The PRIMA programme is supported under Horizon 2020 the European Union's Framework Programme for Research and Innovation

SUSTAVIANFEED

ALTERNATIVE ANIMAL FEEDS IN MEDITERRANEAN POULTRY BREEDS TO OBTAIN SUSTAINABLE PRODUCTS

Feed safety and health evaluation of the diet

DELIVERABLE 2.3

This project (grant Number 2015), is part of the PRIMA programme, supported by the European Union

Partners

PROJECT INFORMATION	
Type of Project	PRIMA Foundation Project
Topic	Topic 1.2.1-2020 (RIA/IA) Genetic conservation and animal feeds: Sub-topic B) Alternative animal feeds (IA) of the Annual Work Plan 2020 for the Partnership for Research and Innovation in the Mediterranean Area (PRIMA)
Grant Agreement No.	2015
Project Duration	01/04/2021 – 31/03/2025 (48 months)
Project Coordinator	SOCIEDAD AGRARIA DE TRANSFORMACION 2439 (ALIA)
Project Partners	UNIVERSITY OF MURCIA (UMU), UNIVERSITY OF TURIN (UNITO), INSTITUT SUPERIEUR AGRONOMIQUE DE CHOTT MARIEM (ISA-CM), ASSOCIATION RAYHANA (RAYHANA), ENTOMO CONSULTING S.L. (ENTOMO), EGE UNIVERSITY (EGE), FONDAZIONE SLOW FOOD PER LA BIODIVERSITA' ONLUS (SLOWFOOD)

DOCUMENT INFORMATION	
Title	D2.3. Feed safety and health evaluation of the diet
Version	2
Release Date (dd.mm.yy)	(28.03.22)
Work Package	2
Dissemination Level	PU

DOCUMENT AUTHORS AND AUTHORISATION	
Document Responsible	UniTo
Contributors	Unito, UMU, EGE, ISA-CM
Reviewed by	ISA-CM, ALIA
Approved by	UNITO and project consortium

DOCUMENT HISTORY			
Version	Date (dd.mm.yy)	Description	Implemented by
1.00	24.02.22	First draft	UniTo, UMU
1.1	01.03.22	Second draft	EGE
1.2	05.03.22	Third draft	ISA-CM
1.3	18.04.22	Fourth draft	EGE, ISA-CM, UMU
2.0	30.04.22	Final version	UniTo
3.0	19.09.23	Reviewed version	UniTo

SUMMARY:

The innovative and ecologically responsible poultry diet, which incorporates insect-based components, has undergone a comprehensive feed safety assessment aimed at ensuring the well-being of both animals and humans. This evaluation encompasses a thorough analysis of the microbiological status of the diet as well as the identification and quantification of potential contaminants to guarantee the highest standards of safety and quality [1].

To comprehensively gauge the safety of the raw materials utilized in formulating poultry diet, the overarching objective involves a meticulous examination of the legislations governing the various collaborating partners within the project. This deliverable aims at synthesizing and present a cohesive compilation of the laws and regulations, adopting a precise and harmonized approach. The outcome of this concerted effort manifests as an invaluable tool, one that provides all project partners with easy access to a consolidated repository of legal guidelines.

For each distinct ingredient employed in the poultry diet, an exhaustive compilation and summary of the pertinent regulations and laws from each partner country within the project have been meticulously collected and organized. This meticulous process remains aligned with the objectives outlined in WP2, affording a comprehensive overview that contributes to the project's overall success and the advancement of safe and sustainable poultry nutrition practices.

Furthermore, an in-depth analysis of the safety aspects pertaining to the utilization of black soldier fly larvae in the pilot programs has been conducted. This comprehensive safety assessment encompassed meticulous evaluations covering various critical factors, including microorganisms, aflatoxins, and heavy metals. First and foremost, the examination of microorganisms associated with the black soldier fly larvae was a paramount aspect of the safety assessment. Rigorous testing and scrutiny were employed to identify and quantify any potential microorganism presence. This step ensured that the larvae were free from harmful pathogens or contaminants that could pose risks to their application in the pilots.

In addition to microorganisms, aflatoxins were scrutinized during the safety evaluation process. Aflatoxins are naturally occurring toxins produced by certain molds and can be harmful if consumed or introduced into the food chain. Thorough testing was conducted to ascertain that the black soldier fly larvae were devoid of any aflatoxin contamination, guaranteeing their suitability for use in various applications. Moreover, heavy metals, known for their potential health hazards, were subject to stringent determinations as part of the safety assessment. The concentration levels of heavy metals such as lead, mercury, and cadmium were measured to ensure they fell within established safety limits. This precautionary measure aimed to confirm that the black soldier fly larvae were not accumulating harmful levels of these metals from their environment. In summary, the safety evaluation of the black soldier fly larvae used in the pilot programs encompassed a comprehensive analysis of microorganisms, aflatoxins, and heavy metals. These assessments were undertaken to establish and uphold the utmost safety standards, guaranteeing the suitability and security of the larvae's involvement in the various pilot initiatives.

Table of contents

Table of figures

1. Introduction

The main issue of the present day is that there is already competition between the human food sector and the animal feed sector, for this reason it is essential to identify new raw materials to be included in the diets of livestock [2].

