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SUSTAVIANFEED

ALTERNATIVE ANIMAL FEEDS IN MEDITERRANEAN POULTRY BREEDS TO OBTAIN SUSTAINABLE PRODUCTS

NUTRITIONAL EVALUATION OF THE DIET

DELIVERABLE 2.5

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SUMMARY

To develop the project, it is necessary to design diets that respond to the objective of evaluating the effects of the inclusion of more sustainable diets on the performance of egg and/or meat poultry production, animal welfare, as well as on the quality of these products. Thus, this deliverable number 2.5 corresponds to the nutritional evaluation of the diets proposed for the development of the project. To evaluate the nutritive value of the poultry diets using more sustainable feeding programs according to the scope of the project, in each pilot (Spain, Italy, Turkey and Tunisia), it was necessary to determine the nutritional value of the usual ingredients used in each area, as well as to nutritionally characterize the alternative ingredients or by-products available. In addition, each pilot according to its study objective (meat and/or eggs production), selected the genetic type of animal adapted to its environment to use in the project, estimating the nutritional requirements of these animals. Each participant created a database with the nutritional value of the ingredients, which was used for the optimization of the diets in order to meet the requirements of the birds according to the different pilots, optimizing balanced diets. A design of isoenergetic and isonitrogenous diets was contemplated according to the production phase and the pilot, in order to compare feeding programs with usual diets (less sustainable) with alternative programs with the inclusion of more sustainable diets (with alternative ingredients and *Hermetia illucens* insect larvae).

The general methodology used for the development of the Task was implemented, according to the following general protocol:

- Nutritional characterization of usual and alternative ingredients
- Nutritional characterization of *Hermetia illucens* insect larvae
- Characterization of birds, type of poultry production and nutritional requirements
- Design and formulation of diets

For nutritional characterization of usual ingredients of the feed for laying hens or/and meat-type chickens, as well as the possible local ingredients (by-products or other alternatives) that could be used in the manufacture of the experimental feeds; the most relevant bibliography, national and international databases, were used to assign the nutritional value of these ingredients. In addition, internationally recognized procedures of analyses were developed, when the ingredients had lack sufficient information about their nutritional (especially the by-products of some area, and the insects usable in the development of the project). Therefore, each pilot has selected the usual and alternative ingredients that could be used in the formulation of the diets by assigning nutritional values from available scientific and technical information, and/or through chemical analysis. ALIA and UMU used a data base with nutritive value of ingredients indicated by the Spanish Foundation for the Development of Animal Nutrition (FEDNA, 2019) and Heuzé et al. (2013). UNITO and ISA-CM used a database of the nutritive value of ingredients according to INRA (2004). EGE analyzed the chemical component (dry matter, crude ash, ether extract, crude protein, starch, total sugar, Ca, P, amino acid and fatty acid profile) and estimated metabolizable energy of used ingredients, and completed the nutritive information with NRC (1994) and Sari et al. (2008).

For the nutritional characterization of the *Hermetia illucens* larva, the chemical composition of the dried larva produced by the company ENTOMO, under conditions

compatible with the production of the insect during the development of the project, was analyzed. ALIA analyzed the basic chemical composition (moisture, crude protein, crude fiber, ether extract, ash, Ca and P); and UMU developed amino acids, and macro and micro-mineral analyzes. Furthermore, EGE has carried out the characterization of a *Hermetia illucens* larva produced in Turkey, determining its basic chemical composition (dry matter, crude ash, ether extract, crude protein, total sugar, Ca and P), lysine and methionine, and fatty acid profile.

The dried larva produced by ENTOMO contains 9% moisture. This larva has a very high crude protein content (42% as-fed basis, 46.15% DM), and also ether extract (21.6% as-fed basis, 23.07% DM), being the contents of Ca (5.0 as-fed basis, 5.49% DM) and P (1.02% as-fed basis, 1.12% DM) appreciable levels. The most prevalent essential amino acids in the ENTOMO larvae were lysine, valine and leucine with levels > 2.3% as-fed basis (> 2.5% DM), observing that histidine (0.87% as-fed basis, 0.95% DM) was the minority essential amino acid. The protein concentration of Turkish larvae was lower than that found in the sample of ENTOMO larva, but the percentage of ether extract was higher. In addition, more than 70% of the fatty acids found in the Turkish larvae were saturated, being lauric acid, the fatty acid with the highest proportion (>40 g/100g lipid). The composition of the larvae varied greatly depending on the origin, possibly due to differences in the substrates used and the rearing system.

The samples of *Hermetia illucens* from Spain and Turkey were analyzed to determine microbiological status, the main analyses were *Enterobacteriaceae*, Total coliforms, *Escherichia coli, Clostridium perfringens, Listeria monocytogenes* and *Salmonella spp.* In both studies, the counts of microbial contaminants were low, highlighting the absence of *Listeria monocytogenes* and *Salmonella spp*.

To estimate the nutritional requirements of the birds, each pilot characterized the type of poultry production, laying hens or/and meat chickens, the breed or hybrid, and the phase and level of production. Thus, nutritional recommendations for poultry, adapted from scientific and technical references was used to assign the requirements of the animals.

The pilot of Spain (UMU) indicated that they will use laying hens, crossing with breeds adapted to western-Mediterranean, for first phase of lay production, meeting the nutritional requirements of these animals according to FEDNA (2018). The pilot of Italy (UNITO) specified that they will use meat chickens, *Bianca di Saluzzo* male (an Italian autochthonous breed), for Grower (d0 – d60) and Finisher (d61 – d150) periods, meeting the nutritional requirements of these birds according to low input diets for slow-growing chickens (Cerolini et al, 2019). The pilot of Turkey (EGE) specified that they also will use meat chickens, in this case the Anadolu-T (ecotype) and Ross 308 as a control strain, for Starter (d0 - d14), Grower (d15 - d28) and Finisher phases (d29 - slaughter age), meeting the nutritional requirements according to Sarıca et al. (2019; 2021).The pilot of Tunisia (ISA-CM and RAYHANA) specified that they will also use meat chickens and laying hens, in this case the autochthonous Tunisian (ecotype), and Géant and Génoise (local) will be used, meeting nutritional requirements according to TECHNA (Tunisian company expert in feed formulation).

For the design and formulation of the diets, a set of general criteria established in the different meetings held during the development of the project was followed. Optimized diets to meet the requirements of birds of each pilot were established to compare a control diet (with usual ingredients, no sustainability criteria), with other diets more sustainable (according to the criteria of deliverable 2.4 about feed impact). Thus, sustainable diets have lower levels of soybean meal and include alternative ingredients (unusual or by-products), and at least one of them incorporates *Hermetia illucens* insect larvae. In addition, at least the control and one alternative diet will be iso-energetic and iso-nitrogenous (for crude protein and/or amino acid). Software was used to calculate the optimized formulation, using the databases of ingredients (with nutritional values estimated or analysis) and the nutritional requirements according to the phase and type of production, in each pilot. Specific technical software MULTIF21 Grupo SETNA v.0.1.0 was used for Spanish pilot, and also for Tunisian pilot (software Allix3 ©2021); and for Italian and Turkish pilot Excel data base – Microsoft) was used.

Finally, for the Spanish pilot (UMU), three experimental treatments were developed: one control (with inclusion of usual ingredients) (Control), and two more sustainable ones: with alternative ingredients, and 3% or 6% of *Hermetia illucens* dried larvae (3-HERM and 6-HERM, respectively).

For the Italian pilot, three experimental treatments have been developed for each productive phase (Grower and Finisher): a control (with inclusion of usual ingredients) (Control), and two more sustainable: diet with alternative ingredients (ALTER); and other with ALTER diet supplemented with *Hermetia illucens*, so a reduction in intake of the alternative diets is expected between 3 and 6% (approximately a mean of 4.5%, 4.5-HERM).

To design of diets for the Turkish pilot (EGE), three experimental treatments were established for each productive phase: one control (with inclusion of usual ingredients) (Control), and two more sustainable ones: one with alternative ingredients, and the other with and 5% of *Hermetia illucens* dried larvae (ALTER and 5-HERM, respectively).

In the design of diets of Tunisian pilot (ISA-CM and RAYHANA) three experimental treatments were developed for each productive phase: a control (with the inclusion of usual ingredients) (Control), and two more sustainable: one with alternative ingredients without insects (ALTER), and other with alternative ingredients and a 5 % of dry larvae of *Hermetia illucens* (5-HERM). In addition, this general design was used for poultry production of meat or eggs.

In conclusion, the objectives of task 2.5 have been achieved, obtaining nutritionally optimally balanced diets for all pilots, incorporating more sustainable ingredients, and including insects. These diets are considered final preliminary diets, since they must be adapted to the specifications in each pilot of the nutritional characterization of the *Hermetia illucens* larva used, and the availability of the ingredients at the moment of formulation for the manufacture of the feeds in the *in vivo* trials, that will be carried out in the following work packages.

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Acronyms and abbreviations

Abbreviation	Description
AOAC	Association of Official Analytical Chemists
C18:2	Linoleic acid
CF	Crude fiber
CP	Crude protein
CVB	Centraal Veevoeder Bureau [Central Animal Feed Bureau]
DDGS	Distiller's Dried Grains with Solubles
DM	Dry matter
EAA	Essential amino acids
EE	Ether extract
FEDNA	Fundación Española para el Desarrollo de la Nutrición Animal [Spanish Foundation for the Development of Animal Nutrition]
INRA	Institut National de la Recherche Agronomique [National Institute of Agronomic Research]
Lys	Lysine
ME	Metabolizable energy
Met	Methionine
Met+Cys	Methionine+cysteine
NRC	National Research Council
Thr	Threonine
Trp	Tryptophan

1 Introduction

To adjust the nutritional value of diets, it is necessary to balance the nutritional contribution of the ingredients to the requirements of the animals, taking into account the genotype, production system, production objectives and the environment (Castrodeza et al., 2005). In each project area, there are common ingredients used in poultry production, and other unusual ones that could constitute an alternative, to achieve balanced and more sustainable feeding programs (Tallentire et al. 2018). However, some of these ingredients are not sufficiently characterized for their use in poultry diets, and must be analyzed in order to evaluate their potential inclusion in poultry diets (Biesek et al., 2020). On the other hand, new ingredients are making their way, which due to their composition, can be considered of high nutritional value, such as insects. In this sense, there are insect species that have a high content of protein and energy, and can be constituted as a strategic ingredient in the inclusion in poultry feeding programs (De Marco et al., 2015). In addition, some species are currently authorized for use in bird feed, as has been regulated in the European Union (Commission Regulation (EU) 2021/1372). However, it is known that depending on the type of breeding substrate, the composition of the insect can vary (Pinotti and Ottoboni, 2021). In our project, the insect of choice has been *Hermetia illucens*, which will be produced in different locations of project following the guidelines of the ENTOMO company, so the nutritional characterization of the larvae of this insect must be carried out within the scope of the project.

On the other hand, to optimize a feeding program, not only the nutritional value of the ingredients must be known, but also the nutritional requirements of the animals. These requirements will depend on the genotype, the type and production system, as well as the environmental conditions. In the scientific and technical literature, the prediction models of nutritional requirements in poultry production are collected. Poultry require that the diets contain the optimal amount of energy, protein, minerals and vitamins, so any deficiency or imbalance could have negative effects on the performance and welfare of the animals (Blair, 2018). For an optimal production of eggs and meat is necessary that poultry diets are easily digested and well-balanced, since these productions are very sensitive to the quality of the diet.

In the scientific and technical literature, the prediction models of nutritional requirements in poultry production have been collected (NRC 1994; FEDNA, 2018). This bibliography is not always directly applicable in all conditions, either because it does not correspond to the objectives of the productions, or that the needs change depending on the production system, management and genetics. The metabolizable energy (ME) system has been usually recognized and widely used for relating bird's energy requirements and available energy of feed ingredients, and for formulating feeds of poultry (Abdollahi et al., 2021). In addition, ME corrected for zero nitrogen balance are often used. Currently, protein requirements are designated in relations of amino acid requirements (Veldkamp et al., 2016; Block y Dekker, 2017), which are essential for the formation of muscle tissue, eggs, skin, feathers, etc. Birds have to meet the needs of some amino acids through diet because these cannot be synthesized metabolically, they are called "essential amino acids" (EAA). A deficit in the consumption of these amino acids causes a decrease in growth and production (Mehri et al., 2016), as well as an excess that is not stored in the body, excreted in the urine. Therefore, the contribution of EAA is of special importance, as well as its presence according to an optimal profile in relation to the type of production. Nowadays, in the formulation of poultry feed, diets are adjusted to the ideal amino acid profile, as indicated by various sources, such as the NRC (1994), CVB (2018), Ajinomoto (2015), Evonik (2016), Rostagno and Becker (2017) or FEDNA (2018), as well as the standards of genetic companies. In addition, the amino acids that normally limit production in birds are lysine and sulfur amino acids (methionine and cysteine), followed by threonine. Also, a minimum of protein is also required in the formulation in order to reduce the possibility that some non-essential amino acids not contemplated in the formulation may limit productivity (Opoola et al., 2017) and a maximum protein must be taken into account to avoid digestive health problems in animals and reduce environmental pollution.

