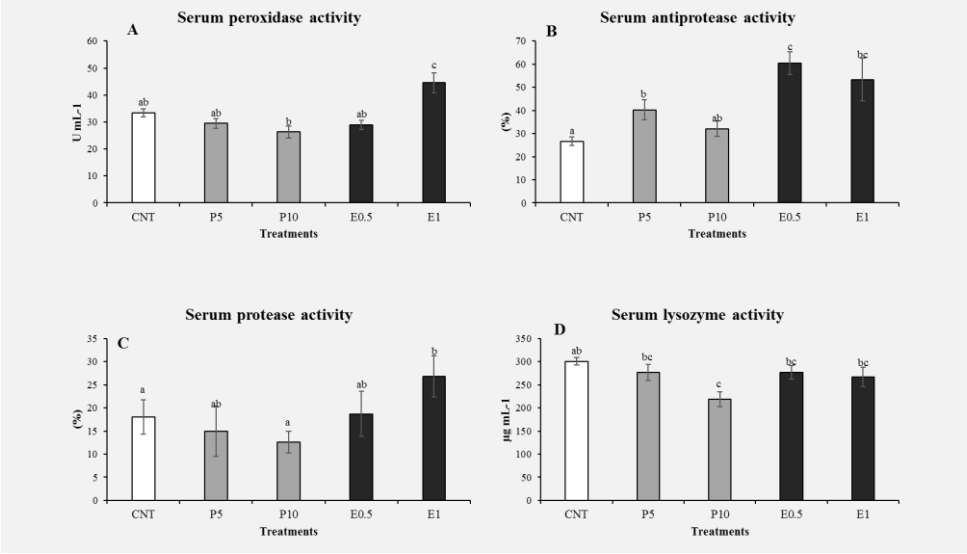


Lowercase letters indicate significant differences between different solvents (a, b, c: P < 0.05).

2.4.3 Serum immune parameters determined in Sparus aurata

At the end of the feeding period, no statistical differences were found among groups for final weight (336 ± 11.35 g) and FCR (2.3 ± 0.7). Fish fed with both diets P5 and P10 showed some inhibitory effects in the immune response, such a decrease of serum peroxidase (Figure 2.5A) and lysozyme activities (Figure 2.5D) respect to the control groups (p<0.05).

Figure 2.5 Immune related parameters determined in the serum of S. aurata fed with honey bee pollen (HBP) and its SFE extract



(CNT: control, P5: pollen 5%, P10: pollen 10%, E0.5: SFE 0.5% and E1: SFE 1%): peroxidase activity (U mg prot−1) (A), antiprotease activity (%) (B), protease activity (%) (C) and lysozyme activity (U mg prot−1) (D). Values are the mean ± SEM (n=9). Statistical differences (P < 0.05) between groups are indicated by different letters.

It is known that the peroxidase enzyme is present in hemocyte granules (Carballal et al., 1997), contributing to the respiratory that involves the production of oxygen metabolites (such as superoxide anion, hydrogen peroxide, and intermediate compounds with high bactericidal activity) (Pruzzo et al., 2005). After the phagocytosis of microorganisms and other microparticles follows a degranulation process by which these radicals are discharged into the phagosome (Klebanoff, 1998). A reduced peroxidase activity may affect the ability to neutralize pathogens (Klebanoff, 1998), suggesting that the inclusion of 5 and 10% of bee pollen in the diet of sea bream may impair defense capacity. Additionally, lysozyme is a bactericidal enzyme present in the lysosome with an important defense role due to its ability to hydrolyze the components of the bacterial walls (Cheng and Rodrick, 1974; Cheng, 1983). The enzyme catalysis the cleavage of β-1-4 bonds between N-acetylglucosamine and N-acetylmuramic acid of bacterial cell wall peptidoglycan, thereby causing bacteriolysis and preventing the growth of bacteria (Cuvillier-Hot et al., 2014; Bulfon et al., 2015). Lysozyme is also known to display anti-viral and anti- inflammatory properties , as well as to activate the complement system and phagocytes by acting like an opsonin (Magnadóttir, 2006; Saurabh and Sahoo, 2008; Bulfon et al., 2015). During phagocytosis, lysozyme is secreted by hemocytes in the hemolymph, thus inactivating pathogens.

Panettieri et al (2020) highlighted how the use of different inclusion levels of HBP in the diet for meagre juveniles led to a worsening of fish growth and nutrients digestibility, along with histological alterations of medium intestine that became more and more severe as the HBP inclusion levels in the diet increased. Such results were supported by immunohistochemistry, hepatic biomolecular markers and blood biochemical analyses. In particular, immunohistochemical detection of TNF-α in medium intestine showed the presence of TNF-α+ cells in the lamina propria and submucosa of bee pollen treated fish, that resulted in accordance with the increased level of the TNF-α protein detected by immunoblotting in the liver. The high hepatic level of HSP70 ($p < 0.05$) in fish fed the diet with the highest inclusion level of HBP (4%) and the linear decrease of total serum protein levels in fish fed the HBP containing diets confirmed the stress situation. The authors attributed these negative effects to the ultrastructure of the bee pollen grains walls that make the bioactive substances unavailable and can irritate the intestine of a carnivorous fish. In particular, the pollen cell walls are composed by stratified concentric layers. The outermost layer is a semi-solid coating composed of neutral lipids, hydrocarbons, terpenoids, and carotenoid

pigments. Inside this wall is the exine a matrix of complex carbohydrate, sporopollenin. The exine greatly resists to monogastric digestion and its structure is similar to lignin. These characteristics of raw pollen may make it irritant for the intestinal mucosa and make the bioactive substances at least partially unavailable for carnivorous fish. These aspects could explain the decrease of serum peroxidase and lysozyme activities in P5 and P10 groups compared to the control group.

There are few studies on the use of bee pollen as feed in aquaculture to improve the immunity and protection of fish against fish pathogens. Geay et al., (2011) observed that lysozyme levels resulted significantly decreased in *Dicentrarchus labrax* fed on a plant-based diet respect to a diet containing fishmeal. An interesting note is that the present study indicates an improvement in lysozyme levels with the inclusion of HBP extract (0.5 and 1%) in the *S. aurata* diet (Figure 2.5D). This difference could be related to the total substitution of fishmeal with vegetable proteins in the case of the study by Geay et al., (2011), or may indicate that the percentages of pollen inclusion (5 and 10%) used in our study are too high, as they may have interrupted the amino acid profile of diets. Likewise, in the study carried out by Abd-El-Rhman (2009) propolis-ethanolic-extract and crude propolis significantly increased the serum lysozyme activity, so it stimulated the immune response in Nile tilapia. The increased lysozyme activity has been reported after supplementing the fish-feed, with non-specific immunostimulants as a mixture of propolis and herba epimedii extracts (Chu, 2006). For this reason, decreased lysozyme activity may suggest immunosuppression in animals, which may lower resistance to pathogenic bacteria (Espinosa Ruiz et al., 2019).

The protease and antiprotease activities were evaluated in serum from sea bream fed the different diets for one month (Figure 2.5B-C). Specimens fed P5, E0.5 and E1 showed significantly increased their antiprotease activities with respect to the control groups ($p<0.05$). On the other hand, animals fed the E1 showed the protease activity significantly increased with respect to the control groups ($p<0.05$).

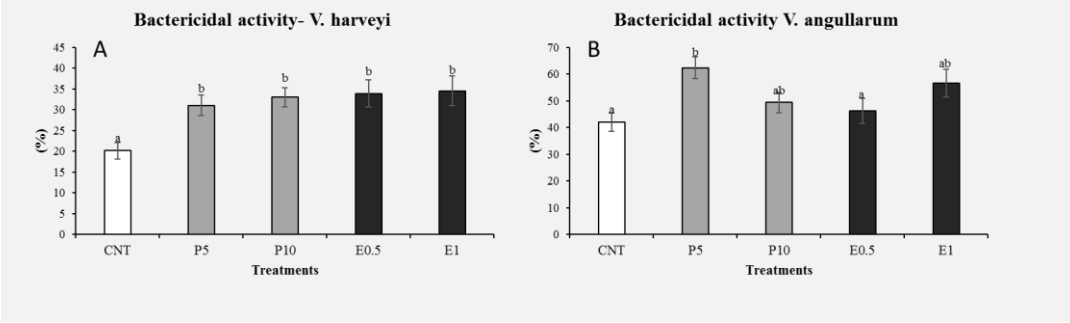
Protease and antiprotease activities activate and improve the development of several immune components such as antimicrobial peptides, complement and immunoglobulins, (Yoshikawa et al., 2001; Cho et al., 2002a, 2002b; Khan et al., 2008). In particular, proteases degrade proteins into either polypeptides or amino acids (Walker and Lynas, 2001), being principally carried out to reduce the pathogenicity of bacteria and parasites.

Antiproteases, instead, are protease inhibitors that are known to be involved in phagocytosis, coagulation, complement activation and fibrinolysis (Walker and Lynas, 2001; Khan et al., 2008). and, moreover, contributes to the innate immunity of animals through its bactericidal and anti-inflammatory properties (Jollès and Jollès, 1984), that restrict the ability of bacteria to invade and grow in fish, by inhibiting their extracellular enzymes (Ellis, 2001; Bulfon et al., 2015).
These results indicate that the pollen extract activated the immune system of sea bream, in accordance with previous studies in which the immunostimulant efficacy of plants, certain parts of the same or even their extracts used in feeding tests on fish has been demonstrated (Chu, 2006; Christybapita et al., 2007; Yin et al., 2009; Zhang et al., 2009; Mansour et al., 2018).

2.4.3.1 Bactericidal activity

Another objective of the present study was to study the effects of the inclusion of pollen in the *S. aurata* diet against two opportunistic pathogenic bacteria, *V. harveyii* and *V. anguillarum*, which were chosen since they are responsible for infections affecting a variety of marine animals, including fish, crustaceans, mollusks and cetaceans and also humans.
Bactericidal activity was evaluated in serum from fish fed the different diets for one month (Figure 2.6 A and B).

Figure 2.6 Bactericidal activity against Vibrio harveyi (A) and Vibrio anguillarum (B) determined in the serum of S. aurata fed with pollen and SFE extract from pollen



(CNT: control, P5: pollen 5%, P10: pollen 10%, E0.5: extract 0.5% and E1: extract 1%). Values are the mean ± SEM (n=9). Statistical differences (P < 0.05) between groups are indicated by different letters.

Regarding the bactericidal activity against *V. harveyii*, all the specimens fed the different doses showed their serum bactericidal activity significantly increased with respect to the control groups ($p<0.05$). On the other hand, only the fish fed with P5 diet showed it bactericidal activity against *V. anguillarum* significantly increased with respect to the control and groups ($p<0.05$). In addition, Vibrionaceae represents the main cause of mortality in farmed marine species (Nguyen and Jacq, 2014; Beltrán et al., 2018). With regard to this, past results show that Gram-positive marine bacteria are usually more susceptible to herbal extracts than Gram-negative marine Vibrionaceae. The results obtained in this study reflect those of similar studies conducted by other authors (Abd-El-Rhman, 2009; Wu et al., 2010; El-Asely et al., 2014). In previous works honey bee pollen or propolis extracts inclusion in the diet reduced the mortality among *A. hydrophila* challenged fish (Abd-El-Rhman, 2009; Wu et al., 2010). This benefit is certainly ascribed to the enhanced non-specific immune responses and the antioxidant effects of pollen or propolis extract through its constituent flavonoids, which have antibacterial activity (Pietta, 2000).

Therefore, we conclude that pollen SFE at 1% seems to be the best option as supplement in the diet of farmed fish, acting as immunostimulant and antioxidant, able to reinforce the humoral response.