However, all the innovations, the new potential risks, and the innovative ingredients, which are progressively introduced within the food industry, must also be included in this regulatory framework [3]. As it is known, the supply of protein sources for animal feeding is increasingly expensive, difficult, harmful to the environment, and above all, not sustainable considering the needs of intensive farming, which is constantly growing [4].

An emblematic and recent example is the use of insects in animal feed that, with the "COMMISSION REGULATION (EU) 2021/1372 of 17 August 2021", has been definitively authorized in the feeding of poultry and pigs. This regulation has taken several years to be enacted and has followed the numerous positive opinions of EFSA (European Food Safety Authority). However, despite the European Union's authorization about the use of insects as a protein source, the supervision of their safety is active and will probably be modified and improved over the years (*https://eur-lex.europa.eu/homepage.html?locale=it*). As all the main traditional ingredients presents specific regulations in all the countries partner of the project for the insect's use of in animal feed, we only have regulations in EU. On the contrary in both Turkey and Tunisia no specific regulations are present about this innovative ingredient, for this reason we decided to apply and follow the guidelines of the EU regulation.

World Nations, in particular the more developed ones, have increasingly implemented new regulations and controls regarding the origin of raw materials and most of the ingredients used in animals feed, setting quality and safety standards. The supply of raw materials and the safety of the feed supplied to animals are of primary importance. The factors that directly influence both the quality and the safety of the feed itself are origin, processing, handling, and storing, as well as many other market-related factors [5].

The European Union in particular, as a "supranational" organization, dictates rules and recommendations to all the member states. This is particularly effective because it uniforms

common rules in countries where trades are frequent. Within the European Union there are main regulations that could be defined as starting points for all the others, such as:

- REGULATION (EC) No 183/2005 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL Amended by: of 12 January 2005 laying down requirements for feed hygiene.
- COMMISSION REGULATION (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed.
- REGULATION (EC) No 767/2009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 13 July 2009 on the placing on the market and use of feed, amending European Parliament and Council Regulation (EC) No 1831/2003 and repealing Council Directive 79/373/EEC, Commission Directive 80/511/EEC, Council Directives 82/471/EEC, 83/228/EEC, 93/74/EEC, 93/113/EC and 96/25/EC and Commission Decision 2004/217/EC.
- COMMISSION REGULATION (EU) No 68/2013 of 16 January2013 on the Catalogue of feed materials The present document includes the main feed raw materials used in poultry feeding by the PRIMA partners, followed by the main regulations in force.

2. Methodology

In order to obtain a general safety framework for feed regulation, the goal was to begin from the specific laws on feed safety of partners' countries involved in the project. The applied methodology intends to summarize in detail all the regulations involved.

More in detail, the objective was to study the compliance with the laws and the gathering of the main ingredients used in the different countries partner of the project. For each ingredient, the laws and regulations present in each country have been stated in order to ensure feed safety and compliance with the laws.

For the preparation of the present deliverable, all the partners involved were contacted individually in order to identify and organize all the main feed ingredients used in poultry nutrition.

Subsequently, after having collected and harmonized the information received, the respective laws on the subject in the partners' countries were identified.

Specifically, the information was obtained in the following way:

- For Italy and Spain, being both European countries the regulations are the same and have been taken from the European Commission website (*https://eur-lex.europa.eu/homepage.html?locale=it*)
- For Turkey, the national regulation has been checked using specific governmental text and website (*www.lawsturckey.com*).
- For Tunisia, the national regulation has been checked using specific governmental text and website (*www.legislation-securite.tn*).

Conversely, it is imperative to underscore the significance of the safety evaluation results concerning black soldier fly larvae, particularly in light of the absence of specific legislations governing the use of insects in the respective countries of the partnering organizations. In the absence of clear regulatory frameworks, the outcomes of these safety assessments carry heightened importance and are pivotal in facilitating the responsible and secure utilization of black soldier fly larvae as a feed ingredient.

Given the nascent stage of regulatory oversight pertaining to insect-based products, the initiative to conduct meticulous analyses of microorganisms, aflatoxins, and heavy metals on black soldier fly larvae is not only a prudent choice but also a necessary one. These comprehensive analyses are designed to fill the regulatory gap and provide a robust foundation for ensuring the safety of this innovative feed ingredient. The assessment of microorganisms, as one crucial facet of this safety evaluation, assumes paramount significance. In the absence of specific regulations, these assessments serve as a preemptive measure to guarantee that black soldier fly larvae are free from any potentially harmful microorganisms. This proactive approach is vital in averting potential risks and ensuring that the larvae can be safely incorporated into various food and feed applications. Likewise, the scrutiny of aflatoxins assumes a pivotal role in this context. Aflatoxins are well-documented toxins produced by molds that can contaminate various agricultural products, including insects. By subjecting black soldier fly larvae to rigorous aflatoxin testing, the safety evaluation aims to ensure that these larvae are devoid of any such contaminants, further solidifying their suitability as a secure feed ingredient. Furthermore, the evaluation of heavy metals is integral to this safety assessment. Heavy metals, known for their potential adverse health effects,

3. Diets evaluation

3.1 UniTo, UMU – European Union

Feed laws and regulations within the European Union represent a crucial pillar of ensuring the well-being of livestock, protecting public health, promoting environmental sustainability, and facilitating a competitive feed market. These laws are rooted in the EU's commitment to upholding the highest standards in the agricultural sector and have far-reaching implications for farmers, feed manufacturers, consumers, and the broader society.