In addition, poultry require at least 14 essential minerals, of which the most limiting in diets are calcium, phosphorus, sodium, copper, iodine, manganese, selenium and zinc. In this sense, calcium and phosphorus are two essential minerals for the development of the bone skeleton, and the formation of the egg shell, so that their imbalances can cause important alterations in poultry production (Blair, 2018). Moreover, other nutrients such as vitamins or essential fatty acids are considered to meet the requirements of birds, and supply them, following scientific and technical recommendations (FEDNA, 2018).

Finally, once the nutritional value of the ingredients has been characterized, and the requirements of the animals have been estimated, according to the production system, the phase, the level and type of production; the formulation of the diets must be optimized in order to meet the requirements accurately. In the case of this project, a specific feeding program design has been carried out in each of the pilots, in order to compare a control diet (with usual ingredients that are not very sustainable), with others more sustainable (according to the criteria of deliverable 2.4): with lower levels of soybean meal and that include alternative ingredients (unusual or by-products), and that at least one of them incorporates *Hermetia illucens* insect larvae. In addition, at least the control and one alternative diet will be iso-energetic and iso-nitrogenous (for crude protein and/or amino acid).

In such a way that in the following sections we will develop the methodology used, the results obtained, as well as the conclusions of deliverable 2.5 (NUTRITIONAL EVALUATION OF THE DIET).

2 Methodology

2.1 Scope

In order to establish more sustainable feeding programs, according to the scope of the project in each pilot, it has been necessary to determine and evaluate the nutritional value of the usual ingredients used in each area, as well as to nutritionally characterize the available alternative ingredients or by-products. On the other hand, each pilot, in relation to its study objective, has selected the genetic type of animal adapted to its environment that will be used in the scope of the project for the production of meat or eggs, estimating the nutritional requirements of these animals in a specific way. Each participant has created a database with the nutritional value of the ingredients, which has been used for the optimization of the diets in order to meet the requirements of the animals according to the different pilots, using, for the optimization of the balanced diets, formulation programs.

This design of feeding programs responds to the objective of evaluating the effects of the inclusion of more sustainable diets on the performance of egg or meat production, animal welfare, as well as on the quality of these products. Therefore, a design of isoenergetic and isonitrogenous diets has been contemplated according to the production phase and the pilot, in order to compare feeding programs with usual diets (less sustainable) with alternative programs with the inclusion of more sustainable diets. Effects that will be evaluated in the scope of the following work packages.

It should be noted that given the highly volatile current situation created by the international situation, and as the countries of Eastern Europe are an important source of materials for animal feeds, the formulas proposed in this milestone could be adapted to future situations although following the sustainability criteria established in the project.

2.2 Methodology

2.2.1 General methodology

The general methodology used for the development of the task have been implemented, according to the following protocol:

- Nutritional characterization of usual ingredients of the feed for laying hens or/and meat-type chickens, as well as the possible local ingredients (by-products or other alternatives) that could be used in the manufacture of the experimental feeds. For this task, the most relevant bibliography, national and international databases have been used to assign the nutritional value of these ingredients.

- The ingredients that lack sufficient information about their nutritional value have been chemically evaluated (especially the by-products of some area, and the insects usable in the development of the project). The analyses have been followed internationally recognized procedures.

- Specification of type of bird, laying hens or/and meat-type chickens, breed or hybrid, and the phase and level of production, to estimate the nutritional requirements of the animals of each pilot or area. For this task, nutritional recommendations for poultry, adapted from scientific and/or technical references have been used to assign the requirements of the animals.

- Formulation of optimized preliminary diets to meet the requirements of birds of each pilot to compare a control diet (with usual ingredients, no sustainability criteria), with other diets more sustainable (according to the criteria of deliverable 2.4 about feed impact). Thus, sustainable diets have lower levels of soybean meal and including alternative ingredients (unusual or by-products), and at least one of them incorporates *Hermetia illucens* insect larvae. In addition, at least the control and one alternative diet will be iso-energetic and iso-nitrogenous (for crude protein and/or amino acid).

- General evaluation of formulated diets.

2.2.2 Nutritional characterization of ingredients (except *Hermetia illucens* insect larvae).

Each pilot has selected the usual and alternative ingredients that could be used in the formulation of the diets by assigning nutritional values from available scientific and technical information, and/or through chemical analysis, according to **Table 1**.

Table 1. References and analysis used for nutritional characterization of ingredients.

Ingredient	Scientific and technical information, and/or through chemical analysis
Spanish pilot (ALIA and UMU)	Database of nutritive value of ingredients of the Spanish Foundation for the Development of Animal Nutrition (FEDNA, 2019) and Heuzé et al. (2013).
Italian pilot (UNITO)	Database of nutritive value of ingredient according to INRA (2004). *Tables de composition et de valeur nutritive des matières premières destinées aux animaux d'élevage: porc, volailles, bovins, ovins, caprins, lapins, chevaux, poissons*.
Turkish pilot (EGE)	Nutritive value of wheat middling was obtained according National Research Council (NRC, 1994), and for sunflower oil of Sari et al. (2008). Chemical analysis[1]: dry matter, crude ash, ether extract, crude protein, starch, total sugar, ME, Ca, P, amino acid and fatty acid profile.
Tunisian pilot (ISA-CM and RYHANA)	Database of nutritive value of ingredients according to INRA (2004), and updated chemical composition provided by the company TECHNA (Tunisia).

[1] Proximate composition (dry matter, crude ash, ether extract, crude protein, starch, total sugar) of feedstuffs was analyzed following AOAC (1995). The crude fiber was determined by the Weende method (Lepper, 1993). The metabolizable energy (ME) was calculated using the following formula (Anonymous, 1991): ME kcal/kg = 38*((1*Crude Protein) + (2.25*Ether Extract) + (1.1*Starch) + (1.05*Total Sugar)) + 53. Phosphorus was estimated by Naumann and Bassler (1993). Ca was determined by ISO 6490-1:1985. Amino acids were determined by HPLC using HPLC Agilent 1260 Infinity II and the protocol of Agilent (2018). The fatty acid composition was determined on lipids extracted from samples according to Folch et al. (1957). Total fatty acid compositions were determined by gas-liquid chromatography (Agilent Technologies 6890 N Network GC System, Anaheim, CA, USA) equipped with a Thermo Scientific TRACE TR-FAME GC Column; 60 m, 0.25 mm ID, 0.25 m thick (Waltham, MA, USA). The fatty acids were identified by comparing their retention time and fragmentation pattern with an established standard (SUPELCO-37-Comp. Fame mix 10 mg/mL in $CH_{12}Cl_2$).

2.2.3 Nutritional characterization of *Hermetia illucens* insect larvae

For the nutritional characterization of the *Hermetia illucens* larvae, the chemical composition of the dried larva produced by the ENTOMO company, under conditions compatible with the production of the insect during the development of the project, has been analyzed (by ALIA and UMU); according to the methods described in **Table 2**. In addition, EGE has carried out the characterization of a *Hermetia illucens* larva produced in Turkey by Germina Agricultural Products company, on dry larvae sample. In addition, references bibliographic had been used to estimate or complete nutritional characterization of *Hermetia illucens*, such as Barragan-Fonseca et al. (2017), Spranghers et al. (2017) and De Marco et al. (2015).

Table 2. Analysis used for nutritional characterization of *Hermetia illucens* insect larvae.

Origin of larvae	Chemical analysis
From ENTOMO company	ALIA[1]: moisture, crude protein, crude fiber, ether extract, ash, calcium and phosphorous. UMU[2]: amino acids and 31 macro and micro-minerals.
From Germina Agricultural Products company	EGE[3]: dry matter, crude ash, ether extract, crude protein, total sugar, ME, Ca, P, amino acids and fatty acid profile.

[1] Procedures AOAC (1995) for moisture, crude protein, crude fiber, ether extract, and ash were performed. Ash samples were diluted in 0.6 N HNO_3 solutions and filtered to analyze the Ca and P. Ca was determined with an atomic absorption spectrophotometer. P was measured by the vanadate–molybdate method according to the official analytical method described in the Commission Regulation (EC) No 152/2009.
[2]The amino acids were determined by HPLC after derivatization, according to the procedure described by Madrid et al. (2013). Tryptophan was not determined. Macro and micro-minerals were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) after microwave acid digestion procedure.
[3]Similar methods to those used for the analysis of the other ingredients from Turkey were utilized.

Furthermore, ALIA and EGE have carried out a microbiological analysis on the larvae samples. The microorganisms identified and quantified, as well as the method of analysis, are indicated in **Table 3**.

Table 3. Microbiological analysis used for nutritional characterization of *Hermetia illucens* insect larvae.

Origin of larvae	Microbiological analysis
From ENTOMO company	ALIA[1]*:* *Enterobacteriaceae*, Total coliforms, Yeasts and moulds, β-glucuronidase positive *Escherichia coli*, *Clostridium perfringens*, Coagulase-positive *Staphylococci*, *Bacillus cereus*, *Listeria monocytogenes* and *Salmonella spp*.

From Germina Agricultural Products company	EGE[2]: *Enterobacteriaceae*, Total coliforms, *Escherichia coli, Clostridium perfringens*, *Bacillus cereus*, Total Bacteria Count, *Listeria monocytogenes*, and *Salmonella spp*.

[1]ALIA: Mesophilic aerobes (plate count of aerobic microorganisms at 30ºC) (method adapted from ISO 4833-1:2013); *Enterobacteriaceae* (plate count of presumptive *Enterobacteriaceae* at 37ºC based on RAPID'Enterobacteriaceae) (method adapted from ISO 21528-2:2004); Total coliforms (plate count of β-glucuronidase positive *Escherichia coli* and total coliforms, based on RAPID'E.coli 2/Agar) (method adapted from ISO 4832:2006); Yeasts and molds (plate count of yeast and molds at 25 ºC, ISO 21527-1,2:2008); β-glucuronidase positive *Escherichia coli* (Plate count of β-glucuronidase positive *Escherichia coli*, method adapted from ISO 16649-2:2001); *Clostridium perfringens* (method adapted from ISO 7937:2004); Coagulase-positive *Staphylococci* (Plate count of coagulase-positive Staphylococci, ISO 6888-2:1999/Amd1:2003); *Bacillus cereus* method adapted from ISO 7932:2004; *Listeria monocytogenes* (Detection of *Listeria monocytogenes* based on RAPID'L. mono) (reference method ISO 11290-1:2017); *Salmonella spp.* (Detection of *Salmonella spp*. based on IRIS SALMONELLA (reference method ISO 6579:2002).

[2] EGE: Similar procedures were performed for the same type of microorganisms analyzed.

2.2.4 Birds, type of poultry production and nutritional requirements

To estimate the nutritional requirements of the birds, each pilot has characterized the type of poultry production, laying hens or/and meat chickens, the breed or hybrid, and the phase and level of production. Thus, nutritional recommendations for poultry, adapted from scientific and technical references have been used to assign the requirements of the animals. The following **Table 4** shows the characterization of the animals, the type of production, genotype and performance, the phase evaluated, and the references used to estimate the nutritional requirements of the animals in each pilot.

Table 4. Characterization of the birds, production and references of nutritional requirements of each pilot.

Pilot	Type of production	Avian breed/hybrid Performances	Phase evaluated for the pilot	Reference/Nutritional requirements
Spain (UMU)	Laying hens	• Crossing with breeds adapted to western-Mediterranean • Body weight (1.5 kg at 18 weeks, and 2.3 kg at 78 weeks) • 290 eggs/year • Egg weight: 61.5 - 62 g	• Lay phase • First phase of lay up to 40 wk approximately	• Diet with ≥16.5% protein and 2750 kcal/kg ME. Spanish Foundation for the Development of Animal Nutrition (FEDNA, 2018).
Italy (UNITO)	Meat chickens	• *Bianca di Saluzzo* male (Italian autochthonous breed) • Body weight (2.8 kg at 25 weeks) • Average daily intake (adult): 140 g/day	• Grower (d0 – d60) • Finisher (d61 – d150) • Slaughter age: 150d	• ≥20.3% protein, ≥2761 kcal/kg ME (from 0 to 60d) • ≥19.5% protein, ≥2831 kcal/kg ME (from 61 to 150 d) Low input diets for slow-growing chickens (Cerolini et al., 2019).