2.5 Conclusions

The supplementation with natural compounds has proved to be a useful tool in aquaculture industry and for this reason, green techniques as SFE, able to reduce the utilization of solvent and to produce extract suitable for nutritional purposes, are encouraged. In the present study, the inclusion of pollen extract in the *S. aurata* diet improved the humoral immunity, as demonstrated by the most common markers related to immunity in fish.

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Chapter 3

Laying performance, blood profiles, nutrient digestibility and inner organs traits of hens fed an insect meal from *Hermetia illucens* larvae

Bovera F., Loponte R., Pero M.E., Cutrignelli M.I., Calabrò S., Musco N., Vassalotti G., Panettieri V., Lombardi P., Piccolo G., Di Meo C., Siddi G., Fliegerova K., Moniello G. (2018) Laying performance, blood profiles, nutrient digestibility and inner organs traits of hens fed an insect meal from Hermetia illucens larvae, Research in Veterinary Science, 120, 86-93, https://doi.org/10.1016/j.rvsc.2018.09.006.

3.1 Abstract

Given probable the increment in the nutritional needs of both humans and animals, animal production will have increased dramatically by 2050. Insect meals could be an alternative protein source for livestock, and they would also be able to reduce the environmental problems related to intensive animal production system. The aim of this study was to evaluate productive performance, blood analysis, nutrient digestibility, and changes in the internal organs of laying hens fed *Hermetia illucens* larvae meal (HI) at two different levels in substitution (25 or 50%) of soybean meal (SBM).
A total of 162 Hy-line Brown hens (sixteen weeks old) were equally ivided into three experimental groups and fed isoprotein and isoenergetic diets.
Egg weight, feed intake, and feed conversion rate were not affected by the soybean meal substitution at both inclusion levels of insect meal. Egg mass was positively affected by the insect meal diets, as was the lay percentage, although only at the lowest inclusion level. Dry matter, organic matter, and crude protein digestibility coefficients were lower for the HI50 diet, probably due to the negative effect of chitin. A reduction in serum cholesterol and triglycerides was observed in both insect-meal fed groups, while serum globulin level increased only at the highest level of insect meal inclusion, and, consequently, the albumin to globulin ratio decreased.
Overall, a protein replacement of 25% with an insect meal from *Hermetia illucens* larvae in the diet of laying hens seems to be more suitable and closer to the optimal level.

3.2 Introduction

The most accredited FAO (FAO, 2013) predicts that, in thirty years, the world's population will have reached 9 billion people. Likewise, in order to satisfy the nutritional needs of both humans and animals, the demand for raw materials, especially protein sources, will have also increased (FAO, 2013). It has been estimated that, by 2050, the production of meat will have increased by 50%, while the demand for fish, milk, and eggs will have grown by 75%. Currently, as regards animal nutrition, soybean meal and fishmeal are the most important conventional protein sources, but their production is linked to severe environmental problems (Flachowsky and Meyer, 2015). For this reason, the use of alternative protein sources with high nutritional value, environmental sustainability and low production cost are required. According to the available literature (Makkar et al., 2014), insects could be a suitable source of protein for many species, including humans.

Józefiak et al. (2016) stated that insects have a high potential as a source of nutrients (proteins) and active substances (polyunsaturated fatty acids, antimicrobial peptides) for poultry. Moreover, Pretorius (2011) reported that the inclusion of larvae in the diet of broiler chickens in ratio of 25% DM didn't highlight negative effects on weight gain, feed intake and feed efficiency, suggesting that it could be a valid alternative protein source. Regarding the metabolizable energy content few data are available, values of 17.9 MJ/kg DM for turkey (Zuidhof et al., 2003) and 14.2 MJ/kg DM for broilers (Pretorius, 2011) have been reported. There are many reasons for considering insects as a sustainable source of protein. First of all, because of their high feed conversion efficiency, due to the lower emission of CO2, CH4, N2O, and NH3 by respiration, metabolism and faecal losses, compared to conventional livestock. In addition, depending of the species, they successfully can grow on organic side streams, converting low-value organic by-products into high-value proteins (van Huis and Oonincx, 2017). At present, insect meals obtained from different insect species are excessively costly and are not competitive if compared to soybean meals. This is due to an inconsistent industrial production of insect meals, in particular in Europe, since Regulation EC 999/2001 banned the use of processed animal proteins (PAP) in animal nutrition. However, European law is evolving. EU Regulation 56/2013 (approved on June 1, 2013) provides that aquaculture animals can be fed animal proteins derived from non-ruminants, and, recently, EU Regulation 2017/893 approved the use of

PAPs derived from seven insect species in aquaculture. As far as poultries are concerned, stakeholders are confident, and the authorization of the use of insect PAPs is expected by 2020–2022. Chickens normally eat worms found in soil, and they can synthesize chitinases, enzymes which are able to hydrolyze chitin (Bökönyi and Gál, 2010), the main compound of an insect's exoskeleton.

Recently, Tabata et al. (2017) reported that acidic chitinase could function as a digestive enzyme, breaking down chitin-containing organisms in chickens' gastrointestinal system.

In previous study, we hypothesized that insects could be an interesting protein source for growing birds, and that chitin stimulates their immune response with no negative effects on the chickens' growth performance, carcass, and meat traits (Bovera et al., 2015, 2016; Loponte et al., 2017). Despite that, the exact amount of insect meal that should be included in the diet in order to guarantee animal health and productive performance, still needs to be established. Indeed, very few studies are available in the literature on the use of insect meals for laying hens, and the results are often contradictory. Recently, Marono et al. (2017) evaluated the effects of a full replacement of soybean meal with a defatted meal from *Hermetia illucens* and found a decrease in feed intake and, consequently, productive performance. Maurer et al. (2016) reported that *Hermetia illucens* larvae meal including up to 100% protein basis in a laying hen's diet did not affect egg production, feed intake and feed conversion efficiency. On the other hand, Cutrignelli et al. (2018) showed that the total replacement of soybean meal with *H. illucens* larvae meal had a negative effect on the layers' nutrient digestibility.

The aim of this study was to evaluate the productive performance, the nutrient digestibility, the blood analysis and the changes in internal organs of laying hens fed *Hermetia illucens* larvae meal at a partial substitution of the soybean meal protein (25 or 50%), in order to establish the most suitable inclusion level of this insect meal in their diets.

3.3 Materials and Methods

3.3.1 Diets and animals

The insect meal tested in this study was obtained from partially defatted *Hermetia illucens* larvae and purchased by a leading European company specialized in insects as a nutritional source (HI, Hermetia Deutschland GmbH & Co. KG, Amtsgericht Potsdam, Germany).

Tab. 3.1 Proximate composition, mineral and essential amino acid composition (% as fed) of the Hermetia illucens larvae meal and soybean meal.

	***Hermetia illucens* larvae meal**	**Soybean meal**
Proximate composition		
Dry matter	92.7	90.0
Crude protein	55.6	43.4
Ether extract	8.34	1.1
ADF	11.5	5.9
ADF-linked protein	4.86	1.78
Ash	7.8	6.0
Mineral composition		
Ca[1]	6.47	2.83
Total P[1]	0.90	0.57
Na[1]	0.12	0.16
Essential Amino Acid composition		
Lysine[1]	4.12	2.92
Methionine[1]	1.09	0.61
Methionine+Cystine[1]	1.32	1.33
Isoleucine[1]	2.97	2.30
Tryptophan[1]	0.30	0.73
Valine[1]	5.02	2.11

Threonine[1]	2.32	1.74

[1] obtained by producers;

The trial was carried out for 20 weeks from February to July 2017 on a private laying hens company located in Sardinia (Italy). A total of 162, sixteen week-old Hy-line Brown hens (average live weight 1.41 kg ± 0.13) were equally divided into three experimental groups each one subject to different dietary treatments.
The control group was fed a corn-soybean meal-based diet (SBM) developed to satisfy the hens' requirements according to the Hy-line Brown commercial line management guide (2016). In the diets of the treated groups (HI25 and HI50), soybean meal was partially replaced by insect meal obtained from the larvae of *Hermetia illucens*, in order to develop three isoprotein and isoenergetic diets. In the HI25 group, 25% of the diet protein content was replaced by the HI protein (inclusion level 7.3%). In the HI50 group, 50% of the diet protein content was replaced by the HI protein (inclusion level 14.6%). An indigestible marker to estimate nutrient digestibility (Celite®, Sigma-Aldrich, St. Louis, Mo) was added to all the diets at the dosage of 20 g/kg.

Tab. 3.2 Ingredients and chemical characteristics of the diets.

	SBM	**HI25**	**HI50**
Ingredients, g/kg			
Maize grain	605.5	597.5	630.5
Soybean meal	265	200	95
Insect meal	-	73	146
$CaCO_3$ grains	80	80	80
Vegetable oil	10	10	-
MinVit*	10	10	10
Methionine	2.5	2.5	2.5
Monocalcium phosphate	5	5	5

Celite	20	20	20
Salt	2	2	2
Chemical-nutritional characteristics			
Dry matter[1], %	91.53	91.39	91.62
Crude protein[1], %	16.45	16.32	17.03
Ether extract[1], %	3.17	3.61	4.06
NDF[1], %	10.38	11.29	12.49
ADF[1], %	5.85	5.90	5.67
ADL[1], %	2.67	2.94	2.29
Lysine[2], %	0.86	0.97	1.00
Methionine[2], %	0.53	0.58	0.61
Metabolizable Energy[2], kcal/kg	2832.3	2845.2	2842.2

SBM: soybean meal based diet; HI25: diet including *Hermetia illucens* as 25 % of replacement of the soybean meal protein; HI50: diet including *Hermetia illucens* as 50 % of replacement of the soybean meal protein;
[1]: determined according to AOAC (2004); [2]: calculated according to NRC (1994)
*Provided 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·

The trial was carried out for 20 weeks from February to July 2017 on a private laying

The hens were housed for 20 weeks in the same building in modified cages (800 cm2/hen), under controlled conditions of temperatures and humidity. For each group, the hens (16 weeks of age) were distributed into three cages (18 hens/cage), and each cage was divided by two internal transects into three equal areas in order to obtain nine replicates of six hens per group. Feed and water were manually distributed, and adequate separations were placed along the trough and the line of the egg collection to control feed

intake and egg production per each replicate. The dark:light cycle was 9:15 h.
After four weeks of adaptation to the new diets, the hens' performances were monitored. Feed intake was measured monthly per replicate, weighing the amount of feed distributed as well as the amount of residual and scattered feed, and it was expressed as individual feed intake per day. The hens' live weight was recorded at the beginning and at the end of the trial. From 20 to 40 weeks of age, the number of eggs produced, and the individual egg weight were recorded per replicate every day. For each replicate of each group, the egg mass was calculated as egg weight x egg production percentage and the feed conversion ratio (FCR) was calculated as the grams of daily feed intake divided by the grams of egg weight per day.

3.3.2 Chemical analysis of diets and excreta

Representative samples of the main protein sources (SBM and HI) and diets were analyzed for chemical composition according to the AOAC (2005) procedures (ID number: 2001.12, 978.04, 920.39, 978.10 and 930.05 for DM, CP, EE, CF and ash respectively). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were also determined according to Van Soest et al. (1991).
The apparent metabolizable energy for HI meal was estimated according to De Marco et al. (2015). The amount of protein linked to ADF was determined (AOAC, 2005) and, for insect meal only, it was used to estimate the amount of chitin, according to Marono et al. (2015), as follows: chitin (%)=ash free ADF (%) – ADF-linked protein (%).
The data regarding amino acids, minerals, and metabolizable energy of all the ingredients were supplied by the respective producers and used to calculate the correspondent contents in the diets. The metabolizable energy of the diets was calculated according to the NRC (1994). In the diet formulation, the contents of the main essential amino acids were optimized according to the Hy-line Brown hens standard (Guide, 2016). The methionine was supplied in all the diet until the minimum required level is reach, while the lysine was not further supplied until the calculated contents in the respective diets were adequate to sustain the layer production (NRC, 1994).
The amount of acid insoluble ash (AIA) in the diets and in the caecal contents was measured according to Vogtmann et al. (1975), using Celite® as an internal marker. Thus, the apparent ileal digestibility coefficients of

dry matter, organic matter, crude protein and ether extract of the diets were estimated using the following equation: 100 – 100 × [(% AIA in the diet / % AIA in the ileal content) × (% nutrient in the ileal content / % nutrient in the diet)].