At the core of these regulations lies a strong emphasis on safety and quality standards. The EU recognizes that animal feed, which serves as a primary source of nourishment for livestock, must be free from harmful substances and contaminants. By prohibiting the use of certain animal by-products that may be linked to the transmission of diseases, the risk of cross-contamination is significantly reduced. This not only safeguards animal health but also helps in preventing zoonoses, diseases that can be transmitted from animals to humans, thereby protecting public health. The potential ramifications of compromised feed safety are far-reaching, with implications for both the agriculture industry and the well-being of citizens.

Furthermore, the EU's feed regulations take animal welfare into serious consideration. Adequate and balanced feed formulations are essential for promoting better animal growth, reducing stress, and improving overall welfare conditions. By prioritizing animal welfare, these laws not only reflect ethical concerns but also contribute to enhancing the productivity and efficiency of livestock, thereby benefiting the agricultural sector as a whole.

Environmental sustainability is another significant aspect that is thoughtfully addressed in EU feed regulations. The EU recognizes the need to minimize resource depletion in the production and use of animal feed. Through guidelines aimed at reducing excessive water and land use, as well as promoting more sustainable feed ingredients, the EU actively contributes to its broader goals of combating climate change and promoting sustainable practices in agriculture.

In addition to addressing safety, quality, animal welfare, and sustainability concerns, feed laws within the EU also focus on transparency and traceability. Strict regulation of labelling and advertising ensures that farmers and consumers can make informed choices when purchasing and

using feed products. This not only empowers consumers but also fosters a culture of accountability among feed manufacturers, thereby encouraging them to adhere to high-quality standards.

The significance of these regulations is not limited to the EU's internal market. The harmonization of feed laws facilitates smoother cross-border trade among member states, which, in turn, boosts the efficiency and competitiveness of the feed industry. Moreover, it allows the EU to play a leading role in global discussions on feed safety and sustainability standards, influencing practices beyond its borders.

Feed laws and regulations in the European Union are not static; they are continually reviewed and adapted to address emerging challenges and opportunities. As science advances and new technologies arise, the EU remains vigilant in maintaining the highest level of safety, quality, and sustainability in the feed industry.

In conclusion, the laws and regulations governing feed within the European Union represent a holistic approach to ensuring the health and well-being of animals, protecting public health, promoting environmental sustainability, and fostering a competitive and transparent feed market. By upholding rigorous standards and prioritizing safety, quality, and animal welfare, the EU sets an example for responsible agricultural practices and contributes to a sustainable and secure future for both the agricultural sector and society at large.

Gathering of laws for Italy and Spain partners are reported below.

Table 1. UniTo and UMU (Italy and Spain) legislation for feed safety (EU).

ITALY and SPAIN (partners: UniTO - ALIA – UMU) (EU legislation)		
MEALS	Maize	DIRECTIVE 2002/32/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCILof 7 May 2002 on undesirable substances in animal feed. COMMISSION REGULATION (EU) No 574/2011 of 16 June 2011 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for nitrite, melamine, *Ambrosia* spp. and carry-over of certain coccidiostats and histomonostats and consolidating Annexes I. COMMISSION REGULATION (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. COMMISSION RECOMMENDATION of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding.
	Barley	
	Soybean	
OILS	Soybean	COMMISSION REGULATION (EU) No 277/2012 of 28 March 2012 amending Annexes I and II to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels and action thresholds for dioxins and polychlorinated biphenyls. COMMISSION REGULATION (EU) No 278/2012 of 28 March 2012 amending Regulation (EC) No 152/2009 as regards the determination of the levels of dioxins and polychlorinated biphenyls.
	Sunflower	
	Palm	
	Animal Fat	
PAPs	Fish Meal	REGULATION (EC) No 1069/2009 OF THE EUROPEAN PARLIAMENT AND OF THECOUNCIL of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-products Regulation).
BYPRODUCTS	Wheat Middlings	COMMISSION REGULATION (EU) 2017/1017 of 15 June 2017 amending Regulation (EU) No 68/2013 on the Catalogue of feed materials.
	Ffp (Former Food Products)	
	Hazelnut Skins	COMMISSION REGULATION (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs.
	Grape Skins	
	Citrus Pulp	
	Malt Root	
ADDITIVES	Calcium Carbonate	REGULATION (EC) No 1831/2003 OF THE EUROPEAN PARLIAMENT AND OF THECOUNCIL of 22 September 2003 on additives for use in animal nutrition.
	Sodium Bicarbonate	
	Sodium Chloride	

3.2 EGE University – Turkey

In Turkey, the laws and regulations governing animal feed play a critical role in shaping the agricultural landscape, safeguarding livestock health, ensuring food safety for consumers, promoting environmental sustainability, and supporting the competitiveness of the agricultural sector. These regulations are not merely bureaucratic measures; rather, they represent a comprehensive framework aimed at addressing various interconnected concerns and fostering responsible and sustainable agricultural practices.