Turkey (EGE)	Meat chickens	• Anadolu-T (ecotype) • Average 1687g in 35 d and 2300 g at 42 d • Feed consumption 4258-4566 g/bird at 42 d	• Starter (d0 - d14) • Grower (d15 - d28) • Finisher (d29 - slaughter age)	• ≥21% protein, ≥3000 kcal/kg ME (from 1 to 15d) • ≥20% protein, ≥3100 kcal/kg ME (from 16 to 28d) • ≥18% protein, ≥3100 kcal/kg ME (from 36d until the end) Sarıca et al. (2019; 2021).
Tunisia	Meat chickens Laying hens	• (ISA-CM)[1] Autochthonous Tunisian (ecotypes) • (RAYHANA)[1] Géant and Génoise (local)	• Starter (d1-d28) • Grower (d29-d66) • Finisher (d67 - slaughter age) • Laying phase	• ≥21.5% protein, ≥2853 kcal/kg ME (from 1 to 289d) • ≥18.2% protein, ≥2933 kcal/kg ME (from 29 to 66d) • ≥16.7% protein, ≥2978 kcal/kg ME (from 67d until the end). • For laying hens, diet with ≥16.68% protein and ≥2636 kcal/kg ME Tunisian TECHNA company[2].

[1] According to the information provided by Bessadok et al. (2003), Raach-Moujahed et al. (2011), Hnia and Hadj Ayed (2016), and unpublished own of ISA-CM results. Tunisian chicken populations are characterized by a light weight not exceeding 1620 g in males and 1206 g in females at 18 weeks of age, low egg production (less than 130 eggs per hen per production cycle) and low laying rate (less than 45%). Unless in improved (crossed with Rhode Island) local hens fed a commercial feed where the laying rate can be up to 76%.
[2]Tunisian company expert in feed formulation.

2.2.5 Design and formulation of diets

Each pilot has designed a feeding program according to the type of genotype, poultry production, expected performance and evaluated period. For the design and formulation of the preliminary diets, a set of general criteria established in the different meetings held during the development of the project have been followed. Such criteria were:

- Formulation of optimized diets to meet the requirements of birds according to type of poultry production and phase of each pilot.
- Implement a diet design, by phase and type of production, to compare a usual control diet (with non-sustainable ingredients), and other diets that include more sustainable ingredients (according to the criteria of deliverable 2.4 about feed impact).
- Sustainable diets should contain less imported soybean meal (or other imported ingredients), and incorporate alternative ingredients (unusual or by-products).
- At least one of sustainable diets must include larvae of the insect *Hermetia illucens*.
- At least the control and one alternative diet will be iso-energetic and iso-nitrogenous (for crude protein and/or amino acids), by phase and type of production, in each pilot.

For the formulation of the preliminary diets of the feeding programs, software has been used to calculate the optimized formulation, using the databases of ingredients (with nutritional values estimated or analysis) and the nutritional requirements according to the phase and type of production, in each pilot. In addition, incorporating the set of criteria established to obtain more sustainable diets.

For the formulation of the diets for the Spanish pilot, a specific technical software MULTIF21 Grupo SETNA v.0.1.0 was used, for Italian and Turkish pilot Excel data base – Microsoft) was used, and for Tunisian pilot the formulate program was Allix3 ©2021. In addition, a global assessment of the formulated diets will be carried out.

3 Nutritional characterization of ingredients (except *Hermetia illucens* insect larvae)

In this section, the results of the nutritional characterization of the ingredients (usual or alternative) used in the formulation of the diets in each pilot are presented, except for the *Hermetia illucens* insect, which will be presented in a separate section.

3.1 Spain

For this pilot, a database based on the nutritional characterization of ingredients of the Spanish Foundation for the Development of Animal Nutrition (FEDNA, 2019) and Heuzé et al. (2013), was developed. The following **Table 5** shows the main nutritional values of the majority ingredients (usual and alternative) selected for the formulation of the diets in this pilot.

The nutritional value of the ingredients proposed in the Spanish pilot covers a range of products, from very energetic, such as soybean oil and animal fat (>8000 kcal/kg ME), and also cereals [maize and wheat (>3000 kcal/kg ME) and barley (>2700 kcal/kg ME)]; to vegetable protein concentrates with excellent nutritional value such as soybean meal (>44% crude protein) with a high lysine content (2.78%), as well as other protein concentrates with less nutritional value [sunflower meal (28% of crude protein and 1% lysine) and rapeseed meal (33% of crude protein and 1.85% lysine)]. In addition, the Spanish pilot also indicates that wheat middling by-products are usually available, but they have moderate energy and protein (about 2025 kcal/kg ME and 14.3% of crude protein). However, in intensive poultry production, the ingredients that are traditionally considered to be of better quality are usually used (mostly cereals with higher nutritional value, and soybean meal).

Table 5. Database of main nutritional values[1] of the ingredients selected for Spanish pilot (as-fed basis)[2].

Usual Ingredients	ME (kcal/kg)	CP (%)	EE (%)	CF (%)	C18:2 (%)	Ca (%)	P (%)	Na (%)	Lys (%)	Met (%)	Met+ Cys (%)	Thr (%)	Trp(%)
Maize	3250	7.3	3.3	2.1	1.66	0.03	0.25	0.01	0.22	0.15	0.31	0.26	0.06
Wheat	3100	11.2	1.4	2.4	0.56	0.05	0.29	0.02	0.31	0.18	0.43	0.32	0.13
Barley	2785	11.3	1.7	4.7	0.67	0.06	0.32	0.02	0.41	0.19	0.43	0.37	0.14
Wheat middlings	2025	14.3	3.5	9.1	1.44	0.12	0.82	0.03	0.57	0.22	0.51	0.45	0.20
Soybean meal	2280	45.5	1.7	5.3	0.64	0.33	0.66	0.03	2.78	0.64	1.33	1.77	0.60
Sunflower meal	1420	28	1.3	26	0.59	0.4	0.9	0.03	1.00	0.63	1.12	1.01	0.36
Rapeseed meal	1680	33	2.6	12.6	0.43	0.7	1.1	0.05	1.85	0.66	1.45	1.46	0.44
Animal fat	8400	0	100	0	9.8	0.78	1.27	-	-	-	-	-	-
Soybean oil	8750	0	100	0	53.46	-	-	-	-	-	-	-	-

Soybean hulls	580	12	2.4	34.3	1.17	0.5	0.15	0.03	0.75	0.14	0.36	0.43	0.14
Alternative ingredients	**ME (kcal/kg)**	**CP (%)**	**EE (%)**	**CF (%)**	**C18:2 (%)**	**Ca (%)**	**P (%)**	**Na (%)**	**Lys (%)**	**Met (%)**	**Met+ Cys (%)**	**Thr (%)**	**Trp (%)**
Bakery by-product	3100	10.7	7.6	3.6	1.49	0.16	0.3	0.42	0.29	0.17	0.39	0.33	0.12
Sunflower cake	2160	31	9.1	20.7	4.14	0.49	1	0.03	1.11	0.70	1.24	1.12	0.40
Carob Pulp	960	4.5	0.7	7.5	-	0.45	0.11	0.03	0.16	0.10	0.16	0.15	0.08
Citrus Pulp	1100	6.1	1.6	11.4	0.30	1.8	0.12	0.08	0.17	0.06	0.14	0.17	0.05
Barley Rootlets	1576[3]	19.1	1.5	12.9	0.50	0.22	0.6	0.04	0.82	0.27	0.55	0.62	0.22
Rapeseed cake	2310	29.7	12.7	11.3	2.24	0.7	1.15	0.06	1.69	0.60	1.31	1.33	0.40
Soybean concentrate	2820	65	1	3.9	0.35	0.28	0.77	0.02	4.12	0.95	1.92	2.60	0.85
Peas	2670	21.5	1	6	0.39	0.08	0.4	0.02	1.54	0.22	0.52	0.82	0.20
Maize DDGS	2330	28	12.5	7.2	5.36	0.03	0.82	0.12	0.83	0.54	1.09	1.04	0.22

[1]ME= Metabolizable energy, CP= Crude protein, EE= Ether extract, CF= Crude fiber, C18:2=Linoleic acid, Lys= Lysine, Met= Methionine, Met+Cys= Methionine+Cysteine, Thr= Threonine, Trp= Tryptophan.
[2]According to FEDNA (2019).
[3]According to Heuzé et al. (2013).

As usable alternative ingredients, energy-rich products such as bakery by-products (>3000 kcal/kg ME) have been evaluated, as well as possible protein alternatives (but with different levels of protein and amino acids), such as sunflower cake (31% of crude protein and 1.11% lysine), rapeseed cake (29.7% of crude protein and 1.69% lysine), peas (21.5% of crude protein and 1.54% lysine), and by-product of cereals such as DDGS (28% of crude protein and 0.83% lysine) or barley rootlets (19.1% of crude protein and 0.82% lysine). Likewise, other products have been evaluated, but due to their poor energy or protein content they have been ruled out (carob pulp and citrus pulp). In addition to nutritional value, it must be considered for formulation that several of these products have maximum levels of incorporation in poultry diets, due to possible negative effects on performances or derived products.

3.2 Italy

For Italian pilot, a database based on the nutritional characterization of ingredients of the Nutritive Value Tables of INRA (2004), was used. The following **Table 6** shows the main nutritional values of the majority ingredients (usual and alternative) selected for the formulation of the diets in this pilot.

Table 6. Database of main nutritional values of the ingredients selected for Italian pilot (as-fed basis)[1, 2].

Usual Ingredients	ME (kcal/kg)	CP (%)	EE (%)	CF (%)	Ca (%)	P (%)	Lys (%)	Met (%)	Thr (%)

Maize	3130	8.10	3.7	2.2	0.04	0.26	0.21	0.16	0.27
Soybean meal	2230	45.3	1.9	6.0	0,34	0.62	2.53	0.58	1.58
Sunflower meal	1500	33.4	1.7	21.2	0.41	1.08	0.98	0.71	1.05
Soybean oil	9000	-	-	-	-	-	-	-	-
Alternative ingredients	**ME (kcal/kg)**	**CP (%)**	**EE (%)**	**FB (%)**	**Ca (%)**	**P (%)**	**Lys (%)**	**Met (%)**	**Thr (%)**
Maize Gluten meal	3500	60.6	2.5	1.1	0.07	0.49	0.96	1.43	1.89
Peas	2500	20.7	1.0	5.2	0.11	0.40	0.20	0.20	0.64
Fava beans	2480	25.4	1.3	7.9	0.14	0.46	1.60	0.20	0.60

[1]ME= Metabolizable energy, CP= Crude protein, EE= Ether extract, CF= Crude fiber, Lys= Lysine, Met= Methionine, Thr= Threonine.
[2] According to INRA (2004).

The nutritional value of the usual ingredients of the Italian pilot ranges from energetic sources (soybean oil and maize) to protein concentrates of high quality, such as soybean meal (45.3% of crude protein and 2.53% lysine); as well as other protein concentrates as sunflower meal, in this case of high crude protein (33.4%) and methionine (0.71%). As alternative ingredients, three protein concentrates are proposed: a by-product of maize with high crude protein (60.6%), lysine (0.96%) and methionine (1.43%); and two legume seeds with moderate-high protein (20.7 and 25.4% of crude protein, respectively).

3.3 Turkey

For the pilot of Turkey, the nutrient characterization database was largely based on chemical analysis of usual and alternative ingredients. **Table 7** shows the results of the analysis of basic chemical composition, and lysine and methionine, of these ingredients. The nutritional value of the usual ingredients indicated in the Turkish pilot covers a range of products, from very energetic, such as sunflower oil (>8000 kcal/kg ME), and also cereals (maize and wheat, >2900 kcal/kg ME); to vegetable protein concentrates with excellent nutritional value such as soybean meal (>43% of crude protein and 1.43% lysine) and fish meal (60.15% of crude protein and 3.69% lysine), as well as other protein concentrates as sunflower meal with high protein (38.72% of crude protein and 1.21% lysine).

Table 7. Database of main nutritional values of the ingredients selected for Turkish pilot (as-fed basis)[1,2].