3.3.3 Blood analysis

In order to evaluate if the nutritional treatments affect animal welfare and health, at 40 weeks of age blood samples were collected from the wing vein of two birds per replicate (a total of 54 blood samples, 18 per group) in plastic tubes. The serum was separated by centrifugation at 1500×g for 15 min and stored at −20 °C until the analysis was performed. All biochemical traits of the blood serum — total protein, albumin, globulin, glucose, cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl-transferase (GGT), alkaline phosphatase (ALP), creatine kinase (CK), lactic dehydrogenase (LDH), lactate, blood urea nitrogen (BUN), creatinine (Crea), uric acid, calcium, phosphorus, magnesium, iron, and chloride — were determined using commercially available kits from Spinreact (La Vall d'en Bas, Girona, Spain) by means of enzymatic colorimetric or kinetic methods according to the manufacturer's instructions. In addition, full diagnostics, calibration and running checks were performed by means of specific checks and calibrators (Spintrol H Normal and calibrator, Spintrol B Normal and calibrator) from the same factory. Spectrophotometric measurements were performed using an automatic biochemical analyzer, AMS AUTOLAB (Rome, Italy). Globulin concentration was estimated as the difference between total protein and albumin. Thus, the albumin to globulin ratio was calculated.

3.3.4 Slaughtering

At 40 weeks of age, two hens randomly chosen per replicate (18 per group) were slaughtered. The digestive tract was excised and weighed, the intestinal tracts were identified, and their length was measured. Other inner organs (reproductive system, liver, spleen) and the abdominal fat were also separated from the carcass and weighed. The ileum was dissected from 20mm after Meckel's diverticulum to 40mm proximal to the ileocecal junction, in order to avoid the contamination of the other intestinal contents, and the digesta were pooled per replicate (one pool from two hens per replicate; 9 pools per group), immediately frozen and subsequently freeze-

dried. The dried ileal digesta was ground enough to pass through a 1-mm sieve and stored at −20 °C until the chemical analysis.

3.3.5 Statistical analysis

The data collected were processed by means of ANOVA using the PROC GLM of SAS (2000). The differences between the groups regarding body weight, weight gain, nutrients digestibility, blood profiles and inner organs traits were analyzed by means of one-way ANOVA according to the following model:

$$Yij = m + Di + eij$$

where: Y is the single observation, m is the general mean, D is the effect of the diet (i=SBM, HI25 or HI50) and e is the effect of the error.
The data regarding egg weight and mass, feed intake, feed conversion ratio and lay percentage were processed by a 2-way ANOVA according to the model:

$$Yijk = m + Di + Mj + DMij + eijk$$

where: Y is the single observation, m is the general mean, D is the diet effect (i=SBM, HI25 or HI50), M is the month of lay effect (j=from 1 to 5), DM is the first-degree interaction effect and e is the error effect. The comparison between the means was performed by the Tukey's test (SAS, 2000). In addition, the orthogonal contrast analysis (SAS, 2000) was performed to test the linear and quadratic effect between the means. The significance level was set at $P < 0.05$.

3.4 Results

The nutritional characteristics of the main protein sources as well as the ingredients and chemical characteristics of the diets are reported in Table 3.1 and 3.2, respectively.
During the investigation no mortality or morbidity of the hens was observed. Dietary treatments did not affect the hens' live weight at the end of the trial (40 weeks). However, during the 20-week trial, the hens from the HI25 group showed a lower ($P < .05$) weight gain compared to those included in the HI50 group (Table 3.3).

Tab. 3.3 Proximate composition, mineral and essential amino acid composition (% as fed) of the Hermetia illucens larvae meal and soybean meal.

	SBM	HI25	HI50	RMSE	Mean comparison, P values		
					Tukey test	Contrast	
						Linear	Quadratic
20 weeks	1499.5	1546.1	1507.0	135.5	0.5419	0.5326	0.3902
40 weeks	1850.2	1796.4	1857.1	121.5	0.4687	0.9246	0.1901
Weight gain	305.7^{ab}	250.3^{b}	350.1^{a}	10.6	0.0362	0.5489	0.0021

SBM: soybean meal based diet; HI25: diet including *Hermetia illucens* as 25 % of replacement of the soybean meal protein; HI50: diet including *Hermetia illucens* as 50 % of replacement of the soybean meal protein;
RMSE: root means square error; a, b: $P < 0.05$

Dietary treatments showed no effect on egg weight, feed intake and feed conversion ratio. However, the hens of the SBM group showed lay percentage value lower ($P < .05$) than the percentage detected in the HI25 group, and the egg mass of the SBM group was lower compared to both HI groups (Table 3.4).

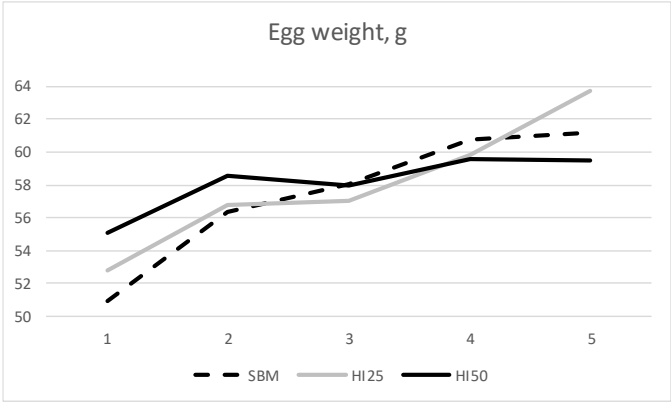
Table 3.4 Effect of the dietary treatments and the month of lay on the productive performance of the hens

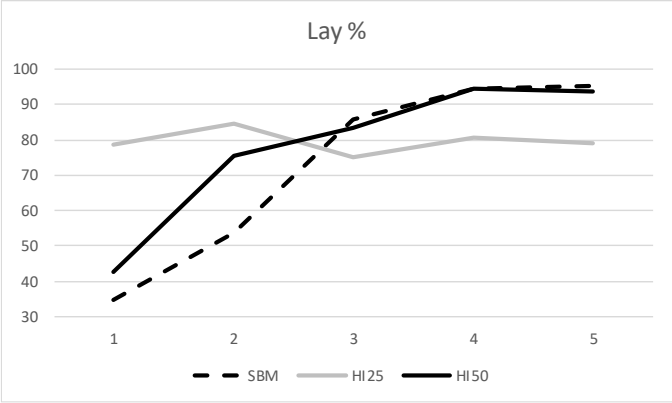
	Egg weight, g	Feed intake, g/d	FCR	Lay %	Egg mass
	Effect of the dietary treatment				
SBM	57.48	99.97	1.74	72.87^{b}	42.80^{b}
HI25	58.04	97.69	1.68	80.38^{a}	46.71^{a}
HI50	58.14	101.96	1.76	77.99^{ab}	45.72^{a}
	Effect of month of collection				
1	52.97^{C}	98.89	1.87^{A}	52.03^{D}	28.00^{D}
2	57.24^{B}	99.37	1.74A^{B}	72.62^{C}	41.58^{C}
3	57.69^{AB}	96.58	1.67^{AB}	81.43^{B}	47.00^{B}
4	60.06^{A}	106.30	1.77^{AB}	90.03^{A}	54.08^{A}
5	61.47^{A}	98.20	1.59^{B}	89.29^{A}	54.76^{A}
RMSE	2.13	8.72	0.15	6.26	3.25
	P value				
Diet effect	0.5792	0.3340	0.3005	0.0431	0.0392
Month effect	<0.0001	0.0865	0.0019	<0.0001	<0.0001
Interaction	0.0429	0.9926	0.9997	<0.0001	<0.0001
	Contrast analysis				
Linear	0.3305	0.4895	0.8133	0.1938	0.4356
Quadratic	0.6950	0.1911	0.1274	0.1473	0.3698

SBM: soybean meal based diet; HI25: diet including *Hermetia illucens* as 25 % of replacement of the soybean meal protein; HI50: diet including *Hermetia illucens* as 50 % of replacement of the soybean meal protein; FCR: feed conversion ratio;
RMSE: root means square error; a, b: P < 0.05; A, B, C, D: P < 0.01

As expected, egg weight and mass as well as lay percentage increased over time ($P < .05$) and the FCR in the last month of the trial was more favorable than that observed at the beginning ($P < .05$). However, a significant interaction dietary treatment per month collection was observed for egg weight ($P < .05$), lay percentage, and egg mass ($P < .05$). In particular, during the first month of the trial, egg weight was higher in the HI50 group followed by the HI25 and SBM groups, while at the end of the trial the HI25 group had a higher egg weight, followed by the control and the HI50 groups (Fig. 3.1, Graph 1). The HI25 group had a higher lay percentage and egg mass during the first month of the trial. However, both values were lower than those of the other two groups during the fifth month (Fig. 3.1, Graphs 2 and 3).

Figure 3.1 Effect of the interaction dietary treatment x month in lay for egg weight, lay percentage and egg mass





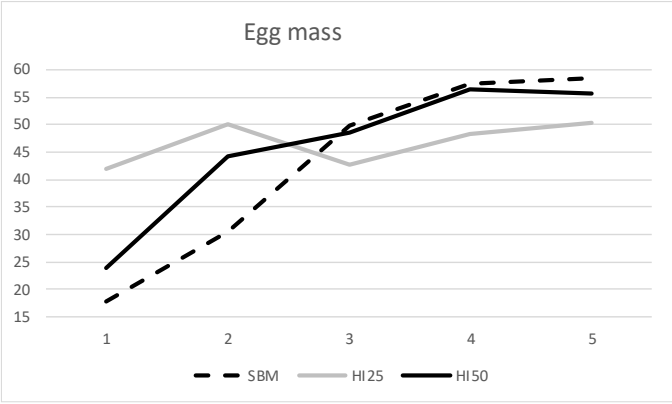


Table 3.5 shows the coefficients of the apparent ileal digestibility of the nutrients measured in the hens at 40 weeks of age.

Table 3.5 Apparent ileal digestibility coefficients (%) of the nutrients measure in the layers at 40 weeks of age

	SBM	**HI25**	**HI50**	**RMSE**	**Mean comparison, P-values**		
					Tukey test	**Contrast**	
						Linear	**Quadratic**
Dry matter	74.98^{Aa}	70.29^{Ab}	64.29^{B}	1.95	0.0031	0.0874	0.1125

Organic matter	75.07^{A}	73.66^{AB}	67.23^{B}	3.39	0.0059	0.0432	0.2156
Crude Protein	86.15^{A}	81.12^{B}	76.06^{C}	1.76	0.0089	0.0012	0.1589
Ether extract	91.41	90.83	89.33	4.06	0.2192	0.0498	0.3998

SBM: soybean meal based diet; HI25: diet including *Hermetia illucens* as 25 % of replacement of the soybean meal protein; HI50: diet including *Hermetia illucens* as 50 % of replacement of the soybean meal protein;
RMSE: root mean square error; a, b: P < 0.05; A, B: P < 0.01.