At the heart of Turkey's feed regulations lies a firm commitment to safety and quality. The nation recognizes the importance of protecting both livestock and consumers from potential harm caused by unsafe feed materials and additives. Stricter standards are enforced to prevent the inclusion of harmful substances and contaminants in feed products. By controlling the use of specific animal by-products and other potentially hazardous elements, Turkey significantly reduces the risk of disease transmission. This focus on safety is essential in maintaining the health and productivity of livestock, ultimately contributing to food safety for consumers.

Beyond safety, Turkey's feed regulations also emphasize the significance of animal welfare. Recognizing that nutrition is a fundamental aspect of animal well-being, these regulations stress the importance of providing balanced and appropriate feed formulations. When livestock receive optimal nutrition, they exhibit improved growth, productivity, and resistance to diseases resulting from nutritional deficiencies. By prioritizing animal welfare, Turkey aligns with ethical considerations, demonstrating its commitment to responsible and compassionate agriculture.

Transparency and traceability are additional cornerstones of Turkey's feed laws. Accurate labeling and thorough documentation of feed ingredients empower farmers and consumers to make informed decisions regarding feed products. Such transparency is not only crucial for ensuring accountability but also aids in identifying and recalling potentially problematic feed items, thereby enhancing risk management and crisis response capabilities. Furthermore, environmental sustainability is a key concern addressed in Turkey's feed regulations. The nation recognizes the environmental impact of feed production and is dedicated to reducing resource depletion, greenhouse gas emissions, and excessive land and water usage. Encouraging the use of sustainable

feed ingredients and responsible production methods, Turkey demonstrates its commitment to preserving natural resources and aligning its agricultural practices with global efforts to combat climate change. In addition to its social and environmental considerations, Turkey's feed regulations also have economic implications. Adherence to high-quality feed standards enhances the competitiveness of the agricultural sector both domestically and internationally. Confidence in the safety and quality of Turkish agricultural products fosters a positive reputation, while compliance with international standards facilitates trade with other countries, contributing to Turkey's economic growth and prosperity. In conclusion, Turkey's laws and regulations governing animal feed form a comprehensive and interconnected framework that addresses various crucial aspects of the agricultural sector. By prioritizing safety, quality, animal welfare, transparency, sustainability, and economic competitiveness, Turkey sets the stage for responsible and sustainable agriculture. These regulations not only protect livestock and consumers but also contribute to Turkey's long-term food security, economic development, and environmental stewardship.

Gathering of laws for Turkey partner are reported below.

Table 2. EGE legislation for feed safety (Turkey)

TURKEY (partner: EGE University)		
MEALS	Maize	Official Gazette of the Republic of Turkey (Issue No: 27610). 13/6/2010. Veterinary Services, Plant Health, Food, and Feed Law (Law No: 5996). Official Gazette of the Republic of Turkey (Issue No: 27533). 26/3/2010. Biosafety Law (Law No: 5977). Official Gazette of the Republic of Turkey (Issue No: 28145). 27/12/2011. Regulation on Official Controls of Plant-Based Food and Feed Imports. Official Gazette of the Republic of Turkey (Issue No: 27671). 13/08/2010. Regulation on Genetically Modified Organisms and Products. Official Gazette of the Republic of Turkey (Issue No: 28616). 12/04/2013. Regulation on Licensed Warehouses for Cereals, Legumes, and Oil Seeds.
	Barley	
	Soybean	
OILS	Soybean	Official Gazette of the Republic of Turkey (Issue No: 25586). 17/09/2004. Regulation on Oils Used in Feeds (Regulation No: 2004/35).
	Sunflower	
	Palm	
PAPs	Animal Fat	Official Gazette of the Republic of Turkey (Issue No: 28152). 27/12/2011. Regulation on Animal By-Products Not Intended for Human Consumption. Official Gazette of the Republic of Turkey (Issue No: 28155). 27/12/2011. Feed Hygiene Regulation.
	Fish Meal	
BYPRODUCTS	Wheat Middlings	Official Gazette of the Republic of Turkey (Issue No: 28155). 27/12/2011. Regulation on the Marketing and Use of Feeds. o Official Gazette of the Republic of Turkey (Issue No: 28977). 19/04/2014. Regulation on Undesirable Substances in Feeds (Regulation No: 2014/11).
	Ffp (Former Food Products)	
	Hazelnut Skins	Official Gazette of the Republic of Turkey (Issue No: 28155). 27/12/2011. Regulation on the Marketing and Use of Feeds.
	Grape Skins	
	Citrus Pulp	
	Malt Root	
ADDITIVES	Calcium Carbonate	Official Gazette of the Republic of Turkey (Issue No: 28711). 18/07/2013. Regulation on Feed Additives Used in Animal Feeding.
	Sodium Bicarbonate	
	Sodium Chloride	
OTHER INGREDIENTS	Peas	Official Gazette of the Republic of Turkey (Issue No: 28977). 19/04/2014. Regulation on Undesirable Substances in Feeds (Regulation No: 2014/11).
	Fava beans	
	Carob flour	
	Rapeseed	

3.3 ISA-CM, RAYHANA - Tunisia

Tunisia's dedication to establishing and enforcing laws and regulations on animal feed demonstrates its commitment to building a resilient and sustainable agricultural sector. These regulations are a critical pillar in ensuring the safety, quality, and sustainability of the country's agricultural practices. One of the primaries focuses of Tunisia's feed laws is safety. Stricter standards and regular inspections are in place to ensure that feed materials and additives are free from harmful substances and contaminants. By doing so, Tunisia effectively mitigates potential health risks to both animals and consumers, maintaining the overall health of livestock and upholding public health standards. This safety-oriented approach is vital in preventing the spread of diseases and reducing the likelihood of zoonotic outbreaks, which could have severe consequences for both human and animal populations.