Usual Ingredients	ME kcal/kg	DM %	Ash %	CP %	EE %	Starch %	Sugar %	Ca %	P %	Met (%)	Lys (%)
Maize	3218	88.84	0.93	7.62	3.17	59.08	3.40	1.20	0.12	0.27	0.16
Wheat	2963	90.07	1.72	12.61	1.55	50.90	4.29	0.44	0.38	0.56	0.36
Soybean meal	2113	88.96	6.49	43.82	0.59	-	8.63	0.19	0.64	1.18	1.43

Sunflower meal (High protein)	1964	88.90	5.93	38.72	1.74	-	7.30	0.39	0.86	0.24	1.21
Fish meal	3187	92.29	11.14	60.15	9.93	-	-	1.61	2.20	1.95	3.69
Sunflower oil	>8000										
Alternative ingredients	**ME kcal/kg**	**DM %**	**Ash %**	**CP %**	**EE %**	**Starch %**	**Sugar %**	**Ca %**	**P %**	**Met (%)**	**Lys (%)**
Cottonseed meal	1961	89.36	8.15	34.21	4.22	-	6.21	1.15	1.01	1.08	0.99
Camelina meal	2407	93.10	4.88	40.50	5.38	-	8.92	0.36	0.70	0.00	1.03
Tomato pulp	825	93.62	6.95	15.11	1.30	-	2.17	1.70	0.37	0.61	0.70
Grape pomace	1235	92.70	14.35	21.51	3.01	-	2.69	1.16	0.58	0.18	0.40
Whey powder (de-mineralized)	1199	94.86	5.30	8.40	0.37	-	19.03	0.65	0.57	0.10	0.34
Brewer´s dried grain	1327	96.98	4.03	23.14	2.43	2.72	1.87	2.31	0.71	0.22	0.86
Wheat middlings	2000	88.0		15.0	3.0			0.12	0.85	0.69	0.21

[1]ME= Metabolizable energy, DM=dry matter, CP Crude protein, EE=Ether extract, Met=Methionine, Lys=Lysine.
[2]Data obtained from chemical analyzes and bibliographic sources (NRC, 1994; Sari et al., 2008).

Among the alternative ingredients, there are ingredients with a high protein content, such as cottonseed meal or camelina meal (34.21 and 40.50% crude protein, respectively). However, these ingredients have important limitations for their use in monogastric, because they contain anti-nutritive factors that can negatively affect performance (Świątkiewicz et al., 2016; Russo and Reggiani, 2017). Other alternative ingredients contain a low energy level (<1300 kcal/kg ME), such as tomato pulp, grape pomace and whey powder (demineralized). On the other hand, brewers' dried grain and wheat middling could reach energy levels >1300 kcal/kg ME, and moderate levels of crude protein (23.14 and 15.0%, respectively).

The characterization of the fatty acids profile of the ingredients should be considered to optimize the adequate contribution to ensure the performance of the animals. In addition to optimizing the effect that they can cause on the quality of the final products, both for meat and eggs. **Table 8** presents the results of the analysis of the fatty acid profile of the usual and alternative ingredients. In all plant-based materials tested (except grape pomace), the main fatty acid found was linoleic acid (>24.9 g/100 g lipid), an omega-6 (*n*-6) essential fatty acid for poultry (Cherian, 2015). It should be noted that camelina meal presented a profile of fatty acids, in which another essential fatty acid stood out, α-linolenic acid (13.45 g/100 g of lipids), an omega-3 (*n*-3) essential fatty acid. Camelina is considered a seed rich in this *n*-3 fatty acid (Kurasiak-Popowska and Stuper-Szablewska, 2020); and although in our case the alternative material is the meal resulting from the extraction of the oil, which has an ether extract content of 5.38%, its contribution should be considered. Regarding materials of animal origin, in whey powder palmitic saturated fatty acid predominates (26.84 g/100 g of lipids), although in fish meal we found long-chain and highly unsaturated *n*-3 fatty acids, such as eicosapentaenoic acid (EPA, 20:5 *n*-3), and fundamentally docosahexaenoic acid (DHA, 22:6 *n*-3).

Table 8. Fatty acid profile of analyzed feedstuffs of pilot of Turkey (g/ 100 g lipid).

Fatty acid	Usual Ingredients					Alternative ingredients					
	Maize	**Wheat**	**Soybean Meal**	**Sunflower meal**	**Fish meal**	**Cottonseed meal**	**Camelin a Meal**	**Tomate Pulp**	**Grape Pomace**	**Beer Pulp**	**Whey powde r**
Butyric (C4:0)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Caproic (C6:0)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Caprylic (C8:0)	0.000	0.000	0.144	0.000	0.027	0.000	0.000	0.019	10.972	0.000	0.000
Capric (C10:0)	0.000	0.091	0.000	0.497	0.046	0.157	0.094	0.034	0.000	0.365	3.499
Undecanoic (C11:0)	0.000	0.000	0.000	0.000	0.004	0.086	0.011	0.000	0.000	0.000	0.000
Lauric (C12:0)	0.459	2.744	1.260	10.590	0.073	0.370	0.067	0.946	12.199	0.589	7.078
Tridecanoic (C13:0)	0.000	0.000	0.000	0.000	0.176	0.000	0.000	0.000	0.000	0.305	0.000
Myristic (C14:0)	0.224	0.665	0.457	3.588	5.191	0.729	0.116	0.342	3.116	0.664	12.746
Myristoleic (C14:1)	0.037	0.180	0.000	0.000	0.905	0.077	0.039	0.053	0.000	0.367	5.457
Pentadecanoic (C15:0)	0.000	0.065	0.192	0.000	0.187	0.000	0.015	0.078	0.000	0.252	0.000
cis-10-pentadecanoic (C15:1)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Palmitic (C16:0)	**13.064**	**16.932**	**14.496**	**10.176**	**22.27**	**22.508**	**7.036**	**12.983**	**14.85**	**22.947**	**26.843**
Palmitoleic (C16:1)	0.161	0.301	0.128	2.234	4.028	0.4743	0.188	0.340	4.319	0.284	4.567
Heptadecanoic (C17:0)	0.151	0.176	0.521	0.754	1.762	0.137	0.058	0.116	0.000	0.087	0.000
cis-10heptadecanoic (C17:1)	0.111	0.120	0.194	0.000	0.589	0.153	0.121	0.638	2.435	0.000	0.000
Stearic (C18:0)	2.255	1.244	4.252	4.022	5.537	2.589	2.802	5.722	2.608	1.865	8.724
Elaidic (C18:1 trans)	0.080	0.000	0.281	0.720	0.452	0.091	0.069	0.074	0.000	0.000	4.983
Oleic (C18:1 cis)	32.54	17.019	19.956	23.277	22.84	16.713	20.163	23.140	17.667	12.315	19.964
Linolelaidic (C18:2 trans)	0.1196 0	0.132	0.208	0.981	0.456	0.0822	0.126	0.105	0.000	0.000	0.000
Linoleic (C18:2 cis)	**47.720**	**53.307**	**49.800**	**37.469**	**6.142**	**53.903**	**24.971**	**49.80**	**15.200**	**51.271**	**6.137**
Arachidic (C20:0)	0.529	0.204	0.559	0.5719	0.802	0.2951	1.939	0.504	0.000	0.303	0.000
γ-Linolenic (C18:3n6)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.642	0.000	0.000
cis-11-eicosenoic (C20:1)	1.193	4.325	0.387	1.523	1.051	0.467	21.812	2.419	2.672	5.253	0.000
Linolenic (C18:3n3)	**0.374**	**1.066**	**6.245**	**1.456**	**0.743**	**0.113**	**13.450**	**0.399**	**0.000**	**0.920**	**0.000**
Heneicosanoic (C21:0)	0.000	0.098	0.068	0.000	1.237	0.054	0.110	0.176	0.000	0.000	0.000
cis-11.14-eicosadienoic (C20:2)	0.139	0.145	0.052	0.000	0.460	0.056	1.621	0.339	0.000	0.440	0.000
Behenic (C22:0)	0.226	0.203	0.580	0.594	0.458	0.237	0.430	0.164	0.000	0.305	0.000
cis-8.11.14-eicosatrienoic (C20:3n3)	0.000	0.000	0.000	0.000	1.359	0.000	0.337	0.000	0.000	0.000	0.000
Erucic (C22:1)	0.000	0.214	0.000	0.000	0.048	0.046	1.681	0.000	0.000	0.145	0.000
cis-11.14.17-eicosatrienoic (C20:3n6)	0.000	0.073	0.000	0.000	0.073	0.264	1.340	0.092	0.000	0.045	0.000
Arachidonic (C20:4)	0.184	0.088	0.203	0.000	0.640	0.084	0.093	0.060	12.319	0.232	0.000
Tricosanoic (C23:0)	0.000	0.000	0.000	0.000	0.000	0.038	0.234	0.087	0.000	0.200	0.000
cis-13.16-docosadienoic (C22:2)	0.000	0.000	0.000	0.000	6.625	0.000	0.129	0.000	0.000	0.070	0.000
Lignoceric (C24:0)	0.000	0.131	0.000	0.000	0.058	0.101	0.119	0.752	0.000	0.219	0.000

cis-5.8.11.14.17-eicosapentaenoic (C20:5)	**0.288**	**0.174**	**0.000**	**0.000**	**0.526**	**0.081**	**0.219**	**0.216**	**0.000**	**0.186**	**0.000**
Nervonic (C24:1)	0.042	0.000	0.000	1.006	0.579	0.000	0.283	0.161	0.000	0.072	0.000
cis-4.7.10.13.16.19-docosahexaenoic (C22:6)	**0.089**	**0.293**	**0.000**	**0.53954**	**14.22**	**0.193**	**0.124**	**0.205**	**0.000**	**0.299**	**0.000**

3.4 Tunisia

For Tunisian pilot (ISA-CM and RAYHANA), a database based on the nutritional characterization of ingredients according to INRA (2004), and updated chemical composition provided by the company TECHNA (Tunisia) was used. The following **Table 9** shows the main nutritional values of the majority ingredients selected for the formulation of the diets in these pilots. The nutritional value of the ingredients indicated in the Tunisian pilot covers a range of materials, from very energetic, such as soybean oil (>9000 kcal/kg ME), and also cereals as maize, barley, wheat and triticale (>2800 kcal/kg ME); to vegetable protein concentrates with excellent nutritional value such as soybean meal or expeller (>44% crude protein); and even extruded soybean with high energy and crude protein (3440 kcal/kg ME, and 34.5% crude protein, respectively). Furthermore, other protein concentrates with less nutritional value as rapeseed meal (30.5% of crude protein), and also Leguminosae seed as fava beans with high-moderate crude protein (23%) and metabolizable energy (>2520 kcal/kg ME) are showed. On the other hand, great variability of wheat by-products is showed, from middlings to bran, which have different energy content (from 3200 to 1800 kcal/kg ME) depending on their starch content. Also, pasta waste by-product is described, it could be considered a high energy ingredient (3125 kcal/kg ME).

Table 9. Database of main nutritional values of the ingredients selected for Tunisian pilot (as-fed basis)[1, 2].

Ingredients	ME*	ME**	CP (%)	Lys (%)	Met (%)	Ca (%)	P (%)	EE (%)	Starch (%)	CF (%)	Lys dig.%	Met dig.%	Thr dig.%
Maize	3300		7.5	0.23	0.16	0.02	0.25	3.7	63.5	2.0	0.19	0.15	0.24
Barley	2850		10.0	0.38	0.14	0.05	0.32	1.7	52	4.5	0.3	0.2	0.29
Wheat	3300		12.5	0.48	0.22	0.05	0.45	2.3	62.5	1.0	0.4	0.2	0.29
Wheat by-product 1	3200		11.0	0.36	0.48	0.05	0.35	2.7	55	1.5	0.4	0.23	0.38
Wheat by-product 2	2976		13.5	0.36	0.21	0.05	0.3	2	56.1	3.19	0.3	0.19	0.33
Wheat bran	1800		14.8	0.58	0.21	0.12	0.92	4	21	8.0	0.43	0.17	0.37
Triticale	3000	3007	10.5	0.34	0.18	0.05	0.32	1.6	58	2.7	0.29	0.16	0.27
Soybean meal	2410		46.7	2.92	0.67	0.3	0.6	1.8	2.7	4.5	2.6	0.61	1.6
Soybean expeller	2917		44.0	2.73	0.63	0.3	0.6	8.3	3	5.3	2.37	0.56	1.44
Extruded soybean	3440		34.5	2.17	0.5	0.3	0.55	18.5	2.9	4.8	1.91	0.43	1.14

Soybean oil	9000							99.7					
Rapeseed meal	2538		30.5	1.68	0.61	0.76	0.85	15.5	2	11	1.34	0.56	1.1
Fava beans	2520	2525	23.0	1.51	0.18	0.13	0.55	1.4	40	7.7	1.36	0.15	0.71
Pasta waste	3125		12.0	0.33	0.19	0.03	0.17	0.5	64	1.0	0.26	0.16	0.27

[1]ME*= metabolizable energy for broiler chicks (kcal/kg), ME**=metabolizable energy for laying hens (kcal/kg), CP= crude protein, Lys= lysine, Met= methionine, EE= ether extract, CF= crude fiber, Lys dig.= lysine digestible, Met dig.= methionine digestible, Thr dig.= threonine digestible.