The coefficients of dry matter digestibility of the SBM and HI25 groups were higher (P < .05) than in the HI50 group; the SBM group also showed a dry matter digestibility higher (P < .05) than that detected in the HI25 group. The organic matter digestibility of the SBM group was higher (P < .05) than that of the HI50 group, and crude protein digestibility was the highest (P < .05) in the SBM group, followed by the HI25 and the HI50 groups. A significant value for the linear contrasts was detected for the organic matter, ether extract (P < .05) and crude protein (P < .05) digestibility.
Table 3.6 reports serum proteins, glucose, and lipids, liver, renal and muscle function markers as well as the electrolytes of the laying hens according to the dietary treatments. Globulin content was higher (P < .05) in the HI50 group compared to the other two groups and, as a consequence, the opposite happened for the albumin to globulin ratio.

Table 3.6 Effect of the dietary treatments on biochemical profiles of the layers at 40 weeks of age

	SBM	**HI25**	**HI50**	**RMSE**	**Mean comparison, P values**		
					Tukey test	**Contrast**	
						Linear	**Quadratic**
TP, g/dl	4.73	5.17	5.02	0.21	0.4190	0.4909	0.8405
Albumin, g/dl	2.08	2.43	2.23	0.25	0.2316	0.7633	0.0491
Globulin, g/dl	2.66^{b}	2.73^{b}	3.41^{a}	0.13	0.0307	0.0167	0.7716

A/G	0.78[a]	0.89[a]	0.67[b]	0.02	0.0259	0.2475	0.1106
AST, U/l	34.55	29.11	45.22	4.84	0.6740	0.5223	0.4561
ALT, U/l	193.2	181.7	171.4	14.20	0.6022	0.1643	0.9600
GGT, U/l	73.00	72.77	98.78	12.81	0.6451	0.1643	0.4077
ALP, U/l	962.7	1035.7	838.1	78.4	0.8614	0.7356	0.6721
Bil, mg/dl	1.12[a]	0.47[b]	0.59[b]	0 .03	0.0275	0.5255	0.0312
Glucose, mg/dl	171.7	166.7	157.3	25.20	0.4390	0.1432	0.5164
Chol, mg/dl	110.87[a]	95.60[b]	95.00 b	6.09	0.0325	0.3047	0.6247
Trig, mg/dl	1788.1 A	979.4[B]	983.9 B	106.9	0.0023	0.3321	0.9023
BUN, mg/dl	9.46[a]	4.82[b]	4.30[b]	0.39	0.0103	0.0217	0.3310
Uricacid, mg/dl	5.02	4.91	4.91	0.16	0.9854	0.2358	0.4839
Crea, mg/dl	0.60	0.49	0.49	0.002	0.5687	0.3608	0.4122
LDH, U/l	3441	3637	3370	132.5	0.8698	0.3823	0.0196
CK, U/l	666	617	625	4.25	0.7785	0.5214	0.6338
Mg, mg/dl	3.73[a]	3.38[a]	2.54[b]	0.14	0.0231	0.0234	0.3900
P, mg/dl	8.00[AB]	10.11[A]	6.71[B]	0.23	0.006	0.2975	0.0140

Ca, mg/dl	10.71	11.26	11.33	1.97	0.1793	0.2205	0.5084
Cl, mmol/l	157.2^{A}	150.5^{AB}	144.9^{B}	7.78	0.0077	0.0020	0.9249
Fe, mcg/l	2591.1	262.6	219.7	59.7	0.1543	0.5166	0.0683

SBM: soybean meal based diet; HI25: diet including *Hermetia illucens* as 25 % of replacement of the soybean meal protein; HI50: diet including *Hermetia illucens* as 50 % of replacement of the soybean meal protein;
RMSE: root means square error; a, b: $P < 0.05$; A, B: $P < 0.01$.
TP: total protein; A/G: albumin to globulin ratio; AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, GGT: Gamma glutamyl-transferase, ALP: Alkaline phosphatase, CK: Creatine kinase, LDH: Lactic dehydrogenase, BUN: Blood urea nitrogen, Crea: Creatinine; Bil: bilirubin; Chol: cholesterol; Trig: triglycerides.

Bilirubin had a higher ($P < .05$) value in the SBM group compared to the other ones. The BUN ($P < .05$), cholesterol ($P < .05$) and triglycerides ($P < .05$) levels were higher in the SBM group compared to the insect groups.
In the HI50 group, a lower level of Mg ($P < .05$) was detected in comparison to the other groups, and P blood levels were lower ($P < .05$) in HI50 than in the HI25 group. In addition, the contrast analysis showed a quadratic relationship for P blood levels. The HI50 group also had a lower ($P < .05$) Cl content than the other groups with a significant ($P < .05$) linear effect of the contrasts.
Table 3.7 shows the weights (full and empty) and the length of the different hens' gastrointestinal tracts expressed as percentage of their live weight. Very few differences were observed among the groups. The HI25 group had a higher incidence ($P < .05$) on the full muscle stomach compared to the HI50 group, and the full intestine and jejunum length of the insect groups were higher ($P < .05$) than those of the SBM. The quadratic component of the variance was significant ($P < .05$) for the jejunum and cloaca length. Concerning the live weight of internal organs, a significant ($P < .05$) effect of the quadratic contrast was detected only for abdominal fat.

Table 3.7 Effect of dietary treatments on gastro-intestinal traits of the hens at 40 weeks of age

	SBM	HI25	HI50	RMSE	Mean comparison		
					Tukey test	Contrast	
						Linear	Quadratic
LW, g	1850.2	1796.4	1857.1	159.4	0.4646	0.9246	0.1901
	Gastro-intestinal weight, % live weigh						
Crop	0.53	0.54	0.61	0.04	0.3956	0.2113	0.6066
Full MS	1.92^{ab}	2.20^{a}	1.81^{b}	0.28	0.0222	0.4224	0.0082
Full GS	0.56	0.66	0.60	0.03	0.2833	0.4944	0.1479
Empty MS	1.62	1.75	1.59	0.20	0.1769	0.7049	0.0236
Empty GS	0.49	0.57	0.56	0.02	0.1439	0.1220	0.2379
Full duodenum	0.99	1.12	1.05	0.09	0.6996	0.7305	0.4358
Empty duodenum	0.93	0.91	0.87	0.03	0.7072	0.5058	0.9429
Full jejunum	1.46	1.53	1.59	0.13	0.7243	0.4277	0.9339
Empty jejunum	1.27	1.37	1.28	0.21	0.9704	0.9085	0.3173
Full ileum	1.24	1.27	1.44	0.12	0.7451	0.6324	0.2159
Empty ileum	1.00	1.05	1.18	0.11	0.1327	0.0498	0.5765

Full caeca	0.90	1.00	0.87	0.09	0.3060	0.6859	0.1404
Empty caeca	0.61	0.67	0.57	0.01	0.2965	0.5163	0.1620
Empty cloaca	1.40	1.33	1.28	0.12	0.5247	0.2682	0.8380
Full whole GIT	9.31	9.84	9.84	0.23	0.6784	0.5597	0.6321
Empty whole GIT	7.80	8.12	7.96	0.53	0.5833	0.6851	0.3433
			Intestinal length, % live weight				
Duodenum	1.49	1.43	1.50	0.37	0.9885	0.9146	0.6892
Jejunum	3.45[b]	4.68[a]	4.64[a]	0.12	0.0265	0.1093	0.0245
Ileum	3.69	3.82	3.91	0.86	0.8907	0.5617	0.9441
Caeca	2.39	2.44	2.58	0.54	0.8069	0.6431	0.6485
Cloaca	0.82	0.98	0.81	0.16	0.0621	0.9347	0.0196
Entire intestine	11.84[b]	13.35[a]	13.44[a]	0.22	0.0324	0.0857	0.2356

SBM: soybean meal based diet; HI25: diet including *Hermetia illucens* as 25 % of replacement of the soybean meal protein; HI50: diet including *Hermetia illucens* as 50 % of replacement of the soybean meal protein;
RMSE: root means square error; a, b: $P < 0.05$.

3.5 Discussion

The feeding of a partially defatted insect meal from *Hermetia illucens* larvae as a partial alternative to the soybean meal affected some productive tracts of the layers used in this trial.

Feed intake, egg weight, and feed conversion ratio were in line with the standard of the Hy-line Brown hens (Guide, 2016) and were not different among the groups. This suggests an adequate palatability of insect meals and a similar dietary efficiency among the groups. These results are in line with the findings of Maurer et al. (2016), who found that egg production, feed intake, and feed conversion ratio in Lohmann Leghorn laying hens fed experimental diets, in which *Hermetia illucens* larvae meal replaced 100% of soybean cake, were unaffected by the dietary treatments. On the contrary Marono et al. (2017) replacing completely soybean meal with defatted insect meal from *H. illucens* larvae observed a decreasing in hens' feed intake and egg weight, thereby an improving of their FCR. This trial is more comparable to that of Marono et al. (2017), for the analyzed laying period (26–42 weeks for Marono et al. and 20–40 for this trial), while the research of Maurer et al. (2016) was performed for 10 weeks at a later laying period (from 64 weeks of age). Probably, a lower level of soybean replacement, as in this trial, avoids the negative effect on feed intake and thus on the hens' productive performance, as reported by Marono et al. (2017) and ascribed to the different color and flavor of the insect-based diet.

The lay percentage showed a higher value in the HI25 group compared to the control. This affected the corresponding egg mass, which was also higher than the control in the HI50 group. These results could be evaluated considering the different level of the lysine, in the hens' diets as well as the different protein digestibility coefficients estimated at the ileum level. All diets are in line with the standards indicated in the Brown Hy-line hens' management guide (2016), which reports an amount of 0.88 and 0.42%, respectively for lysine and methionine, with a feed intake of around 100 g/d. The insect meal used in this trial had higher contents of methionine and lysine than the soybean meal.

Methionine levels were balanced among HI groups supplementing the diets, while lysine amounts in the HI25 and HI50 were higher than the control diet (+13 and+16%, respectively). Lysine is generally considered the second limiting amino acid (Fakhraei et al., 2010; Fouad et al., 2017) in avian species and Fakhraei et al. (2010) observed that the lysine requirement for the most favorable egg mass was higher than the lysine requirement based

on egg production (around +15% than the recommended values). Thus, it is reasonable to suppose that a higher amount of lysine, exceeding the optimum suggested by breeding standards, can have a positive effect on egg mass. However, the effect of dietary treatments on lay percentage and egg mass was more evident during the first month of the trial and the group with the lowest inclusion of insect meal had the most interesting results. This aspect needs further investigation and could be affected by the different protein digestibility of the diets. The HI50 diet contained a higher amount of total lysine, but it is also likely that a higher amount of this essential amino acid was unavailable for digestion. The reduction of dry matter and organic matter digestibility observed in the HI50 group was mainly due to the reduction in protein digestibility (11.7% reduction), while in the HI25 group protein digestibility diminished by approximately 5.8%.

This is due to chitin content, which, due to its protein binding activity, makes it unavailable for digestion (Longvah et al., 2011). The linear decrease in ether extract digestibility based on the increase in insect meal inclusion level in the diet, as shown in the contrast analysis, is probably tied to chitin's ability to reduce fat absorption along the gastrointestinal tract by binding bile acids (Ylitalo et al., 2002).