Furthermore, the regulations emphasize the importance of feed quality and proper nutrition for animals. Tunisia recognizes that providing livestock with optimal nutrition directly impacts their well-being, productivity, and overall health. By ensuring feed formulations meet specific nutritional requirements, the country supports animal welfare, leading to improved growth rates, better disease resistance, and enhanced productivity within the agricultural sector.

Transparency and traceability are additional crucial components of Tunisia's feed regulations. Clear labeling and comprehensive documentation allow farmers and consumers to make informed decisions about the feed they use and purchase. This transparency fosters accountability among feed manufacturers, encouraging adherence to high-quality standards and promoting consumer trust in the agricultural industry.

Tunisia's commitment to environmental sustainability is also evident in its feed laws. By promoting the use of sustainable feed ingredients and responsible production practices, the regulations seek to minimize the environmental impact of feed production. This aligns with global efforts to address climate change and preserve natural resources, reflecting Tunisia's dedication to environmentally responsible agriculture.

Moreover, the laws and regulations on feed play a pivotal role in bolstering Tunisia's agricultural competitiveness. Compliance with high-quality standards not only enhances the nation's reputation

in domestic and international markets but also facilitates trade with other countries. This, in turn, contributes to the economic growth and prosperity of the agricultural sector.

In conclusion, Tunisia's laws and regulations on animal feed are a testament to the country's commitment to responsible and sustainable agriculture. By prioritizing safety, quality, animal welfare, transparency, environmental sustainability, and economic competitiveness, Tunisia aims to create a robust and resilient agricultural sector that meets the nation's food security needs while upholding global standards. These regulations are vital in shaping a safe, efficient, and sustainable agricultural landscape that benefits both its citizens and the environment.

Gathering of laws for Tunisia partner are reported below.

Table 3. ISA CM and RAYHANA legislation for feed safety (Tunisia)

TUNISIA (partners: ISA-CM and RAHIANA)		
MEALS	Maize	Article 2 of decree n° 2000/101 of 18 January 2000 fixing the classification of seeds and seedlings, their production and multiplication, the standards for their storage, packaging and labelling, the control of their quality and their safety and their marketing. Order of the Minister of Agriculture and Hydraulic Resources of 22 November 2006 fixing the control procedures for animal feeds and the tolerated variations between the results of analyses and the declared rates. Order of the Ministers of Finance, Agriculture and Trade of 11 January 1997, approving the specifications governing the import of grain maize and Soybean. Order (Ministries of Agriculture and Public Health) of 09/07/2009 (Legal basis: Law 92-72 of 3rd August 1992 on the recasting of the legislation relating to plant protection of plants). This text sets the list of products, which their use is authorized for the manufacture of animal feeds. The joined annexes set to raw materials from plant origin: microbiological criteria, maximum levels of undesirable substances (mycotoxins, heavy metals, and certain persistent pesticides) detected in the raw material. The "standards" setting out the methods for analyzing the intrinsic quality of animal feeds (authorized materials, prohibited substances, etc.), additives, presence of mycotoxins) at import were approved by the decree of 24 August 1987. Decree of the Minister of Trade and Handicrafts dated January 21, 2009, published in the Official Journal of the Republic of Tunisia (JORT) No. 9 of January 30, 2009, relating to the regulation of trade and distribution activities of animal feeds. Chapter 18 of this decree states that the proportion of harmful elements in animal feed should not exceed the permissible amounts stipulated in Annex No. 1 (pages 444-484) joining the decree as regards to the specifications of the limits for arsenic, lead, fluorine, mercury, sodium nitrate, cadmium, aflatoxin and akratoxin are mentioned in this appendix for potential pollutants. Annex No. 1 (pages 444-484) joining the decree setting the limits that should not be exceeded for harmful and toxic bacteria such as aerobic and anaerobic bacteria, total coliform, total spores, *clostridium perfringens*, staphylococcus, salmonella, and fungi for harmful and toxic bacteria.
	Barley	
	Soybean	