[2] Nutritive values of ingredients according to INRA (2004), and updated chemical composition provided by the company TECHNA (Tunisia).

4 Nutritional characterization of *Hermetia illucens* insect larvae

4.1 Spain

The nutritional characterization of the dried larvae of *Hermetia illucens* produced by ENTOMO was carried out by ALIA and UMU. **Table 10** shows the basic chemical composition, amino acids, and macro and micro-minerals of this larva biomass. As a result of the analysis of the basic chemical composition, the dried larva produced by ENTOMO contains 9% moisture. This larva has a very high crude protein content (42% as-fed basis, 46.15% DM), and also ether extract (21.6% as-fed basis, 23.07% DM), being the contents of Ca (5.0% as-fed basis, 5.49% DM) and P (1.02% as-fed basis, 1.12% DM) appreciable levels.

In the literature, the composition of *Hermetia illucens* larvae, despite having high protein and fat content, could be variable depending on the substrate. Spranghers et al. (2017) indicated a range of 39.9-43.1% crude protein in dry matter, using four different substrates to rear *Hermetia illucens* prepupae. However, these protein differences appear to be in a narrow range in *Hermetia illucens* prepupae composition (Oonincx et al., 2015). Although, the fat content can vary greatly, thus in a review of the literature by Barragan-Fonseca et al. (2017) on the nutritional quality of the larva, different fat levels were found in more than 20 percentage units, attributing these differences to the different substrates used. Spranghers et al. (2017) showed that a high correlation was also found between the EE content of *Hermetia illucens* prepupae and non-fibrous carbohydrates, although they did not find correlations between insect fat or protein and the composition of the substrates.

The most prevalent essential amino acids in the larvae were lysine, valine and leucine with levels > 2.3% as-fed basis (>2.5% DM), observing that histidine (0.87% as-fed basis, 0.95% DM) was the minority essential amino acid. Likewise, Romano et al (2021) found that *Hermetia illucens* larvae reared with sweet potato, past coffee or dough for two weeks, contained lysine, valine and leucine as major essential amino acids, but in this case tryptophan and methionine were the minor essential amino acids.

Regarding the proportion of lysine (5.90% of CP), threonine (3.40% of CP) and methionine (2.69% of CP) in protein content of ENTOMO larvae were close to profile described by Barragan-Fonseca et al. (2017) of 6.6, 3.7 and 2.1% of CP for lysine, threonine and methionine of larvae reared with manure. Thus, Makkar et al. (2014) indicated that larvae of *Hermetia illucens* had a high proportion of lysine, being able to reach a value similar to the proportion of this amino acid in soybean meal protein, which can be around approximately 6% of crude protein content as indicated by FEDNA (2019). In other works, the decreasing profile of the proportion of lysine, threonine and methionine in the protein of the larvae is maintained, although the percentage magnitude differs compared to our results, fundamentally with lower values in methionine, as found by Crosbie et al. (2020) with 1.76% of this amino acid in the protein content of *Hermetia illucens* larvae meal.

Table 10. Basic chemical composition, amino acids and macro and micro-minerals of analyzed dried larvae produced by ENTOMO (as-fed basis).

Basic composition[1]	%	Macro and micro-minerals[2]	Amount	Macro and micro minerals[2]	Amount
Moisture	9.0	Al (mg/kg)	377.96	S (g/100g)	0.50
Crude Protein	42.0	As (mg/kg)	0.35	Sr (mg/kg)	168.70
Crude Fiber	9.9	Be (mg/kg)	0.03	Ti (mg/kg)	8.70
Ether extract	21.6	Bi (mg/kg)	<0.01	Tl (mg/kg)	232.35
Ash	14.0	B (mg/kg)	5.13	V (mg/kg)	0.86
P	1.02	Ca (g/100g)	3.93	Zn (mg/kg)	250.38
Ca	5.00	Cd (mg/kg)	0.51		
Amino acids[2]	**%**	Co (mg/kg)	0.45		
Lysine(%)	2.48	Cr (mg/kg)	4.14		
Methionine(%)	1.13	Cu (mg/kg)	20.97		
Threonine(%)	1.43	Fe (mg/kg)	881.54		
Isoleucine%)	1.62	K (g/100g)	1.53		
Valine(%)	2.31	La (mg/kg)	<0.01		
Histidine (%)	0.87	Li (mg/kg)	1.01		
Phenylalanine (%)	1.46	Mg (g/100g)	0.412		
Leucine (%)	2.50	Mn (mg/kg)	300.20		
Arginine (%)	1.60	Mo (mg/kg)	2.38		
Cysteine(%)	0.38	Na (g/100g)	0.121		
Serine (%)	1.68	Ni (mg/kg)	1.27		
Tyrosine(%)	2.22	Pb (mg/kg)	0.79		
Glycine(%)	1.95	P (g/100g)	1.21		
Aspartic acid (%)	3.51	Rb (mg/kg)	6.37		
Glutamic acid (%)	4.04	Sb (mg/kg)	<0.01		
Alanine (%)	2.75	Se (mg/kg)	<0.01		
Proline (%)	2.02	Si (mg/kg)	357.88		

[1]According analyses of ALIA.
[2]According analyses of UMU.

In addition, a wide range of macro and micro-minerals have been analyzed. Regarding Ca and P, two analytical techniques have been used for ENTOMO larval biomass, obtaining close values. It should be noted that the levels using both techniques were >3.9% for Ca, and >1% for P (as-fed basis).

Barragan-Fonseca et al. (2017) indicated that Ca, P, Fe, Mn, Zn and Cu are found in high concentrations, compared to the content of these minerals in other insects. ENTOMO larvae obtained concentrations comparable to those described by these authors. It should be noted that the high content of Ca in *Hermetia illucens* larvae could also be due by the secretion of calcium carbonate through their epidermis, indicated by Newton et al. (1977). However, after moulting, adults contain low levels of Ca (Finke, 2013), since it remains in the pupal casing. Also, mineral levels could be conditioned by their quantity and availability in the substrate, thus Newton et al. (2005) described differences in the mineral content of larvae using different types of manure, poultry or pigs.

From the trace mineral analysis, it should be noted that the levels of heavy metals found (as-fed basis): As (0.35 mg/kg), Cd (0.51 mg/kg) and Pb (0.79 mg/kg), are within the levels allowed by European feed legislation. Thus, Regulation (EU) No. 1275/2013 of the Commission limited maximum limits (values expressed relative to a feed with a moisture content of 12%) in feed materials, for As 2 mg/kg; for Cd 1 or 2 for materials of plant or animal origin, respectively; and for Pb of 10 mg/kg in general for feed materials.

In addition, microbiological analyzes were performed to determine the level of microbial contamination (**Table 11**). From the microbiological analyzes performed on the ENTOMO larval samples, the counts of *Enterobacteriaceae* (5.4 x 10^3cfu/g), Total coliforms (5.6 x 10^1cfu/g), *Escherichia coli* (3.0 x 10^1cfu/g) and *Clostridium perfringens* (1.0 x 10^1cfu/g) are low. In addition, *Listeria monocytogenes* and *Salmonella spp* were not detected. In general, the colony count was close to low or acceptable microbiological levels, in relation to the limits established by various specialized laboratories in raw materials and feeds (Manrique, 2020).

Table 11. Microbiological analyzes of dried larvae produced by ENTOMO.

Microorganism	cfu/g
Mesophilic aerobes	2.8 x 10^6
Enterobacteriaceae	5.4 x 10^3
Total coliforms	5.6 x 10^1
Yeasts and moulds	<1.0 x 10^2
β-glucuronidase positive *Escherichia coli*	3.0 x 10^1
Clostridium perfringens	1.0 x 10^1
Coagulase-positive *Staphylococci*	<1.0 x 10^1
Bacillus cereus	1.0 x 10^2
Listeria monocytogenes	Not detected /25 g
Salmonella spp	Not detected /25 g

4.2 Turkey

A nutritional characterization of a *Hermetia illucens* sample from Turkey (different from the one produced by ENTOMO in Spain) was carried out by EGE. Basic chemical composition, amino acids and fatty acid profile are shown in **Table 12**. As a result of the analysis of the basic chemical composition, the sample larvae of Turkey contained <5% of moisture. This larva has a very high crude protein content (36.91%), and also ether extract (32.97%). However, the protein concentration was lower than that found in the sample of ENTOMO larva, although the percentage of ether extract was higher in the case of the sample of Turkey. The sample from Turkey had a lower protein level, the concentration of lysine and methionine were also lower than sample from Spain (1.29 and 0.62% versus 2.48 and 1.13% for lysine and methionine of samples of Turkey and Spain, respectively). In addition, the levels of Ca (1.62%) and P (0.54%) were lower than those found in ENTOMO sample.

These results indicated that the composition of the larvae varied depending on the origin, possibly due to differences in the substrates used and the rearing system, factors that have been reflected in the consulted bibliography (Newton et al., 2005; Spranghers et al., 2017; Barragan-Fonseca et al., 2017). It should be noted that the drying and transformation process of the larvae could also affect the quantity and availability of nutrients, as indicated by DiGiacomo and Leury (2019). Also, the fatty acid profile has been analyzed, the main fatty acids were: lauric acid (41.44 g/100g lipid), palmitic acid (17.58 g/100g lipid), oleic acid (13.041 g/100g lipid), linoleic acid (9.708 g/100g lipid), myristic acid (9.173 g/100g lipid) and stearic acid (2.398 g/100g lipid). On the other hand, it is remarkable that more than 70% of the fatty acids found in the larvae are saturated, being lauric acid, the fatty acid with the highest proportion (>40 g/100g lipid).

Table 12. Basic chemical composition, methionine, lysine and fatty acid profile of analyzed dried larvae from Turkey (as-fed basis).

Basic composition[1]	**Amount**	**Fatty acids**	**g/ 100 g lipid**
Dry matter (%)	95.54	cis-10-heptadecanoic (C17:1)	0.222
Ash (%)	5.27	**Stearic (C18:0)**	2.398
Crude protein (%)	36.91	Elaidic (C18:1 trans)	0.329
Ether extract (%)	32.97	**Oleic** (C18:1 cis)	13.041
Starch (%)	-	Linolelaidic (C18:2 trans)	0.141
Total sugar (%)	1.71	**Linoleic** (C18:2 cis)	9.708
ME (kcal/kg)[1]	4331	Arachidic (C20:0)	0.179
Ca (%)	1.62	γ-Linolenic (C18:3n6)	0.000
P (%)	0.54	cis-11-eicosenoic (C20:1)	0.674
Amino acids	**Whole-larvae**	Linolenic (C18:3n3)	0.905
Methionine (%)	0.62	Heneicosanoic (C21:0)	0.016
Lysine (%)	1.29	cis-11.14-eicosadienoic (C20:2)	0.078
Fatty acids	**g/ 100 g lipid**	Behenic (C22:0)	0.062
Butyric (C4:0)	0.000	cis-8.11.14-eicosatrienoic (C20:3n3)	0.068
Caproic (C6:0)	0.000	Erucic (C22:1)	0.020
Caprylic (C8:0)	0.131	cis-11.14.17-eicosatrienoic (C20:3n6)	0.000
Capric (C10:0)	0.785	Arachidonic (C20:4)	0.098
Undecanoic (C11:0)	0.000	Tricosanoic (C23:0)	0.000

Lauric (C12:0)	41.44	cis-13.16-docosadienoic (C22:2)	0.059
Tridecanoic (C13:0)	0.000	Lignoceric (C24:0)	0.018
Myristic (C14:0)	9.173	cis-5.8.11.14.17-eicosapentaenoic (C20:5)	0.000
Myristoleic (C14:1)	0.250	Nervonic (C24:1)	0.042
Pentadecanoic (C15:0)	0.106	cis-4.7.10.13.16.19-docosahexaenoic (C22:6)	0.025
cis-10-pentadecanoic (C15:1)	0.000		
Palmitic (C16:0)	17.58		
Palmitoleic (C16:1)	2.382		
Heptadecanoic (C17:0)	0.132		

As a result of the bibliographic review carried out by Barragan-Fonseca et al. (2017), it was indicated that most of the authors found that the fraction of saturated fatty acids in the fat of *Hermetia illucens* was majority (>58%) with lauric acid stood out among them; being lower in the fat larvae, the monounsaturated fatty acids and polyunsaturated (<40%). As for lauric acid, it should be noted that this fatty acid has antibacterial properties, so it could modulate the gut microbiota and health status (El-Hack et al., 2020). Another outstanding compound in the composition of *Hermetia illucens* larvae is chitin. The chitin is a long-chain polymer of N-acetylglucosamine, and it is a component of insect exoskeletons. Finke (2013) indicated that these larvae contained about 5.41% DM. This compound also has potentially positive antimicrobial and immunomodulatory properties, although it could affect the availability of nutrients (El-Hack et al., 2020). From the microbiological analyzes performed on the Turkish sample (**Table 13**), the counts of *Enterobacteriaceae* (5.3 x 10^3cfu/g), Total coliforms (5.8 x 10^3cfu/g*), Escherichia coli* (<10 cfu/g) and *Clostridium perfringens* (<10 cfu/g) are low. In addition, *Listeria monocytogenes* and *Salmonella spp* were not detected. These results were similar to those obtained by the sample produced by ENTOMO.