Chitin's positive effects on poultry's health and metabolism are clearly confirmed by the hens' blood analysis. Hens fed a higher amount of insect meal showed higher values of globulin and a lower albumin / globulin ratio compared to the other groups. Griminger and Scanes (1986) stated that high globulin concentrations and low albumin / globulin ratios indicate a better disease resistance and immune response in birds. In a recent study on the use of Tenebrio molitor larvae meal to feed broilers from 30 to 62 d of age, Bovera et al. (2015) also found a lower albumin / globulin ratio in the group which was fed an insect meal and the same result was reported by Marono et al. (2017) in laying hens which were fed *H. illucens* larvae meal, as a total replacement of soybean meal. The lack of effects on the albumin to globulin ratio in hens fed the HI25 diet could be due to a lower amount of ingested chitin. In fact, based on our analysis, the amount of chitin estimated in insect meal was 6.64% as feed; thus, considering the level of inclusion of insect meal in the diets and the hens' average feed intake, the HI25 group ingested around 0.47 g/d of chitin, while the HI50 group ingested 0.99 g/d. Probably, the lowest amount of chitin was not sufficient to act on the hens' immune system. In a recent research on laying hens, Borrelli et al. (2017) showed that the increases in SCFAs concentrations due to the feeding of *Hermetia*

illucens larvae meal as total replacement of soybean meal promoted both gut and overall health in birds.

The amount of chitin was sufficient to reduce bilirubin in the blood of both groups fed insect meal, probably because of its cytoprotectivity or an inhibitory effect on the cytochrome P450 (Santhosh et al., 2007). levels in blood and these results are in line with the previous findings (Marono et al., 2017). According to Hossain and Blair (2007), the lower serum cholesterol and triglycerides contents can be attributed to chitin.

These authors, including a commercial chitin in the broiler diet from 1 to 21 d of age at 0, 25, 50, and 75 g/kg of the diet, showed a cholesterol and triglycerides reduction at all inclusion levels. The effect of chitin on lowering cholesterol and triglycerides could be ascribed to its positive charge, which is able to attract negatively charged bile acids and free fatty acids (Prajapati and Patel, 2010). As reported by El-Gobary et al. (2016), chitin decreased lipid absorption in the intestine by binding to the lipids and fatty acids directly or binding bile acids and fatty acids and lowered the plasma cholesterol.

A decreased amount of BUN was detected in the hens fed the insect meal. In quails, Riaz et al. (2014) considered higher values of serum urea concentrations as indicative of kidney damage and impaired glomerular function in removing the non-protein nitrogenous and metabolic substance of protein from the body. Actually, uric acid is the most prevalent poultry nitrogenous waste product (Harr, 2002), and its level in the serum reflects protein catabolism. In our study, no differences in uric acid levels were found between the groups, suggesting that the tested diets had no effect on protein metabolism. Indeed, such an apparent contrast between BUN and uric acid levels deserves to be further explored, mainly because feeding chitin may result in reducing ammonia levels, partly by acting on BUN levels (Khempaka et al., 2011).

The contents of Mg, Cl and P are reduced in the HI50 group, and this is partially consistent with the finding of Marono et al. (2017), who detected a significant decrease in chloride in the blood of hens, which were fed insects. However, even if not significant, the authors also recorded a reduction of around 25% and 10%, respectively in Mg and P in the blood of the hens fed *H.* illucens. An increase in the full intestinal length of hens in both insect groups was mainly due to the increase in the jejunum length. Bovera et al. (2016), studying the full replacement of soybean meal with insect meal from Tenebrio molitor in broilers, also observed an increased intestinal length in the insect group, mainly resulting from an elongated ileum.

The effect of the dietary treatment on intestinal length could be mainly due to the low digestibility coefficients observed in the layers, which were fed the insect meal. As it is well known, low feed digestibility in poultry increases their intestinal length (Smits and Annison, 1996) as a consequence of their body's compensatory mechanism increasing their available nutrient absorption surface (Borin et al., 2006). It is well known that the jejunum is the section of the small intestine in which most of the nutrients absorption occurs. More specifically, simple sugars, water soluble vitamins (with the exception of Vit C), and amino acids are absorbed in this tract, while the rest of the feeds passes into the ileum. Thus, the diminished protein digestion could affect the elongation of the jejunum. Cutrignelli et al. (2018) did not found changes in the intestinal length of Lohmann Brown hens fed insect meal from *Hermetia illucens*, as a total replacement of soybean meal. Probably, the discrepancy between these findings is related to the animals' age. In fact, in the trial conducted by Cutrignelli et al. (2018), hens aged 24 to 45 weeks were used, while the hens used in this trial started to fed insect meal at 16 weeks of age. This might have triggered chitin's negative effect, as the animals were at an earlier growth stage.

In conclusion egg weight, feed intake, and feed conversion rate were not affected by the substitution of soybean meal at both inclusion levels. Egg mass was found to be positively influenced by the insect meal diets, as well the lay percentage, only in the HI25 diet. These results are probably due to the higher availability of methionine and lysine in the HI25 diet.

Dry matter, organic matter, and crude protein digestibility decreased, as the percentage of insects in the hens' diet increased, due to chitin's negative effect, which was more remarkable in the HI50 diet. The results obtained confirm that the inclusion of insect meal in the diet lowered serum cholesterol and triglycerides contents at both inclusion levels, due to the effect of chitin, which decreases nutrient absorption in the intestine. The serum globulin level increased only at the highest level of insect meal inclusion. As a result, the albumin to globulin ratio decreased.

Our results indicate that protein replacement at 25% with insect meal from *Hermetia illucens* larvae in the diet of laying hens is more suitable and closer to the optimal level than replacement at 50%.

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Chapter 4

Intestinal morphometry, enzymatic and microbial activity in laying hens fed different levels of a *Hermetia illucens* larvae meal and toxic elements content of the insect meal and diets

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4.1 Simple Summary

Recently, several studies have focused on the use of insect larvae meal as an alternative to soybean meal in poultry diets. In this regard, it is crucial to understand all the possible aspects related to the chemical-nutritional characteristics, the effects on the animals' health and welfare, and the safety of different insect meals. This study aimed to evaluate volatile fatty acids production in the caeca, the intestinal morphometry, and the brush border enzymatic activity of hens fed different levels of *Hermetia illucens* (Linnaeus) (Diptera: *Stratiomyidae*) larvae meal for 24 weeks.
The research also aimed to contribute to the knowledge of the concentration of toxic elements in insect meals. Overall, the insect meal inclusion affected the small intestine morphometry, the enzymatic activity, and the caecal microbial activity. The soybean meal group showed the highest intestinal functionality, while a compensatory mechanism, probably mediated by the chitin, led to a positive increase of volatile fatty acids and butyrate in the 50% protein replacement diet with potential positive effects on gut healthiness. The levels of toxic elements in the diets and insect meal were lower than the maximum levels of heavy metals set by the EU Commission for the feed.

4.2 Abstract

To evaluate the effects of feeding a *Hermetia illucens* (HI) larvae meal on the different intestinal traits of hens, and to determine the toxic elements' concentration in the insect meal and diets, 162 hens were randomly allotted to three groups. The control received a corn-soybean meal-based diet (SBM); the HI25 and HI50 groups received two diets in which the 25% and 50% of the dietary protein were replaced by the HI protein, respectively. The duodenal and jejunal villi height and villi/crypt were higher ($p < 0.01$) in the SBM than in the HI groups. The ileal villi height was higher ($p < 0.05$) in the SBM and HI25 groups than the HI50. The HI50 group exhibited a lower duodenal maltase activity. The intestinal alkaline phosphatase (IAP) activity linearly decreased in the duodenum and jejunum as the dietary insect meal inclusion increased. The HI50 group had a higher acetate and butyrate level than the SBM. The levels of cadmium (Cd), lead (Pb), mercury (Hg), and arsenic (As). In the diets and insect meal were lower than the maximum values established by the EU Commission.
The 25% soybean protein replacement with Hermetia illucens larvae meal in the diet of laying hens was more suitable and closer to the optimal level than 50%.

4.3 Introduction

The EU approval for the use of the insect meals in poultry nutrition is “not so distant in the future”, and the approval by the EU Member States could be possible during the first quarter of 2019 (IPIF, 2018; All about Feed, 2019). Thus, it is mandatory to understand all the possible aspects related to the chemical-nutritional characteristics, the effects on the animal health and welfare, the impact of feed and food safety of the different meals deriving from insects. This goal is not easy to reach as some characteristics can be modified according to the species and, within the species, in relation to the harvesting stage, the growth substrate, etc. In recent years, several studies pointed out the attention on the use of insect meals in growing broiler (Bpvera et al., 2015; Bovera et al., 2016; Schiavone et al 2018; Loponte et al., 2018), quail (Cullere et al., 2018), and barbary partridge (Loponte et al., 2017; Secci et al., 2018). In laying hens, the available literature on the effects of insect meals on laying performance, egg quality, metabolic and nutritional effects is limited and very often the results are conflicting due to the different species of insects used, the different strains and age of lay of the hens utilized in the trials (Al-Qazzaz et al., 2016; Marono et al., 2017; Bovera et al., 2018; Secci et al, 2018).

An important aspect concerning the use of insect meal as a feed ingredient, related to human and animal health, is the possible accumulation of mineral elements in the insect body during the growing cycle. Some elements (Cu, Se, Cr, Fe, Mn, Ni) are essential for biological functions, but the heavy metals like Cd, Pb, Hg, and as can induce adverse effects due to their potential toxicity and bioaccumulation in the food chain (Migliarini et al., 2005). To regulate animal dietary exposure to heavy metals, the EU Commission established maximum levels (MLs) for different undesirable substances in animal feeds (EC No 2002/32). Data on the transfer of chemical contaminants from different substrates to the insects are very limited (EFSA, 2015).

Therefore, monitoring of toxic elements concentrations, in the insect meal and diets, is necessary from the point of view of both nutrition and contamination. The few studies on the mineral profile of insect meals (Cullere et al., 2018; Liland et al, 2017; Irungu et al., 2018) often showed different minerals with a very wide range of variation according to the composition of the substrate used for the insects growing. For example, increasing the inclusion level of the brown algae, Ascophyllum nodosum, in the growing substrate led to increased calcium, sodium, and magnesium and

decreased phosphorus, manganese, and copper concentrations in *Hermetia illucens* larvae (Liland et al, 2017). This study represents the continuation and completion of a previous trial (Bovera et al., 2018) in which the laying performance, blood profiles, and nutrient digestibility of hens fed Hermetia illucens (HI) larvae meal from 16 to 40 weeks of age were investigated. Thus, the aim of the present research was to evaluate the effect of the inclusion of a partially defatted meal from HI larvae in the diet on the volatile fatty acids (VFAs) production in the caeca, intestinal morphometry, and brush border enzymatic activity of 40 weeks old layers. In addition, the research aimed to contribute to the knowledge of the trace and toxic elements' concentration in insect meals.

4.4 Materials and Methods

4.4.1 Animals and Experimental Treatments

All the animals were treated according to the principles of the animal welfare stated by the EC Directive 63/2010/EEC regarding the protection of the animals used for experimental and other scientific purposes. The experimental procedures were approved by the Ethical Animal Care and Use Committee of the Department of Veterinary Medicine and Animal Production of the University of Napoli Federico II, Italy (prot. N. 2017/0017676).
The trial was carried out in a private laying hens farm located in Sardinia (Italy) for 24 weeks, from February to July 2017. A total of 162, sixteen weeks old Hy-line Brown hens (average live weight 1.41 kg ± 0.13 standard deviation) were equally divided into three experimental groups, differing for the dietary treatment.
The control group was fed a corn-soybean meal-based diet (SBM group) formulated to meet the hens' nutrients requirements according to the Hy-line Brown commercial line management guide 2016 (Hy-Line Brown Commercial Layers Management Guide, 2016). In the other groups, the soybean meal was partially replaced by a partially defatted insect meal obtained from Hermetia illucens larvae (HI, Hermetia Deutschland GmbH & Co KG, Amtsgericht Potsdam, Germany) in order to formulate two isoproteic and isoenergetic diets. In the HI25 group, the 25% of the dietary protein was replaced by the HI protein (inclusion level of the HI in the diet 7.3%); in the HI50 group, the 50% of the dietary protein was replaced by the protein of the HI (inclusion level 14.6%). The hens were housed in the same building in modified cages (800 cm2/hen), under controlled temperature (25°C) and humidity (64% ± 1.2%) conditions. For each group, the hens were distributed into 3 cages (18 hens/cage), and each cage was divided by 2 internal transects into 3 equal areas, to obtain 9 replicates of 6 hens per group. Feed and water were manually distributed, and appropriate separations were placed along the trough and the line of the egg collection to control the feed intake and the egg production per each replicate. The dark : light cycle was 9 : 15 hours.