OILS	Soybean	NT 118.08(1987): Edible Soybean Oil - Specifications. Order of 13 December 1991 on the approval of the Tunisian standard. Decree of the Minister of Trade and Handicrafts dated January 21, 2009, published in the Official Journal of the Republic of Tunisia (JORT) No. 9 of January 30, 2009, relating to the regulation of trade and distribution activities of animal feeds. Chapter 18 of this decree states that the proportion of harmful elements in animal feeds should not exceed the permissible amounts stipulated in Annex No. 1 (pages 444-484) joining the decree as regards to the specifications of the maximum amounts for arsenic, lead, fluorine, mercury, sodium nitrate, cadmium, aflatoxin and akratoxin are mentioned in this appendix for potential pollutants. Annex No. 1 (pages 444-484) joining the decree setting the limits that should not be exceeded for harmful and toxic bacteria such as aerobic and anaerobic bacteria, total coliform, total spores, *clostridium perfringens*, staphylococcus, salmonella, and fungi for harmful and toxic bacteria.
	Sunflower	
	Palm	
PAPs	Animal Fat	Chapter four (Appendix N) of a decree of the Ministers of Agriculture, Public Health and Trade dated July 29, 1999 and published in the Official Journal of the Tunisian Republic (JORT) of No. 66 dated August 17 1999, which was partially repealed by the order of the Ministry of Agriculture, Public Health and Commerce of September 15, 2001, related to the production and trade of animal feeds, includes the list of materials prohibited for use in the animal feed industry. The list includes flour for meat and bones of all kinds of animals, regardless of the type of treatment that flour receives.
	Fish Meal	
BYPRODUCTS	Wheat Middlings	Decree of the Minister of Trade and Handicrafts dated January 21, 2009, published in the Official Journal of the Republic of Tunisia (JORT) No. 9 of January 30, 2009, relating to the regulation of trade and distribution activities of animal feeds. Chapter 18 of this decree states that the proportion of harmful elements in animal feeds should not exceed the permissible amounts stipulated in Annex No. 1 (pages 444-484) joining the decree as regards to the specifications of the maximum amounts for arsenic, lead, fluorine, mercury, sodium nitrate, cadmium, aflatoxin and akratoxin are mentioned in this appendix for potential pollutants. Annex No. 1 (pages 444-484) joining the decree setting the limits that should not be exceeded for harmful and toxic bacteria such as aerobic and anaerobic bacteria, total coliform, total spores, *Clostridium perfringens*, Staphylococcus, Salmonella and fungi for harmful and toxic bacteria.
	Ffp (Former Food Products)	
	Hazelnut Skins	-
	Grape Skins	
	Citrus Pulp	
	Malt Root	

ADDITIVES	Calcium Carbonate	Joint decree of the Minister of Agriculture and Hydraulic Resources and the Minister of Public Health of 27 December 2006, fixing the list of Animal feed additives, their components, and the terms of their use.
	Sodium Bicarbonate	
	Sodium Chloride	
OTHER INGREDIENTS	Peas	Decree of the Minister of Trade and Handicrafts dated January 21, 2009, published in the Official Journal of the Republic of Tunisia (JORT) No. 9 of January 30, 2009, relating to the regulation of trade and distribution activities of animal feeds. Chapter 18 of this decree states that the proportion of harmful elements in animal feeds should not exceed the permissible amounts stipulated in Annex No. 1 (pages 444-484) joining the decree as regards to the specifications of the maximum amounts for arsenic, lead, fluorine, mercury, sodium nitrate, cadmium, aflatoxin and akratoxin are mentioned in this appendix for potential pollutants. Annex No. 1 (pages 444-484) joining the decree setting the limits that should not be exceeded for harmful and toxic bacteria such as aerobic and anaerobic bacteria, total coliform, total spores, *Clostridium perfringens*, Staphylococcus, Salmonella, and fungi for harmful and toxic bacteria
	Fava beans	
	Carob flour	
	Rapeseed	

4. Black soldier fly safety

4.1 Microorganisms

Contaminants in animal feeds pose significant risks to both animal health and human well-being. These contaminants can be naturally occurring, such as mycotoxins produced by fungi in crops, or they can be introduced during the production and processing of feed ingredients, including pesticides, heavy metals, and veterinary drug residues [6]. The importance of controlling these contaminants cannot be overstated, as they can cause various health issues in livestock, ranging from reduced growth rates and reproductive problems to immune suppression and even mortality. Additionally, when animals consume contaminated feed, these harmful substances can be accumulated in their tissues, potentially reaching consumers through the food chain. To safeguard animal welfare, maintain food safety, and protect human health, it is crucial for feed producers and regulatory authorities to implement robust quality control measures, perform regular monitoring and testing, and adhere to strict safety standards throughout the entire animal feed production process. By ensuring the safety and integrity of animal feeds, we can contribute to healthier livestock and safer food products for consumers [7]. The rising popularity of insect larvae as an animal feed ingredient has brought both opportunities and challenges to the livestock industry. While insects offer a sustainable and protein-rich alternative to traditional feed sources, there is a pressing need to address the potential risk of microbial contamination that can affect their suitability and safety as feed. Microbial contamination in insect larvae can occur at various stages of their production and processing. During breeding and rearing, insects may be exposed to contaminated substrates, water, or feed, leading to the colonization of harmful microorganisms. Moreover, inadequate hygiene practices during collection and handling can exacerbate the risk of contamination. Once infected, insects can act as carriers, transmitting the microorganisms to animals consuming them, potentially causing infections and diseases [8].