Table 13. Microbiological analyzes of dried larvae from Turkey.

Microorganism	cfu/g
Total Bacteria Count	6.6 x 10^5
Enterobacteriaceae	5.3 x 10^3
Total coliforms	5.8 x 10^3
Escherichia coli	<10
Clostridium perfringens	<10
Bacillus cereus	2.0 x 10^1
Listeria monocytogenes	Not detected /25 g
Salmonella spp	Not detected /25 g

5 Diet formulation

Each pilot has considered a feeding program according to genotype, poultry production (meat or/and laying eggs), expected performance and studied period. A set of general criteria was established in order to design and formulate of the diets to obtain comparable and more sustainable diets. In addition, during this period the diets have been subjected to a feedback process for their reassessment and revision of the established criteria. The following sections will describe the results obtained from this task in each pilot.

5.1 Optimized diets of Spain

5.1.1 Adjustment to the requirements of formulated diets

The pilot of UMU will be carried out in laying hens, using an adapted ecotype (Crossing with breeds adapted to western-Mediterranean). The production phase to evaluated will be the first phase of lay, up to 40 weeks of life, approximately.

In the design of preliminary diets, three experimental treatments have been developed: one control (with inclusion of usual ingredients) (Control), and two more sustainable ones: with alternative ingredients, and 3% or 6% of *Hermetia illucens* dried larvae (3-HERM and 6-HERM, respectively).

The optimized formulation of the diet was carried out using the database of the nutritional characterization of the ingredients, and the nutritional requirements of hens for the first phase of egg production, following the inclusion criteria of ingredient. **Table 14** shows the adjustment of preliminary formulation to the requirements for laying hens.

Table 14. Adjustment to laying hens' requirements in the **final preliminary formulations** for Spanish pilot (as-fed basis).

Treatment	ME[2] kcal/kg	CP (%)	EE (%)	CF (%)	Ca (%)	P (%)	Met (%)	Lys (%)	Met + Cys (%)	Trp (%)	Thr (%)	Na (%)	Linoleic Acid (%)
Control[1]	2750	16.50	5.68	4.44	3.83	0.51	0.42	0.82	0.70	0.19	0.62	0.15	2.81
3-HERM	2750	16.70	6.04	4.84	3.83	0.54	0.42	0.82	0.71	0.19	0.62	0.16	2.73
6-HERM	2750	16.80	5.78	5.00	3.83	0.55	0.42	0.82	0.72	0.20	0.62	0.16	2.32

[1] Treatment: Control = feed with inclusion of usual ingredients; 3-HERM = diet with alternative ingredients and 3% dried larvae; 6-HERM = diet with alternative ingredients and 6% dried larvae.
[2]ME = metabolizable energy; CP = crude protein; EE = ether extract; CF = crude fiber, Met= Methionine, Lys= Lysine, Met+Cys= Methionine+Cysteine, Trp= Tryptophan.

The nutritional values of diets were close to be iso-energetic and iso-nitrogenous (crude protein, lysine, methionine, methionine+cysteine, tryptophan, and threonine). Furthermore, the content of minerals, such Ca, P and Na was similar. The fiber and ether extract were higher in sustainable diet than control diets to balance energy.

5.1.2 Ingredients of formulated diets

As a result of the formulation of the preliminary diets, the content of ingredients in the formulas of the proposed dietary treatments for laying hens were those indicated in **Table 15**.

Table 15. Main ingredients of diets of pilot of Spain of laying hens in the **final preliminary formulation**.

Treatment	Imported Maize (%)	National Wheat (%)	Soybean meal (%)	Sunflower meal (%)	DDGS (%)	Peas (%)	Soybean oil (%)	Dried larvae (%)
Control[1]	55.00	1	22	6.29			3.20	-
3-HERM	42.49	8.03	16.05	6.5	2	6.68	3.13	3
6-HERM	25.40	25.03	11.16	6.5	1.5	10	2.67	6

[1]Treatment: Control = feed with inclusion of usual ingredients; 3-HERM = diet with alternative ingredients and 3% dried larvae; 6-HERM = diet with alternative ingredients and 6% dried larvae. All diets include minerals, vitamins and trace minerals-premix, synthetic amino acids or other additives.

The ingredient content of the diets for the Spanish pilot is in accordance with the recommended formulation criteria, as follows:

- Control diets (usual ingredients): high amounts of imported maize (55 percentage units) and soybean meal (22 percentage units).

- 3-HERM diet (with sustainable ingredients and insect):
  - Reduction imported maize (-12.51 percentage units) and soybean meal (-5.95 percentage units).
  - Introduction of alternative ingredients: more national wheat (+7.03 percentage units); DDGS (+2 percentage units); peas (+6.68 percentage units) and dried larvae at 3%.

- 6-HERM diets (with sustainable ingredients and with insect):
  - Reduction imported maize (-29.60 percentage units) and soybean meal (-10.84 percentage units).
  - Introduction of alternative ingredients: more national wheat (+24.03 percentage units); DDGS (+1.5 percentage units); peas (+10 percentage units) and dried larvae at 6%.

It should be noted that the use of peas to partially substitute soybean in diets for laying hens could be limited due to the possible negative influence (on the performances) of antinutritive factors present in this seed, or its limited content of sulfur amino acids; although a limit in its incorporation, different treatments, or the inclusion of exogenous enzymes could improve its results in this production. However, Ciurescu and Paná (2017) indicated that untreated peas could be included in the diets of laying hens at a level of up to 35%, replacing soybean meal throughout the laying cycle, without a change in the production, or in the egg quality, except for the color of the egg yolk. Although, FEDNA (2019) marked maximum inclusion level of pea in laying hens of 10%. Another ingredient used in the alternative diets of the Spanish pilot has been maize DDGS. Abd El-Hack et al. (2015), in a literature review, indicated that the maximum levels of inclusion of DDGS should be between 10-15% for laying hens, although higher levels of DDGS could be used with success, if the diet is balanced in amino acids as lysine and methionine. Regarding the use of *Hermetia illucens* in laying hens, several works have been carried out with promising results, although it is necessary to increase the knowledge on its incorporation level, since high levels of incorporation could decrease intake (Marono et al., 2017). In the case of the Spanish pilot, foreseeable limits of incorporation of these alternative ingredients have been taken into account.

5.1.3 General evaluation of formulated diets

The diets of Spanish pilot meet the requirements of laying hens, according to the type of poultry production. The formulated preliminary diets are close to be iso-energetic and iso-nitrogenous. The design of dietary treatments has applied the contrast between usual diet (with non-sustainable ingredients), and other two diets that contain more sustainable ingredients (according to the criteria of deliverable 2.4 about feed impact). Thus, these diets include less imported cereal and soybean meal, and incorporate more alternative ingredients (unusual or by-product). In addition, these sustainable diets include larvae of insect, at 3 or 6%, respectively.

5.2 Optimized diets of Italy

5.2.1 Adjustment to the requirements of formulated diets

The pilot of UNITO will be carried out in poultry meat production, using *Bianca di Saluzzo* male of low growing chickens. The production phases will be two: grower period (from 0 to 60 days) and finisher period (from 61-150 days).

In the design of preliminary diets, three experimental treatments have been developed for each productive phase: a program control (with inclusion of usual ingredients) (Control), and two more sustainable ones: diets with alternative ingredients (ALTER); and other with ALTER diets plus *Hermetia illucens* dried larvae, so a reduction in intake of the alternative diets is expected between 3 and 6% (approximately a mean of 4.5%, 4.5-HERM). The optimized formulation of the diet was carried out using the database of the nutritional characterization of the ingredients, and the nutritional requirements of meat chickens for two phases of production, following the inclusion criteria of ingredient. **Table 16** shows the adjustment of preliminary formulation to the requirements for meat chickens. The nutritional values of control and ALTER diets were close to be iso-energetic and iso-nitrogenous for crude protein, by phase of production. Also, the content of minerals, such Ca and P, was similar per period. Nutritional content of 4.5-HERM diets will be dependent of the percentage of substitution of ALTER feeds by *Hermetia illucens*, and since these larvae of insect has a high concentration of energy and protein, this third program will cover the requirement of the birds.

Table 16. Adjustment of the meat chickens' requirements in the **final preliminary formulations** for Italian pilot (as-fed basis).

Phase	Treatment[1]			Ether extract (%)	Crude fiber (%)	Ca (%)	P (%)	Met (%)	Lys (%)	Thr (%)

		ME[2] kcal/kg	Crude protein (%)							
Grower (0-60d)										
	Control	2761.51	20.52	4.08	3.39	1.20	0.60	0.40	1	0.71
	ALTER	2796.40	20.31	2.73	3.92	1.15	0.60	0.47	0.84	0.64
	4.5-HERM	Plus[3]	plus	plus	plus	plus	plus	plus	plus	plus
Finisher (61-150d)										
	Control	2831.60	19.50	4.89	3.28	1.19	0.59	0.38	0.94	0.67
	ALTER	2837.72	19.61	3.30	3.89	1.19	0.59	0.39	0.87	0.61
	4.5-HERM[3]	plus	plus	plus	plus	plus	plus	plus	plus	plus

[1]Treatment: Control = feed with inclusion of usual ingredients; ALTER = diet with alternative ingredients; 4.5-HERM = a diet with alternative ingredients (ALTER) plus extra-supplemented with *Hermetia illucens* dried larvae; a reduction in intake of the alternative diet is expected between 3 and 6%. All diets include minerals, vitamins and trace minerals-premix, synthetic amino acids or other additives.
[2]ME= metabolizable energy, Met= Methionine, Lys= Lysine, Thr= Threonine.
[3]plus=nutritional content dependent on the percentage of substitution of ALTER feed for *Hermetia illucens.*

5.2.2 Ingredients of formulated diets

As a result of the formulation of the preliminary diets, the content of ingredients in the formulas of the proposed dietary treatments for meat chickens were those indicated in **Table 17**.

Table 17. Main ingredients of diets of pilot of Italy of birds in the **final preliminary formulation**.

Phase	Maize (%)	Soybean meal (%)	Fava beans (%)	Pea (%)	Sunflower meal (%)	Maize gluten (%)	Soybean oil (%)	Dried larvae (%)
Grower (0-60d)								
Control[1]	60.0	34.57	-	-	-	-	1.2	
ALTER	54.70	6.57	8.6	8.6	5.0	12.0	-	
4.5-HERM	52.24[2]	6.27	8.21	8.21	4.78	11.46	-	4.5
Finisher (60-150d)								
Control	61.77	32.0	-	-	-	-	2.0	
ALTER	54.50	4.035	9.6	9.6	5.0	12.0	0.6	
4.5-HERM	52.05[2]	3.85	9.17	9.17	4.78	11.46	0.57	4.5

[1]Treatment: Control = feed with inclusion of usual ingredients; ALTER = diet with alternative ingredients; 4.5-HERM = a diet with alternative ingredients (ALTER) plus extra-supplemented with *Hermetia illucens* dried larvae; a reduction in intake of the alternative diet is expected between 3 and 6%. All diets include minerals, vitamins and trace minerals-premix, synthetic amino acids or other additives.

The ingredient content of the diets for the Italian pilot is in accordance with the recommended formulation criteria, as follows:

- Control diets (usual ingredient): high amount of maize (60.0 and 61.77 for grower and finisher, respectively), high amount of soybean meal (34.57 and 32.0% for grower and finisher, respectively) and soybean oil (1.2 and 2.0% for grower and finisher, respectively).