4.4.2 Chemical Composition of the Ingredients and Diets

The main protein sources (soybean and insect meal) and the diets were analyzed for the chemical composition according to the AOAC official methods (AOAC, 2005). The metabolizable energy of the diets was calculated according to the NRC estimation procedure (NRC, 1994), while the apparent metabolizable energy of the insect meal used in the present trial was obtained from the studies of De Marco et al. (2015). The data on the amino acids, minerals, and metabolizable energy values of all the ingredients were supplied by the respective producers and used to calculate the correspondent contents in the diets. The amount of protein linked to the acid detergent fiber (ADF) was determined (AOAC, 2005); and only for the insect meal, it was used to estimate the amount of chitin, according to Marono et al. (2015) as follows: chitin (%) = ash free ADF (%) - ADF-linked protein (%). The chemical-nutritional characteristics of the two protein sources are reported in Table 4.1, while the ingredients and chemical-nutritional characteristics of the diets are indicated in Table 4.2.

Table 4.1 Proximate composition, mineral, and essential amino acid composition (% as fed) of the Hermetia illucens larvae defatted meal and soybean meal.

	Hermetia illucens **larvae meal**	**Soybean meal**
Proximate composition		
Dry matter	92.7	90.0
Crude protein	55.6	43.4
Ether extract	8.34	1.1
ADF	11.5	5.9
ADF-linked protein	4.86	1.78
Ash	7.8	6.0
Mineral composition		
Ca[1]	6.47	2.83
Total P[1]	0.90	0.57

Na[1]	0.12	0.16
Essential Amino Acid composition		
Lysine[1]	4.12	2.92
Methionine[1]	1.09	0.61
Methionine+Cystine[1]	1.32	1.33
Isoleucine[1]	2.97	2.30
Tryptophan[1]	0.30	0.73
Valine[1]	5.02	2.11
Threonine[1]	2.32	1.74

ADF: Acid Detergent Fiber; 1 obtained by the producers.

Table 4.2 Ingredients and chemical characteristics of the three diets: control (SBM), HI25, and HI50.

	SBM	HI25	HI50
Ingredients, g/kg			
Maize grain	605.5	597.5	630.5
Soybean meal	265	200	95
Insect meal	-	73	146
$CaCO_3$ grains	80	80	80
Vegetable oil	10	10	-
MinVit*	10	10	10
Methionine	2.5	2.5	2.5
Monocalcium phosphate	5	5	5

Celite	20	20	20
Salt	2	2	2
Chemical-nutritional characteristics			
Dry matter[1], %	91.53	91.39	91.62
Crude protein[1], %	16.45	16.32	17.03
Ether extract[1], %	3.17	3.61	4.06
NDF[1], %	10.38	11.29	12.49
ADF[1], %	5.85	5.90	5.67
ADL[1], %	2.67	2.94	2.29
Lysine[2], %	0.86	0.97	1.00
Methionine[2], %	0.53	0.58	0.61
Metabolizable Energy[2], kcal/kg	2832.3	2845.2	2842.2

SBM: soybean meal-based diet; HI25: diet including HI as 25% of replacement of the soybean meal protein; HI50:bdiet including HI as 50% of replacement of the soybean meal protein; NDF: Neutral Detergent Fiber; ADF: Acid Detergent Fiber; ADL: Acid Detergent Lignin; 1: determined according to AOAC (2004); 2: calculated according to NRC (1994); * Provided per kilogram: vitamin A (retinyl acetate) 20,000 IU, vitamin D3 (cholecalciferol) 6000 IU, vitamin E (dl--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 chloride 800 mg, 4-6-phytase 1800 FYT, D.L. methionine 2600 mg, canthaxanthin 8 mg.

4.4.3 Trace/Toxic Elements Determination

The trace elements contained in the protein meals and the toxic elements in the insect meal and in the diets were also determined. The samples were digested in ultrapure 65% HNO3 and H2O2 in a microwave digestion system (Ariano, 2015). Trace elements concentrations were determined by

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) technique using a Perkin Elmer Optima 2100 DV instrument coupled with a CETAC U5000AT. The calibration curve and two blanks were run during each set of analyses, to check the purity of the chemicals. A reference material (CRM DORM-4, National Research Council of Canada (NRC-CNRC), Ottawa (Ontario), Canada) was also included for quality control. All the values of the reference materials were within the certified limits.
The instrumental detection limits are expressed as wet weight (w.w.) and were determined following the protocol described by Perkin Elmer ICP application study number 57 (Barnard, 1993).

4.4.4 Villus and Crypt Morphometry

At 40 weeks of age, two hens, randomly chosen per replicate (18 per group), were slaughtered, the different digestive tracts were identified, and each of them was excised and weighed.
A tissue sample of the small intestine tracts was rinsed with an iced saline buffer (pH 7) blotted with absorbent paper and divided into three segments, duodenum, jejunum, and ileum. The intestinal samples (0.5 cm) from duodenum, jejunum, and ileum were fixed by immersion in 4% phosphate-buffered paraformaldehyde for 48 h. The samples were then washed in a phosphate-buffered saline solution, dehydrated in graded ethanol series, and embedded in paraffin according to Vargas et al. (2018). Cross sections (5 μm) at an interval of 200 μm were stained with Mayer's hematoxylin and eosin and examined under Zeiss Axio Imager. A2 microscope for the histopathological assay. For the morphometric analysis of villus and crypts, ten randomly chosen microscopic fields from each section of the duodenum, jejunum, and ileum were acquired using a microscope combined color digital camera Axiocam 503 (Zeiss, Oberkochen, Germany), and the measurements were performed using the Zen 2.3 lite software.

4.4.5 Brush Border Membrane Enzymes Activity

The Brush BorderMembrane (BBM) enzymes were obtained according to Shirazy-Beechey et al. (1991) with some modifications as detailed in Messina et al. (2019). The hydrolysis of sucrose and maltose by the mucosal maltase and sucrase-isomaltase (SI) was determined according to Tibaldi et al. (2006). The intestinal alkaline phosphatase (IAP) and γ-glutamyl transpeptidase (γ-GT) activities were determined on the supernatant using

two commercial kits (Paramedical, Pontecagnano Faiano, SA, Italy) as indicated by the manufacturer. The total protein concentration was determined according to Bradford (1976) (Sigma-Aldrich cat. no. B6916), and bovine serum albumin (Sigma-Aldrich cat. no. 0834) was used as a standard. One unit (U) of enzyme activity corresponded to the amount of enzyme that transforms or hydrolyzes 1 µmole of substrate mL^{-1} min^{-1}.
The specific enzymatic activity was calculated as U of enzyme activity per mg of protein.

4.4.6 Volatile Fatty Acids (VFAs)

Both the caeca of each slaughtered hen were tied at the ends, separated from the rest of the
gastrointestinal tract, put in plastic bags in a pre-warmed thermos (37 °C), and transported in about 1 h to the laboratory. Two aliquots of the caecal content (~5 mL each) were used to measure the VFAs production. Each aliquot was diluted with oxalic acid (1:1, v/v), and VFAs were determined by a gas chromatography method (Stanco et al., 2003), using an equipment (Thermo-Electron mod. 8000top, FUSED SILICA Gaschromatograph, ThermoElectron Corporation, Rodano, Milan, Italy) with an OMEGAWAX 250 fused silica capillary column (30 m x 0.25 mm x 0.25 mm film thickness), a flame ionization detector (185°C), the helium as gas carrier (1.7 mL/min), and under isothermal condition (125°C).

4.4.7 Statistical Analysis

The data were processed by ANOVA using the PROC GLM in SAS (SAS, 2000). The differences among the groups regarding the VFAs in the caeca, the intestinal morphometry, and enzymatic activity were analyzed by the one-way ANOVA according to the following model: $Yij = m + Di + eij$, where Y is a single observation, m is the general mean, D is the effect of the diet (i = SBM, HI25 or HI50), and e is the error.
The comparison among the means was performed by the Tukey's test (SAS, 2000); in addition, the orthogonal contrast analysis was performed to test the linear and quadratic effect among the means (SAS, 2000).

4.5. Results

4.5.1 Trace/Toxic Elements

The data on the concentration of trace elements of the protein sources are reported in Table 4.2, and the toxic elements' concentrations in the HI meal and in the experimental diets are reported in Table 4.3.

Table 4.3 Toxic elements' content (mg/kg) in the insect meal (HI meal) and in the three diets (SBM, HI25, and HI50).

	HI meal	SBM	HI25	HI50
As	0.23	0.95	0.86	0.81
Cd	0.06	0.001	-	0.007
Pb	0.03	-	-	-
Hg	0.01	-	-	-

4.5.2 Villus and Crypt Morphometry

Table 4.4 shows the morphometry traits (villi height, crypt depth, and villi/crypt ratio) for each of the three tracts of the small intestine of the hens according to dietary treatments. In the duodenum, villi height and villi/crypt ratio were higher ($p < 0.01$) in the SBM than in both HI groups. The contrast analysis showed a significant ($p < 0.01$) linear effect for villi height, while the quadratic effect was significant ($p < 0.01$) both for the villi height and villi/crypt. In the jejunum, the villi height and villi/crypt ratio of the hens fed SBM was higher ($p < 0.01$) than that of both HI diets. The contrast analysis showed a significant effect for the linear component for the villi height, villi/crypt ($p < 0.01$), and for the crypt depth ($p < 0.05$). In the ileum, the HI25 group had a higher villi height than the HI50 ($p < 0.05$), and the only significant contrast was a linear effect ($p < 0.054$) for the villi height.

Table 4.4 Effect of the dietary treatments on the villi height and crypt depth of the different small-intestine tracts.

	SBM	HI25	HI50	RMSE	P-values		
					ANOVA	Contrast analysis	
		Duodenum				Linear	Quadratic
Villi height	1394^{A}	1031^{B}	1006^{B}	117.5	<0.0001	<0.0001	<0.0001
Crypt depth	350.8	381.3	395.8	90.56	0.4739	0.2345	0.8059
Villi/crypt	3.99^{A}	2.69^{B}	2.90^{B}	0.79	0.0006	0.0018	0.1023
		Jejunum					
Villi height	1149^{A}	825^{B}	790^{B}	114.2	<0.0001	<0.0001	<0.0001
Crypt depth	273.3^{b}	275.5^{b}	315.9^{a}	26.29	0.0461	0.0329	0.2684
Villi/crypt	4.27^{A}	3.09^{B}	2.68^{B}	0.84	<0.0001	<0.0001	0.1285
		Ileum					
Villi height	1006^{ab}	1013^{a}	843^{b}	161.2	0.0317	0.0162	0.1541
Crypt depth	321.3	335.9	328.7	32.11	0.8308	0.7601	0.6151
Villi/crypt	3.31	3.12	2.77	0.36	0.2351	0.0918	0.7821

SBM: soybean meal group; HI25 and HI50: HI groups in which the soybean protein was replaced by 25% and 50% of the HI larvae meal protein, respectively. A, B: $p < 0.01$; a, b: $p < 0.05$; RMSE: root mean square error.