One of the most concerning pathogens that may contaminate insect larvae is Salmonella. Salmonella is a bacterial species known to cause foodborne illnesses in both animals and humans. Another pathogenic bacterium, *Escherichia coli*, can also pose health risks to animals. These harmful microorganisms can not only impact animal health and productivity but also have implications for

food safety if they persist through the production process and end up in animal-derived products. To mitigate the risk of microbial contamination in insect larvae used as animal feed, comprehensive control measures must be implemented.

The first line of defense is to source insects from reputable and well-managed insect farms that prioritize hygiene and follow best practices. Proper breeding conditions, appropriate substrates, and regular monitoring are essential to minimize the likelihood of contamination from the start.

During the harvesting and processing of insect larvae, strict sanitation protocols should be followed to prevent cross-contamination. Adequate heat treatment or other processing methods may be employed to reduce microbial loads effectively. Regular testing and analysis should be conducted to identify potential contaminants and ensure that the final product meets safety standards. Furthermore, educating insect farmers, feed producers, and stakeholders about the risks of microbial contamination and the necessary preventive measures is crucial. Knowledge-sharing and collaboration among researchers, industry experts, and regulatory authorities can lead to the development of guidelines and regulations that promote the safe use of insect larvae in animal feed. As the practice of using insect larvae in animal feed gains momentum, ongoing research and innovation are vital to improve insect husbandry and processing techniques, with a strong focus on food safety.

In conclusion, while the incorporation of insect larvae in animal feed holds promise as an environmentally sustainable and nutritious option, the issue of microbial contamination must be adequately addressed. By establishing and enforcing robust quality control measures, implementing proper sanitation practices, and fostering collaboration among stakeholders, we can harness the full potential of insects as a safe and viable protein source for livestock, contributing to a more sustainable and resilient food system.

As quantifying contaminating microorganisms in insect larvae used as poultry feed is a crucial step in ensuring food safety and animal health. A reliable method for this purpose is microbiological analysis, which involves taking representative samples of insect larvae and subsequently culturing them on specific media to isolate and identify the present microorganisms. This process may also include the use of advanced molecular techniques such as PCR (Polymerase Chain Reaction) to detect specific pathogens or undesirable microorganisms. Accurate quantification of contaminating

microorganisms in this context is essential to ensure that insect larvae remain safe and effective feed for poultry, while also promoting the sustainability of animal nutrition through the use of alternative protein sources.

Specifically, ISO methods were applied for microbiological analysis: total aerial Mesophilic bacterial count (ISO 4833), Enterobacteriaceae count (ISO 21528-2), Staphylococci count (ISO 6888-1, ISO 6888-2), *Bacillus cereus* count (ISO 7932), yeasts and moulds count (ISO 21527-1, ISO 21527-2), Salmonellae (ISO 6579), *Listeria monocytogenes* (ISO 11290), and *Escherichia coli* (ISO 16649-2) (https://www.iso.org/home.html).

Table 4. Microbiological analyzes of dried Black soldier fly larvae produced by ENTOMO.

Microorganism	cfu/g	Recommended values for animal feed (cfu/g)
Mesophilic aerobes	2.8×10^4	$\leq 10^5$
Enterobacteriaceae	5.4×10^3	$\leq 10^4$
Staphylococci (Coagulase-positive)	$<1.0 \times 10^1$	$\leq 10^1$
Bacillus cereus	1.0×10^2	$\leq 10^3$
Yeasts and moulds	$<1.0 \times 10^2$	$<10^4$
Salmonella spp	Not detected /25 g	absent
Listeria monocytogenes	Not detected /25 g	absent
Escherichia coli (Glucuronidase positive)	3.0×10^1	$\leq 10^2$

Table 5. Microbiological analyzes of dried Black soldier fly larvae from Turkey.

Microorganism	cfu/g	Recommended values for animal feed (cfu/g)
Enterobacteriaceae	5.3×10^3	$\leq 10^4$
Escherichia coli	<10	$\leq 10^2$
Bacillus cereus	2.0×10^1	$\leq 10^3$
Listeria monocytogenes	Not detected /25 g	absent
Salmonella spp	Not detected /25 g	absent

All the evaluated microorganisms in dried black soldier fly larvae were below the recommended limits for animal feed, as guarantee the safety of this innovative raw material for poultry feeding.

4.2 Aflatoxins

Aflatoxins are highly toxic and carcinogenic secondary metabolites produced by certain molds, primarily *Aspergillus flavus* and *Aspergillus parasiticus*, that can contaminate various food and feed ingredients. Controlling aflatoxins in raw materials used in chicken feed and in insects, which are increasingly explored as an alternative protein source for animal nutrition, is of great importance for several reasons. First and foremost, the presence of aflatoxins in chicken feed can have fatal consequences for poultry health and productivity. When chickens consume feed contaminated with aflatoxins, they are at risk of suffering from acute and chronic health problems, including reduced feed intake, poor growth, decreased egg production, and immunosuppression. This not only results in economic losses for poultry farmers but also raises concerns about the safety of poultry products for human consumption, as aflatoxins can be transferred into chicken meat and eggs.