- ALTER diets:

  - Reduction maize (-5.3, and -7.27 percentage units for grower and finisher, respectively), soybean meal (-28 and -27.97 percentage units for grower and finisher, respectively) and oil soybean (-1.2, and -1.4 percentage units for grower and finisher, respectively).
  - Introduction of alternative ingredients: fava beans (+8.6, and +9.6 percentage units for grower and finisher, respectively), pea (+8.6, and +9.6 percentage units for grower and finisher, respectively), sunflower meal (+5 percentage units for grower and finisher) and maize gluten (+12 percentage units for grower and finisher).

- 4.5-HERM diets (ALTER diets plus insect larvae):

  - With an expected *Hermetia illucens* substitution approximately of 4.5%, of ALTER diet: reduction maize (-7.76, and -9.72 percentage units for grower and finisher, respectively), soybean meal (-28.3 and -28.15 percentage units for grower and finisher, respectively) and oil soybean (-1.2, and -1.43 percentage units for grower and finisher, respectively).
  - With an expected *Hermetia illucens* substitution approximately of 4.5%, of ALTER diet: introduction of alternative ingredients, such as fava beans (+8.21, and +9.17 percentage units for grower and finisher, respectively), pea (+8.21, and +9.17 percentage units for grower and finisher), sunflower meal (+4.78 percentage units for grower and finisher) and maize gluten (+11.46 percentage units for grower and finisher).

Pea and fava bean seeds contain antinutritive factors, so the two seeds have an inclusion limit of <10% in the diets of the Italian pilot. Gous (2011) observed that when fava bean was offered in a feed with mash form, growth rate and feed conversion efficiency of broilers decreased but when the feed was pelleted, performance was not negatively affected. Thus, it may be that the pelleting process destroyed some heat labile anti-nutritional factor present in the fava bean. In addition, Perez-Maldonado et al. (1999) indicated that a maximum inclusion limit of this seed should be applied in the feeding of poultry. Also, it has been indicated that the hulling and micronization of fava beans could optimize its use (Laudadio et al., 2011). Another ingredient, such as maize gluten meal, is used in Italian diets, a by-product resulting from starch extraction, that has a high level of protein (60%) and an appreciable amount of available sulfur amino acids (Giannenas et al., 2017), so it could be a complementary ingredient to other alternative sources of protein. Similarly, sunflower meal could be a balancing protein ingredient, although its fiber level limits its use.

5.2.3 General evaluation of formulated diets

The diets of Italy pilot meet the requirements according to the type of poultry production. The formulated preliminary diets by phase are close to be iso-energetic, and iso-nitrogenous for crude protein, at least the controls and alternative without insect. In the design of preliminary diets, three experimental programs have been developed for each productive phase: a control (with inclusion of usual ingredients), and two more sustainable ones (according to the criteria of deliverable 2.4 about feed impact), that include a program with alternative ingredients; and other with alternative diets plus *Hermetia illucens* dried larvae, expecting a reduction of intake of the alternative diets between 3 and 6% (approximately a mean of 4.5%).

5.3 Optimized diets of Turkey

5.3.1 Adjustment to the requirements of formulated diets

The pilot of EGE will be carried out in poultry meat production, using an ecotype of adapted broiler and Ross 308 strain as a control. The production phases will be three: starter (from 0 to 14 days), grower (from 15-28 days) and finisher (from 29 days to slaughter age). In the design of diets, three experimental treatments have been developed for each productive phase: one control (with inclusion of usual ingredients) (Control), and two more sustainable: one with alternative ingredients, and other with and 5% of *Hermetia illucens* dried larvae (ALTER and 5-HERM, respectively). After the optimized formulation of the diets, taking into account the database of the nutritional assessment of ingredients, the nutritional requirements of the birds, and the inclusion criteria of ingredient; preliminary formulations were obtained, thus the adjustment to the requirements by phase and dietary treatment is shown in **Table 18**.

Table 18. Adjustment to the bird requirements of Turkish diets by phase and dietary treatment in **final preliminary formulations** (as-fed basis).

Phase	Treatment[1]	ME[2] kcal/kg	Crude protein (%)	Ether extract (%)	Crude fiber (%)	Ca (%)	P (%)	Methionine (%)	Lysine (%)
Starter (0-14d)									
	Control	3004.95	21.15	6.95	2.93	1.28	0.48	0.69	1.20
	ALTER	3003.58	21.05	9.94	5.24	1.24	0.57	0.69	1.21
	5-HERM	3000.28	21.00	9.44	5.94	1.32	0.57	0.64	1.21
Grower (15-28d)									
	Control	3102.39	20.07	8.10	2.78	1.28	0.47	0.67	1.17
	ALTER	3102.15	20.01	10.00	4.50	1.29	0.52	0.67	1.17
	5-HERM	3102.42	20.00	9.95	5.24	1.34	0.53	0.63	1.17
Finisher (29d-slaughter)									
	Control	3111.38	18.11	7.42	2.51	1.31	0.45	0.63	1.10

	ALTER	3102.34	18.00	9.98	5.14	1.31	0.53	0.59	1.10
	5-HERM	3100.99	18.01	9.95	5.37	1.39	0.53	0.57	1.11

[1]Control = feed with inclusion of usual ingredients; ALTER= diet with alternative ingredients; 5-HERM = diet with alternative ingredients and 5% dried larvae. All diets include minerals, vitamins and trace minerals-premix, synthetic amino acids or other additives.
[2] ME = metabolizable energy.

In this case diets by phase were close to be iso-energetic, iso-protein, iso-lysine and iso-methionine. As for minerals, their calcium and phosphorus content were similar within each period. Only fiber and ether extract were different between diets, due to the necessary adjustment to balance energy, since diets with higher fiber contain higher ether extract.

5.3.2 Ingredients of formulated diets

A study of the main ingredients present in the preliminary diets of the Turkish pilot has been carried out. These ingredients by phase and dietary treatment are shown in **Table 19**.

Table 19. Main ingredients of Turkish diets by phase and dietary treatment in **final preliminary formulations**.

Phase	Maize (%)	National Wheat (%)	Soybean meal (%)	Brewers´ dried grain (%)	Wheat middling (%)	Sunflower meal (%)	Sunflower oil (%)	Dried larvae (%)
Starter (0-14d)								
Control[1]	48.473	7	36	-	-	-	5.3	-
ALTER	32.823	10.5	25	5	7	8	8.35	-
5-HERM	36.473	10.5	17.6	5	4	12	6.1	5
Grower (15-28d)								
Control	48.273	9	33	-	-	-	6.5	-
ALTER	43.123	6	24.8	3	4	7.4	8.35	-
5-HERM	44.623	6	17.5	3	3	11	6.55	5
Finisher (29d-slaughter)								
Control	49.073	15.5	26.5	-	-	-	5.7	-
ALTER	39.573	13.9	13.5	3.9	5.7	11.9	8.2	-
5-HERM	44.173	10	10	5	5	11.1	6.4	5

[1] Control = feed with inclusion of usual ingredients; ALTER= diet with alternative ingredients; 5-HERM = diet with alternative ingredients and 5% dried larvae. All diets include minerals, vitamins and trace minerals-premix, synthetic amino acids or other additives.

The formulation of the diet in terms of the ingredients used adequately meets the following formulation criteria:

- Control diets (usual ingredient): high amount of imported maize (48.473, 48.273 and 49.073% for starter, grower and finisher, respectively) and high amount of soybean meal (36, 33, 26.5% for starter, grower and finisher, respectively).

- ALTER diets:
    - Reduction maize (-15.65, -5.15 and -9.5 percentage units for starter, grower and finisher, respectively) and soybean meal (-11, -8.2 and -13 percentage units for starter, grower and finisher, respectively).
    - Introduction of alternative ingredients: Brewers´ dried grain (+5, +3 and +3.9 percentage units for starter, grower and finisher, respectively), wheat middling (+7, +4 and +5.7 percentage units for starter, grower and finisher, respectively) and sunflower meal (+8, +7.4 and +11.9 percentage units for starter, grower and finisher, respectively).

- 5-HERM diets:
    - Reduction maize (-12, -3.65 and -4.9 percentage units for starter, grower and finisher, respectively) and soybean meal (-18.4, -15.5 and -16.5 percentage units for starter, grower and finisher, respectively).
    - Introduction of alternative ingredients: Brewers´ dried grain (+5, +3 and +5 percentage units for starter, grower and finisher, respectively), wheat middling (+4, +3 and +5 percentage units for starter, grower and finisher phases, respectively) and sunflower meal (+12, +11 and +11.1 percentage units for starter, grower and finisher phases, respectively), and dried larvae at 5%.

Turkish diets have incorporated a by-product such as brewers´ dried grain, which has a moderate energy and protein content, although its fiber level may limit its diet addition, having been limited to 5% inclusion. Abd El-Hack et al. (2019) indicated that this by-product could be a better source of protein or amino acids than corn. However, for broiler chickens, they recommended an inclusion of 5-10% in the diets. Also, Turkish alternative diets include another by-product, wheat middling, which contains moderate levels of nutrients, although it does not exceed 5% addition to diets. There are notable variations in the composition of wheat middling by-products, both in starch and fiber levels, thus maximum incorporation levels of 17-20% are recommended in broilers (FEDNA, 2019). In addition, sunflower meal was used as other ingredients in alternative diets, thus this product was added as a protein source in Turkish pilot diets, not exceeding 12%, since this ingredient should be limited by its fiber content (Alagawany et al., 2015).

5.3.3 General evaluation of formulated diets

The preliminary formulas of the Turkish pilot meet the requirements of birds according to the type of poultry production and phase, and the diets are close to being iso-energetic and iso-nitrogenous. Also, the diet design has implemented the comparison between usual diets (with non-sustainable ingredients), and other diets that include more sustainable ingredients (according to the criteria of deliverable 2.4 about feed impact). Sustainable diets contain less imported soybean meal and incorporate alternative ingredients (unusual or by-product). In addition, at least one sustainable program includes larvae of the insect.

5.4 Optimized diets of Tunisia

5.4.1 Adjustment to the requirements of formulated diets

The pilots of ISA-CM and RAYHANA will be carried out trials in poultry meat and laying hens production, using ecotypes of adapted birds. For the meat production cycle (slow-growing chicken) there will be three phases: starter (from 1 to 28 days), grower (from 29 to 66 days) and finisher (from 67 days to slaughter age). In addition, the period of production of laying hens will be evaluated.

In the design of diets three experimental treatments have been developed for each productive phase: a control (with the inclusion of usual ingredients) (Control), and two more sustainable ones: with alternative ingredients without insects (ALTER) and another with alternative ingredients and a 5 % of dry larvae of *Hermetia illucens* (5-HERM). This general design was used for poultry production of meat or eggs.

To formulate the diets, the database of the nutritional value of the ingredients was used, adjusting the requirements of the animals to the type of production and phase implemented, including the criteria of general formulation. **Table 20** shows the adjustment of preliminary formulation to the requirements by phase and dietary treatment for meat production.

Table 20. Adjustment to the meat bird requirements of Tunisia diets by phase and dietary treatment in **final preliminary formulations** (as-fed basis).

Phase	ME[2] (kcal/kg)	CP (%)	EE (%)	CF (%)	Ca (%)	P (%)	Met (%)	Lys (%)	Met +Cys(%)	Trp (%)	Thr (%)	Na (%)
Starter (1-28d)												
Control[1]	2853.18	21.56	2.86	2.83	1.13	0.69	0.49	1.22	0.85	0.25	0.83	0.16
ALTER	2857.96	21.55	5.09	3.81	1.14	0.72	0.49	1.19	0.88	0.25	0.82	0.16
5-HERM	2856.79	21.54	4.63	3.85	1.11	0.70	0.49	1.19	0.85	0.24	0.82	0.16
Grower (29-66d)												
Control	2933.88	18.28	3.03	2.63	1.04	0.64	0.39	0.98	0.72	0.2	0.71	0.16
ALTER	2937.11	18.2	4.62	3.37	1.02	0.64	0.38	0.94	0.73	0.2	0.68	0.16
5-HERM	2933.53	18.19	4.86	3.57	1.05	0.64	0.39	0.93	0.72	0.19	0.68	0.17
Finisher (67d-slaughter)												
Control	2980.32	16.71	3.12	2.54	0.93	0.58	0.33	0.88	0.63	0.18	0.64	0.16
ALTER	2978.79	16.7	5.15	3.39	0.94	0.59	0.31	0.83	0.64	0.18	0.61	0.16
5-HERM	2979.91	16.69	4.99	3.49	0.96	0.59	0.32	0.83	0.63	0.17	0.62	0.17

[1]Treatment: Control = feed with inclusion of usual ingredients; ALTER = diet with alternative ingredients; 5-HERM = diet with alternative ingredients and 5% dried larvae.
[2]ME = metabolizable energy; CP = crude protein; EE = ether extract; CF = crude fiber, Met= Methionine, Lys= Lysine, Met+Cys= methionine+ cysteine, Trp= tryptophane, Thr= Threonine.