4.5.3 Brush Border Membrane Enzymes Activity

Table 4.5 reports the activities of brush border enzymes in each of the three tracts of the hen's small intestine, according to dietary treatments. In the duodenum, the maltase was higher ($p < 0.05$) in the SBM and HI25 than the HI50 group and the contrast analysis indicated a significant linear effect for the maltase and IAP ($p < 0.05$). In the jejunum, the IAP of the SBM was higher than that of both the HI groups and the contrast analysis showed a significant linear effect ($p < 0.05$) for the IAP and γ-GT.
In the ileum, the γ-GT showed higher values in the SBM than both the insect meal groups ($p < 0.05$), with a quadratic significant effect ($p < 0.05$).

Table 4.5 The specific activity of the maltase, sucrose-isomaltase (SI), intestinal alkaline phosphatase (IAP), γ-glutamyltransferase (γ-GT) measured in the different digestive tracts of the hens fed the test diets over 21 weeks.

	SBM	**HI25**	**HI50**	**RMSE**	**P-values**		
					ANOVA	**Contrast analysis**	
	Duodenum					Linear	Quadratic
Maltase	70.46[a]	74.96[a]	50.82[b]	19.47	0.0498	0.0465	0.1044
Saccarase	12.12	13.02	11.54	3.49	0.7001	0.7422	0.4419
IAP	4.39	3.87	2.75	1.15	0.3165	0.0422	0.7479
γGT	129.4	159.2	119.9	59.9	0.2888	0.5582	0.1457
	Jejunum						
Maltase	71.39	69.94	76.77	16.97	0.7025	0.5325	0.5799
Saccarase	17.49	14.65	16.21	4.97	0.5308	0.6118	0.3194
IAP	5.48a	3.07b	2.87b	2.23	0.0436	0.0295	0.2680
γGT	206.4	199.1	154.9	70.42	0.3064	0.0435	0.5515
	Ileum						
Maltase	77.33	55.92	64.19	22.25	0.1780	0.2505	0.1385
Saccarase	17.34	14.51	17.20	3.90	0.2840	0.9445	0.1172
IAP	3.29	3.09	3.06	1.35	0.9351	0.7379	0.8899
γGT	145.0[a]	94.09[b]	132.73[ab]	37.0	0.0307	0.513	0.0108

SBM: soybean meal group; HI25 and HI50: HI groups in which the soybean protein was replaced by 25% and 50% of the HI larvae meal protein, respectively. a, b: $p < 0.05$; RMSE: root mean square error.

4.5.4 Volatile Fatty Acids

Table 4.6 reports the effect of the dietary treatments on the VFAs production in the hen's caeca. The production of acetate and total VFAs (mmol/L) was higher in the caecal content of the hens fed the HI50 diet than in that of the other groups ($p < 0.05$). The butyrate content was higher ($p < 0.05$) in the HI50 than in the SBM group, while the valerianic acid of the hens fed the SBM diet was higher ($p < 0.01$) than those fed both the HI diets. The contrast analysis showed a significant linear effect ($p < 0.05$) for the isobutyrate, butyrate, isovalerianic, and valerianic acids.
In Table 4.6, the VFAs content is also expressed as a percentage of the total VFAs. In this case, the isobutyrate had a higher production in the caeca of the hens fed the HI diets compared to the control ($p < 0.05$), the butyrate in the HI50 group was higher ($p < 0.05$) than the control, while the valerianic acid in control was higher ($p < 0.01$) than both the HI groups. The contrast analysis showed a significant linear effect for butyrate and isovalerianic acids ($p < 0.05$) and for the valerianic acid ($p < 0.01$). For valerianic acid, the quadratic effect was also significant ($p < 0.01$).

Table 4.6 Volatile fatty acid production in the caecal content of the hens fed the test diets over 21 weeks.

	SBM	**HI25**	**HI50**	**RMSE**	**P-values**		
					ANOVA	**Contrast analysis**	
	Mmol/l					**Linear**	**Quadratic**
Acetate	58.24^{b}	57.54^{b}	65.99^{a}	6.59	0.0242	0.1565	0.3406
Propionate	20.62	20.31	21.42	2.50	0.9169	0.7718	0.7735
Isobutyrate	1.78	2.11	2.34	0.17	0.1442	0.0107	0.8381
Butyrate	7.25^{b}	7.72^{ab}	8.81^{a}	0.59	0.0125	0.0326	0.6576
Isovalerianic	2.78	3.00	3.42	0.25	0.1274	0.0453	0.7919
Valerianic	4.38^{A}	2.99^{B}	3.28^{B}	0.31	0.0021	0.0176	0.3777
Total VFA	95.06^{b}	93.67^{b}	105.0^{a}	6.69	0.0392	0.5826	0.4620
	% total VFA						

Acetate	61.27	61.43	62.85	5.38	0.8571	0.5743	0.9914
Propionate	21.69	21.68	20.40	2.54	0.8092	0.6036	0.7088
Isobutyrate	1.87^{b}	2.25^{a}	2.22^{a}	0.15	0.0212	0.1375	0.3203
Butyrate	7.62^{b}	8.24^{ab}	8.39^{a}	0.53	0.0456	0.0289	0.8072
Isovalerianic	2.92	3.20	3.26	0.12	0.0953	0.0423	0.8112
Valerianic	4.61^{A}	3.19^{B}	3.12^{B}	0.31	<0.0001	<0.0001	0.0014

SBM: soybean meal group; HI25 and HI50: HI groups in which the soybean protein was replaced by 25% and 50% of the HI larvae meal protein, respectively. A, B: $p < 0.01$; a, b: $p < 0.05$; RMSE: root mean square error.

4.6. Discussion

The trace minerals content of the insect meals is still poorly investigated, but it is an important goal to formulate appropriate diets for poultry and avoid a possible excessive excretion into the environment. In addition, the knowledge of the potential accumulation of toxic elements is mandatory to guarantee humans and animals safety. A recent study showed that the concentration of many minerals (magnesium, calcium, iodine, iron, odium, and potassium) in black soldier fly larvae increased linearly as the level of the correspondent mineral in the growing substrate increased, while manganese remained stable in the larvae, despite varying concentrations in the media (Liland et al., 2017)

The data available in the literature on the mineral concentration of the black soldier fly larvae showed a high variability (Cullere et al., 2018; Liland et al., 2017; Irungu et al., 2018), and thus it is difficult to compare our results to the available data. The discrepancy of the results is tied to the different kind of substrates used for the insects' growth but also, for some other elements as Zn, to the technological process following the larvae harvesting. However, the concentrations of Co, Ni, and Se found in the edible insect of our trial were lower than those of the conventional food. The insect meal had a higher amount of Zn, considering the inclusion level, so the only insect meal supplied a daily amount of Zn (331.1 and 662.3 mg/kg, respectively, for HI25 and HI diets) exceeding the correspondent requirements indicated by Hy-Line Brown commercial layers management guide (Hy-Line Brown Commercial layers Management Guide, 2018) (minimum required 80 mg/kg in a complete diet during the laying period).

Concerning the toxic elements, the concentration of Cd, Pb, and Hg was negligible in all the analyzed samples. The mean value of As was 0.23 mg/kg in the HI meal, approximately comparable to the data reported in the literature and lower than in the complete diet samples.

Compared to the MLs of heavy metals set by the EU Commission (Directive (EC) No 2002/32), Cd, Pb, Hg, and As levels in the diets and insect meal were always lower than the maximum values established for the feeding stuff and feed materials. In fact, the EU regulation establishes the following MLs of heavy metals content in mg/kg (ppm) relative to a feed with a moisture content of 12%: the Cd MLs in the feed materials and complete feed are 2.0 and 0.5 mg/kg, respectively; the Pb MLs in the feed materials and complete feed are 10.0 and 5.0 mg/kg, respectively; the Hg MLs in the feed materials and complete

feed are both 0.1 mg/kg; the As MLs in the feed materials and complete feed are both 2 mg/kg. proteins had several effects on the small intestine morphometry and enzymatic activity, as well as on the caecal microbial activity. The morphometry changes mainly occurred in the duodenum and jejunum, but there were also some interesting modifications in the ileum. In general, the villi height decreased as the inclusion level of the insect meal increased in the diet, this is in accordance with the decreased nutrient ileal digestibility recorded in the first part of this trial (Bovera et al., 2018). The crypt depth was unchanged among the groups, or it tended to decrease (in the jejunum) as the level of the HI increased in the diet. The small intestine is involved in the digestion and absorption of almost all the dietary nutrients (Svihus, 2014): the duodenum digests around 95% of the fats (Sklan et al., 1975); jejunum digests and absorbs fats, starch, and protein (Reisenfeld et al., 1980; Sklan et al., 1980); the ileum is mainly involved in water and mineral absorption, but it also digests and absorbs fats, proteins, and starch (Svihus, 2014). The morphological studies of the small intestine are often used to assess its functionality and, in general, an increased villi height is indicative of an improved intestinal function (Awad et al., 2011). Another important consideration is that the ileal villi in chickens are smaller and lower than those of the previous tracts of the small intestine, as in hens fed corn-soybean based diets, very little amount of nutrients are available beyond the jejunum (Imondi et al., 1965; Yamauchi et al., 1995; Yamauchi et al., 1966). In the present trial, the height of the ileal villi was lower than that of the duodenum, but higher compared to the jejunum for hens fed both the insect diets. The effect of the diets on the intestinal villi height can be affected by the nutrient digestibility of the diets. As recorded in the first part of this trial (Bovera et al., 2018), the dry matter digestibility of the SBM and the HI25 diets (75.0% and 70.3%, respectively) in hens was higher ($p < 0.01$) than that of the HI50 group (64.3) and the result was mainly attributable to the crude protein digestibility (86.2 vs. 81.1 vs. 76.1%, respectively, for SBM, HI25, and HI50, $p < 0.01$). The low nutrient digestibility in the hens fed the insect meal is tied to the chitin, present in the insect exoskeleton (Bovera et al., 2015).

Thus, our hypothesis is that higher availability of nutrients in the duodenum and jejunum of the hens fed the SBM, increased the intestine functionality, improving the villi height. In insect fed hens, the lower nutrient digestibility induced an increased amount of the potential digestible nutrients in the ileum. Yamauchi et al. (2007) stated that an increased load of nutrients deriving directly from the duodenum to the ileum (both for jejunum

dissection or different diets) might stimulate the ileal absorptive function, resulting in a compensatory development of the villi. In general, longer villi are the result of activated cell mitosis in the crypts (Samanya et al., 2001); thus, a larger crypt area indicates a more intensive cell production. In the present trial, the only crypt depth was recorded, and this was unchanged among the groups in the duodenum and ileum tracts. However, the villi height to crypt depth ratio is strongly related to the epithelial cell turnover (Wang et al., 2007). In our trial, the cell turnover was higher in the SBM than in the insect meal groups for the duodenum and jejunum, while no differences were observed among the groups in the ileum.

The presence of HI meals in the diet did not affect the activity of both the disaccharases except for the maltase in the duodenum of the hens fed the highest level of HI meal. Recently, Khol et al. (2017) showed that the activity of maltase in the small intestine of mallard, chicken, and quail varied depending on the species and, in the mallard, on the intestinal tract. Working with geese, they also demonstrated an effect of the interaction with the protein and the fiber content of the diet, with the highest activity of maltase registered in the Low Protein-Low Fiber group. Likewise, in the present study, the limiting action of the chitin inside the HI meal on the availability of starch during the digestion process led to lower availability of substrate for the maltase activity.