In this case diets were close to be iso-energetic, iso-protein, iso-lysine and iso-methionine. Also, minerals (Ca, P, and Na) were similar within each period. In this case, small differences in fiber and ether extract content were found in these diets, due to the energy adjustment.

On the other hand, **Table 21** shows the adjustment of preliminary formulation to the requirements by phase and dietary treatment for laying hens.

Table 21. Adjustment to the laying hens' requirements of Tunisia diets in **final preliminary formulations** (as-fed basis).

Phase	ME[2] kcal/kg	CP (%)	EE (%)	CF (%)	Ca (%)	P (%)	Met (%)	Lys (%)	Met +Cys(%)	Trp (%)	Thr (%)	Na (%)	K (%)	CL (%)
Control[1]	2636.5 3	16.72	2.8 6	2.8 6	3.9 2	0.6 1	0.3 4	0.8 8	0.6 4	0.1 9	0.6 4	0.1 5	0.78	0.26
ALTER	2638.0 8	16.68	2.3 0	2.5 8	3.9 0	0.5 7	0.3 4	0.8 8	0.6 4	0.1 9	0.6 2	0.1 5	0.74	0.28
5-HERM	2639.0 8	16.73	4.0 4	3.2 3	3.8 8	0.6 2	0.3 5	0.8 8	0.6 4	0.1 9	0.6 1	0.1 6	0.74	0.3

[1]Treatment: Control = feed with inclusion of usual ingredients; ALTER = diet with alternative ingredients; 5-HER = diet with alternative ingredients and 5% dried larvae.
[2]ME = metabolizable energy; CP = crude protein; EE = ether extract; CF = crude fiber, Met= Methionine, Lys= Lysine, Met+Cys= methionine+ cysteine, Trp= tryptophane, Thr= Threonine.

In this case, diets for laying hens were close to be iso-energetic and iso- nitrogenous (for crude protein, lysine, methionine, methionine+cysteine, tryptophan and threonine). In addition, the content of mineral such as Ca, P, Na, K and Cl were similar for these diets. Also, for the case of meat chickens, the diet with the highest fiber content had the highest ether extract level, to balance of energy.

5.4.2 Ingredients of formulated diets

A study of the main ingredients present in the preliminary diets of the Tunisian pilot has been carried out. These ingredients by phase and dietary treatment for meat production are shown in **Table 22**.

Table 22. Main ingredient of dietary Tunisia for meat production, by phase and dietary treatment, in **final preliminary formulations**.

Phase	Imported Maize (%)	National Triticale (%)	Soybean meal (%)	Rapeseed meal (%)	Fava beans (%)	Pasta waste (%)	Soybean oil (%)	Dried larvae (%)
Starter (1-28d)								
Control[1]	59.7	0	36.3	-	-	-	-	-
ALTER	33.24	15	26.45	10	5	5	1.5	-
5-HERM	33.24	15	23	5	10	5	-	5

Grower (29-66d)								
Control	68.3	-	28	-	-	-	-	-
ALTER	36	14	18	7	7	13	1.5	-
5-HERM	34.8	17	13.7	7	7	12	-	5
Finisher (67d-slaughter)								
Control	72.7	-	24	-	-	-	-	-
ALTER	27	30.55	13.1	7	7	10	2.2	-
5-HERM	40.75	16.0	10.1	7	7	11	-	5

[1]Treatment: Control = feed with inclusion of usual ingredients; ALTER = diet with alternative ingredients; 5-HERM = diet with alternative ingredients and 5% dried larvae. All diets include minerals, vitamins and trace minerals-premix, synthetic amino acids or other additives.

The formulation of the diet in terms of the ingredients used adequately meets the following formulation criteria:

- Control diets (usual ingredient): high amounts of imported maize (59.7, 68.3 and 72.7% for starter, grower and finisher, respectively); and soybean meal (36.3, 28, and 24% for starter, grower and finisher, respectively).

- ALTER diet s(with sustainable ingredients without insect):
    - Reduction imported maize (-26.46, -32.3 and -45.7 percentage units for starter, grower and finisher, respectively) and soybean meal (-9.85, -10 and -10.9 percentage units for starter, grower and finisher, respectively).
    - Introduction of alternative ingredients: national triticale +15, +14 and +30.55 percentage units for starter, grower and finisher, respectively); rapeseed meal (+10, +7 and +7 percentage units for starter, grower and finisher, respectively); Fava beans (+5, +7 and +7 percentage units for starter, grower and finisher, respectively); and pasta waste (+5, +13 and +10 percentage units for starter, grower and finisher, respectively).

- 5-HERM diets (with sustainable ingredients and with insect):
    - Reduction imported maize (-26.46, -33.5 and -31.95 percentage units for starter, grower and finisher, respectively) and soybean meal (-13.3, -14.3 and -13.9 percentage units for starter, grower and finisher, respectively);
    - Introduction of alternative ingredients: national triticale +15, +17 and +16 percentage units for starter, grower and finisher, respectively); rapeseed meal (+5, +7 and +7 percentage units for starter, grower and finisher, respectively); Fava beans (+10, +7 and +7 percentage units for starter, grower and finisher, respectively); pasta waste (+5, +12 and +11 percentage units for starter, grower and finisher, respectively) and dried larvae at 5% for all periods.

On the other hand, these ingredients of dietary treatment of Tunisia for laying hens are shown in **Table 23**.

Table 23. Main ingredient of dietary Tunisia for eggs production in **final preliminary formulations**.

Phase	Imported Maize (%)	National Triticale (%)	Soybean meal (%)	Wheat bran (%)	Pasta waste (%)	Dried larvae (%)
Control[1]	57	-	24	8	-	-
ALTER	40	10	22	5	12	-
5-HERM	31.3	10	16.5	14	12.3	5

[1]Treatment: Control = feed with inclusion of usual ingredients; ALTER = diet with alternative ingredients; 5-HERM = diet with alternative ingredients and 5% dried larvae. All diets include minerals, vitamins and trace minerals-premix, synthetic amino acids or other additives.

The formulation of the diet in terms of the ingredients used adequately meets the following formulation criteria:

- Control diets (usual ingredient): high amounts of imported maize (57 percentage units); and soybean meal (24 percentage units).

- ALTER diets (with sustainable ingredients without insect):
  - Reduction imported maize (-17 percentage units) and soybean meal (-2 percentage units).
  - Introduction of alternative ingredients: national triticale (+10 percentage units); and pasta waste (+12 percentage units).

- 5-HERM diets (with sustainable ingredients and with insect):
  - Reduction imported maize (-25.7 percentage units) and soybean meal (-7.5 percentage units).
  - Introduction of alternative ingredients: national triticale (+10 percentage units); pasta waste (+12.3 percentage units) and dried larvae at 5% for all periods.

The alternative diets of the Tunisian pilot include an alternative cereal of national production, triticale (hybrid between wheat and rye) with levels that do not exceed 17%. Hermes and Johnson (2004) indicated that the incorporation of 15 or 30% of a variety of triticale in broilers or layers respectively, did not affect performances. However, FEDNA (2019) recommends more moderate levels when exogenous enzymes are not used. Another alternative ingredient used in Tunisian meat chicken diets was rapeseed meal, reaching maximum values of 10%. Biesek et al. (2020) studied the effect of the inclusion of various protein alternatives (legume seeds and rapeseed meal) to soybean meal in broiler production, finding that an incorporation of 25% rapeseed meal could decrease the performance of the animals, although the effects on meat quality were less pronounced. On the other hand, the Tunisian diets, both for broiler chickens and for layers, included a by-product such as pasta waste. For broiler, Rostagno and Becker (2017) indicated that this ingredient has a maximum level of 15-20% in broiler diets, although Baghbanzhafar et al. (2013) suggested a maximum inclusion limit of 10%, due to the possible effects that the thermal treatments of pasta production could have on the availability of certain nutrients.

5.4.3 General evaluation of formulated diets

The preliminary formulas of Tunisian pilot meet the requirements of birds according type of poultry production (meat or eggs), and the diets are close to be iso-energetic and iso-nitrogenous. Also, the diet design has implementing the comparation between usual diet (with non-sustainable ingredients), and other diets that include more sustainable ingredients (according to the criteria of deliverable 2.4 about feed impact). Sustainable diets contain less imported soybean meal and incorporate alternative ingredients (unusual or by-product). In addition, the one sustainable diet includes larvae of the insect at 5%.

6 Conclusions

The development of task 2.5 has achieved a design of isoenergetic and isonitrogenous diets according to the production phase evaluated in each pilot, which will be able to compare feeding programs with habitual diets (less sustainable), with alternative programs of more sustainable diets, which alternative ingredients and *Hermetia illucens* larvae will be incorporated. To achieve this objective, it is necessary to implement in each pilot a general protocol for nutritional characterization, nutritional requirements and diet design.

1. It was necessary to determine the nutritional value of the usual ingredients employed in each area, as well as nutritionally characterize the alternative ingredients or by-products available. In addition, nutritive value of *Hermetia illucens* larvae is required. Therefore,

    - For nutritional characterization of usual ingredients of the feeds, as well as the possible local ingredients (by-products or other alternatives), the most relevant bibliography, from national and international databases, was used to assign the nutritional value of these ingredients (NRC, 1994; INRA, 2004; Sari et al., 2008; Heuzé et al., 2013; FEDNA, 2019). In addition, EGE analyzed the chemical components, and estimated metabolizable energy of some Turkish ingredients.
    - The dried larva produced by ENTOMO has a very high crude protein content (42% as-fed basis, 46.15% DM), and also ether extract (21.6% as-fed basis, 23.07% DM), being the contents of Ca (5.0 as-fed basis, 5.49% DM) and P (1.02% as-fed basis, 1.12% DM) appreciable. The most prevalent essential amino acids in the ENTOMO larvae were lysine, valine and leucine with levels > 2.3% as-fed basis (> 2.5% DM). The protein concentration of Turkish larvae was lower than that found in the sample of ENTOMO larva, but the percentage of ether extract was higher. The composition of the larvae varied greatly depending on the origin, possibly due to differences in the substrates used and the rearing system.
    - Both ENTOMO and EGE larval biomass have low microbial contaminants, highlighting the absence of *Listeria monocytogenes* and *Salmonella spp*.

2. To establish the nutritional requirements of the birds in each pilot, it is necessary to set up, according to the study objective (meat and/or egg production), the genetic type, evaluation phase and expected production. Therefore,

    - The pilot of Spain (UMU) indicated that they will use laying hens, crossing with breeds adapted to western-Mediterranean, for the first phase of lay production, meeting the nutritional requirements of these animals according to FEDNA (2018).
    - The pilot of Italy (UNITO) specified that they will use meat chickens, *Bianca di Saluzzo* male (an Italian autochthonous breed), for Grower (d0 – d60) and Finisher (d61 – d150) periods, meeting the nutritional requirements of these birds according to low input diets for slow-growing chickens (Cerolini et al, 2019).
    - The pilot of Turkey (EGE) specified that they will also use meat chickens, in this case the Anadolu-T (ecotype) and a commercial fast-growing strain (Ross 308), for Starter (d0 - d14), Grower (d15 - d28) and Finisher phases

(d29 - slaughter age), meeting the nutritional requirements according to Sarıca et al. (2019; 2021).

- The pilot of Tunisia (ISA-CM and RAYHANA) specified that they will also use meat chickens and laying hens, in this case the autochthonous Tunisian (ecotype), and Géant and Génoise (local) will be used.

3. For the design and formulation of the diets, a set of general criteria, established by the project, must be followed. Optimized diets to meet the requirements of birds of each pilot have been established to compare a control diet (with usual ingredients, no sustainability criteria), with other diets more sustainable (according to the criteria of deliverable 2.4 about feed impact). Thus,

    - All pilots have achieved a design of three feeding programs that met the established criteria. Sustainable diets have lower levels of soybean meal and including alternative ingredients (unusual or by-products), and at least one of them incorporates *Hermetia illucens* insect larvae. In addition, at least the control and one alternative diet have been iso-energetic and iso-nitrogenous (for crude protein and/or amino acid).
    - These diets are considered final preliminary diets, but they must be adapted, in each pilot, to the nutritional characterization of the *Hermetia illucens* larva used, and to the availability of ingredients at the formulation moment for the *in vivo* trials, that will be carried out in the following work packages.

It should be noted that given the highly volatile situation, created by the international circumstances, and as the countries of Eastern Europe are an important source of materials for animal feeds, the formulas proposed in this milestone could be adapted to future situations although following the sustainability criteria established in this milestone.

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