The linear decrease of IAP in the duodenum and jejunum of the hens as the dietary insect meal inclusion increased, shows that the SBM group presents the highest intestinal functionality. Similar results have also been observed by Cutrignelli et al. (2018), when the inclusion of HI larvae meal as 50% substitution of soybean meal protein in laying hens decreased the IAP levels in the jejunum and ileum. This enzyme is considered an excellent marker for the crypt-villus differentiation in chicken (Sabatakou et al, 2017), and, in the present study, the inclusion of HI meal resulted in a negative effect in the jejunum on both the villi/crypt ratio and the IAP specific activity. The γ-glutamyl transpeptidase plays an essential role in the final digestion and absorption of the dietary proteins being involved in the amino acid transport in the intestine [48,49]. Overall, the effect of the inclusion of HI meal on the activity of the γ -GT in the ileum seems to be in contrast with the increased ileal villi height, while it is in agreement with the weight gain results reported by Bovera et al. (2018).

The inclusion of the insect meal in the hens' diet induced several modifications in the microbial activity in the caeca, as showed by the VFAs

production, but the effects were particularly evident with the highest inclusion level (HI50 group). The increased total VFAs production in the latter was mainly due to a higher production of butyrate (+21.5%) and acetate (+13.3%) than in the SBM group, while the valerianic acid decreased in the two groups fed the insect meal. These results completely agree with the finding of Cutrignelli et al. (2018), in which the hens fed an HI meal in total replacement of the soybean proteins showed an increased production of butyrate (+62.6%) and acetate (+36.1%) than the control. Similar to our results, Loponte et al. [6] found an increased amount of the total VFAs (+45.6%), acetate (+40.3%), and butyrate (+64.6%) in broilers fed a Tenebrio molitor larvae meal as a complete replacement of the soybean meal. The increased activity of the microbial population in the caeca can be related to the chitin level of the HI diet, confirming the hypothesis of Loponte et al. (2018) and Cutrignelli et al. (2018). However, another important point emerging from our research is that the chitin needs to be at a sufficient level to act as "prebiotic", stimulating the intestinal microbial activity.

Based on our analysis and taking into account the formula proposed by Marono et al. (2017) for the estimation of the insect chitin from the chemical composition, the amount of the chitin in the *H. illucens* larvae meal used in this trial was 6.64% as feed. The feed intake of the hens involved in this trial, and reported by Bovera et al. (2018), was 99.97, 97.69, and 101.9 g/day, respectively, for the SBM, HI25, and HI50 groups. Thus, considering the inclusion level in the diets, the HI25 group ingested around 0.47 g/d of chitin, while the HI50 group ingested around 0.99 g/d. Our hypothesis is that the lowest level of chitin is not sufficient to modulate the microbial population activity in the hens. The butyric acid is considered to be the main enterocytes energy source (Bovera et al., 2019), and it is also necessary for the proper development of the Gut-Associated Lymphoid Tissue (Mroz et al., 2005). It is reported that the VFAs, in general, have a bacteriostatic effect against some enteric bacteria, including *Salmonella typhimurium*, and, in particular, the butyrate is related to the decreased amounts of *Enterobacteriaceae* in chickens (Van DerWielen et al., 2000).

Thus, both the increases in the total VFAs and butyric acid can improve, through different mechanisms, the health of the hens' intestine.

Very interesting are the significant changes in the mutual proportions of the butyric, isobutyric, and valerianic acid observed, which might indicate that not only the activity but also the interactions among the different microbial species have been modified. This is in accordance with the findings by

Borrelli et al. (2017), who observed changes in the gut microbiota of hens fed HI larvae meal. In particular, these authors found a strong correlation between levels of the bacteria strains *Flavonifractor plautii*, C*hristensenella minuta*, and *Alkaliphilus transvaalensis* and high production of propionate, butyrate, and total VFAs; these bacteria are the principal contributors to -N-acetylhexosaminidases and N-acetylglucosamine 6-phosphate deacetylase production, and these enzymes represent the key enzymes responsible for a higher VFAs production.

4.7 Conclusions

The inclusion of an insect meal from HI larvae as 25 or 50% substitution of the proteins from the soybean meal influenced the small intestine morphometry and enzymatic activity, as well as the caecal microbial activity. The SBM group had the highest intestinal functionality, while some compensatory mechanism, probably mediated by the chitin, led to a positive increase of the VFAs and butyrate in the HM50 diet with potential positive effects on the gut healthiness. However, considering the results of Bovera et al. (2018), to which these results are strongly linked, it is possible to conclude that the 25% soybean protein replacement with the Hermetia illucens larvae meal in the diet of laying hens is more suitable and closer to the optimal level than 50% replacement. Finally, the levels of Cd, Pb, Hg, and As in the diets and insect meal were always lower compared to MLs of heavy metals set by the EU Commission for the feeding stuff and feed materials. This latter aspect provides important information on the safe use of these alternative ingredients in animal feeding.

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Our results can be considered as a small step towards the improving of animals rearing conditions and the sustainability of animal productions.
The first trial, conducted on meagre juveniles, opens a window on the complexity of identifying nutraceuticals compatible with the breeding of a given species that really can improve welfare conditions of animals. Despite the intrinsic qualities of a substance and despite the data in the literature, the real effects that a substance may have should be always preventively scientifically proven. In our case, the results showed that the addition of honey bee pollen on meagre juveniles diets not only did not have the positive effects described in the literature for other fish species, but it even had negative effects on both growth performance and diet digestibility. Moreover, the inflammatory state of the intestine progressively worsened as the level of HBP inclusion increased. These effects could be ascribed to the ultrastructure of the bee pollen grains walls (exine and intine) that makes the bioactive substances unavailable for the fish, while the intestinal inflammation could be due to the bee pollen grains chemical composition rich of lignin and suberin-like substances that can irritate the intestine of monogastric animals such as carnivorous fish. These HBP negative effects could be overcome using an extraction method able to concentrate the bioactive substances and eliminate the indigestible fractions as demonstrated by the results derived from the second trial in which we decided to compare the effects of a raw pollen and of a pollen extract in another carnivorous fish species.

For the extraction of many compounds, also phenolic compounds, it is often chosen as an extraction method the supercritical fluid extraction (SFE) technology. Extraction carried out with CO_2 in supercritical conditions constitutes an alternative compared to traditional separation systems, such as fractional distillation, extraction in vapor stream, extraction with solvents or thermal desorption. CO_2 is in fact, free of toxicity, inert, non-flammable, inexpensive, recyclable and therefore free of impact on the environment. CO_2 SFE extraction is a modern technology of extraction of lipophilic plant components made according to an extremely respectful process and without the release of residual solvent substances. It can replace many traditional extraction processes from plant matrices to obtain dry extracts or essential oils with certain characteristics. The extraction of substances from complex mixtures, in particular, can be made highly selective by adequately modifying the pressure and temperature conditions at which it operates, to adapt them to the solubility of the various components of specific interest.

In our second trial, the indigestible fractions of the pollen were eliminated, and the antioxidant substances were extracted with this technique chemical solvent free, making the antioxidant substances easily available for a monogastric species, such as gilthead sea bream (*Sparus aurata*), one of the two most reared fish species in the Mediterranean area. The results demonstrated that the pollen extract inclusion in diet for *S. aurata* improved the fish humoral immunity.The supplementation with natural compounds has proved to be a useful tool in aquaculture industry and for this reason, green techniques as SFE, able to reduce the utilization of solvent and to produce extract suitable for nutritional purposes, are encouraged.
Although we await the results of further analyses on organoleptic quality and gene expression, we can certainly affirm, in the light of the results of this second trial, that pollen extract has a positive action on carnivorous fish and can be used as nutraceuticals to improve welfare in aquaculture

In support of sustainability, as introduced in the previous chapters, it was decided to test the effect of *Hermetia illucens* meal on laying hens in two different trials. The obtained results are very interesting, and the conclusions are that, in light of the low environmental impact of the insect meals and of the effect obtained on the tested animals, the substitutions with HI meal is an excellent method to improve the poultry rearing sustainability.
In the first trial the productive performance, the nutrient digestibility, the blood analysis and the changes in internal organs of laying hens fed *Hermetia illucens* larvae meal as a partial substitute of the soybean meal protein (25 or 50%) were analysed in order to establish the most suitable inclusion level of this insect meal in their diet.
We obtained that feed intake, egg weight, and feed conversion ratio were not different among the groups. This suggests an adequate palatability of insect meals and a similar dietary efficiency among the groups. The reduction of dry matter and organic matter digestibility observed in the HI50 group was mainly due to the reduction in protein digestibility (11.7% reduction), while in the HI25 group protein digestibility diminished by approximately 5.8%. This was due to chitin content, which, due to its protein binding activity, makes it unavailable for digestion (Longvah et al., 2011). Chitin's positive effects on poultry's health and metabolism are clearly confirmed by the hens' blood analysis. Hens fed a higher amount of insect meal showed higher values of globulin and a lower albumin/globulin ratio compared to the other groups. The amount of chitin was sufficient to reduce bilirubin in the blood of both groups fed insect meal. Furthermore, both diets

including insect meal, reduced triglycerides and cholesterol levels in blood because chitin decreased lipid absorption in the intestine by binding to the lipids and fatty acids directly or binding bile acids and fatty acids and lowered the plasma cholesterol. An increase in the full intestinal length of hens in both insect groups, mainly due to the increase in the jejunum length, was also registered. The effect of the dietary treatment on intestinal length could be mainly due to the low digestibility coefficients observed in the layers, which were fed the insect meal. As it is well known, low feed digestibility in poultry increases their intestinal length (Smits and Annison, 1996) as a consequence of their body's compensatory mechanism increasing their available nutrient absorption surface (Borin et al., 2006). Egg mass was found to be positively influenced by the insect meal diets, as well the lay percentage, only in the HI25 diet. These results are probably due to the higher availability of methionine and lysine in the HI25 diet. Dry matter, organic matter, and crude protein digestibility decreased, as the percentage of insects in the hens' diet increased, due to chitin's negative effect, which was more remarkable in the HI50 diet. The serum globulin level increased only at the highest level of insect meal inclusion. Our results indicate that protein replacement at 25% with insect meal from Hermetia illucens larvae in the diet of laying hens is more suitable and closer to the optimal level than replacement at 50%. More generally, the substitution of soy bean meal with insect meal not only did not have negative effects on production, on the contrary it has several beneficial effects.

This first study was followed by a second one, in which was evaluated the effect of the inclusion of a HI meal in the diet on the volatile fatty acids (VFAs) production in the caeca, intestinal morphometry, and brush border enzymatic activity of 40 weeks old layers. In addition, the research aimed to contribute to the knowledge of the trace and toxic elements' concentration in insect meals. Very interesting were the significant changes in the mutual proportions of the butyric, isobutyric, and valerianic acid observed, which might indicate that not only the activity but also the interactions among the different microbial species have been modified. This is in accordance with the findings by Borrelli et al. (…), who observed changes in the gut microbiota of hens fed HI larvae meal. The inclusion of an insect meal from HI larvae as 25 or 50% substitution of the proteins from the soybean meal influenced the small intestine morphometry and enzymatic activity, as well as the ceacal microbial activity. The SBM group had the highest intestinal functionality, while some compensatory mechanism, probably mediated by the chitin, led to a positive increase of the VFAs and butyrate in the HM50

diet with potential positive effects on the gut healthiness. However, considering the results of the previous study, to which these results are strongly linked, it is possible to conclude that the 25% soybean protein replacement with the Hermetia illucens larvae meal in the diet of laying hens is more suitable and closer to the optimal level than 50% replacement. Finally, the levels of Cd, Pb, Hg, and in the diets and insect meal were always lower compared to MLs of heavy metals set by the EU Commission for the feeding stuff and feed materials. This latter aspect provides important information on the safe use of these alternative ingredients in animal feeding and clarify another aspect under consideration in the dilemma on the use of insect meals.

My job can be considered therefore a small step towards the growing universe represented by the protection of animal welfare and the development of the concept of sustainability in animal production. The results obtained, in fact give us good hopes on the possibility of finding strategies suited to satisfy the future food demands, and at the same time the need to protect the animals and the environment in which we live.