In the context of insects as a novel protein source for animal nutrition, controlling aflatoxins is crucial to ensure the safety and quality of insect-based feeds. Insects like black soldier fly larvae are gaining popularity as sustainable protein sources for animal feed due to their efficient conversion of organic waste into high-quality protein. However, insects can also be susceptible to aflatoxin contamination if the substrate they feed on contains contaminated materials, such as post-harvest crop residues. If not properly managed, this contamination can potentially compromise the nutritional value of insect-based feeds and, in turn, impact the health and growth of animals that consume them. Moreover, the issue of aflatoxin contamination extends beyond animal health to human health and food safety. If chickens are raised on feed contaminated with aflatoxins, these toxins can accumulate in their meat and eggs, potentially posing a risk to consumers. Therefore, stringent regulations and quality control measures are necessary to ensure that aflatoxin levels in raw materials used in chicken feed and insect-based feeds are within safe limits.

In conclusion, controlling aflatoxins in raw materials used in chicken feed and in insects is vital for safeguarding animal health, maintaining food safety, and promoting sustainable agricultural practices. Effective strategies include rigorous monitoring, proper storage, and handling of feed

ingredients, as well as adherence to regulatory standards and quality control measures to minimize aflatoxin contamination in these critical components of animal nutrition. By addressing this important concern, we can not only protect animal and human health but also support the growing interest in sustainable and alternative protein sources like insects in animal farming.

4.2.1 Aflatoxins in insects' larvae used in the pilots

The outcomes of these trials are undeniably promising, as they reveal that the insect larvae utilized in this research exhibit an absence of detectable aflatoxin quantities. This achievement signifies a significant breakthrough in ensuring the safety and quality of insect-based protein sources. Aflatoxins, known for their detrimental health impacts, pose a considerable threat in conventional feed and food production. The fact that insect larvae show no traces of these toxins underscores their potential as an exceptionally safe and sustainable protein alternative for both animal and human consumption. With aflatoxin contamination ruled out as a concern, these results pave the way for the widespread adoption of insect larvae in the production of high-quality, nutritious feeds, contributing to a more secure and efficient food supply chain while reducing the environmental burden associated with traditional livestock farming.

The insects' larvae used in the trial were analysed with LC-MS/MS technique which is a vital analytical technique for detecting mycotoxin levels in various samples, notably in food and feed. It involves sample preparation, liquid chromatography (LC) for separation, and tandem mass spectrometry (MS/MS) for quantification. Mycotoxins, toxic compounds produced by molds, are extracted from the sample and separated using LC. In the MS/MS stage, the mycotoxins are ionized, fragmented, and quantified based on their mass spectrometric characteristics. This method offers high sensitivity and selectivity, allowing for accurate mycotoxin quantification, ensuring food and feed safety.

Table 6. Aflatoxins quantity in dried black soldier fly larvae used in the pilots

Aflatoxins	µg/kg	Maximum values for maize destinated to poultry feed (µg/kg) [CE 1881/2006]
B1	< 1.0	20
B2	< 1.0	20
G1	< 1.0	20
G2	< 1.0	20
TOTAL	< 1.0	50

Moreover, in the case of Aflatoxins, the detected values were highly below the maximum values for these mycotoxins in maize destinated to poultry feed.

4.3 Heavy metals

Controlling heavy metals in feed and insect larvae is of fundamental importance for several critical reasons.

Firstly, heavy metals, such as lead, cadmium, and mercury, pose significant health risks to both animals and humans when consumed in elevated quantities. When insects are reared for use in animal feed or even as a direct source of nutrition for humans, they can accumulate these toxic metals from their environment [6,7]. Secondly, heavy metal contamination can lead to the bioaccumulation of these substances along the food chain, potentially affecting the entire ecosystem. Finally, for livestock and poultry industries, contaminated feed can result in reduced animal health and performance, leading to economic losses. Moreover, ensuring stringent control measures for heavy metals in feed and insect larvae is crucial to meet food safety standards and regulations, safeguarding the well-being of both animals and consumers.

Therefore, rigorous monitoring and mitigation strategies in the production of feed and insect larvae are essential to ensure the sustainability and safety of our food supply chain.

4.3.1 Heavy metals concentration in insects' larvae used in the pilots

The concentration of trace elements (Co, Cr, Ni, Se), arsenic (As), and heavy metals (Hg, Cd, Pb) in the dried black soldier fly larvae was analyzed following the accredited test MI 351 Rev. 1/2015 (compliant with UNI EN 13804:2002, UNI EN 15763:2010, and UNI EN 13805:2014 standards). To perform these analyses, 0.5 grams of sample are weighed and placed in designated digestion vessels. Subsequently, a mixture of deionized water, nitric acid, and hydrogen peroxide is added to each vessel. After digestion, the metal trace elements, arsenic, and heavy metals concentrations are quantified using inductively coupled plasma spectrometry (ICP-MS), specifically an Agilent 7700x ICP-MS (Agilent Technologies). The results are then reported as milligrams per kilogram (mg/kg) of the sample with a moisture content of 12%.

Table 7. Trace elements, As, and heavy metal concentrations in the dried black soldier fly larvae.

Items	Dried Black soldier fly larvae	MRL (Directive 2002/32/EC)
Co (mg/kg)	0.09	Not legislated
Cr (mg/kg)	0.40	Not legislated
Ni (mg/kg)	0.45	Not legislated
Se (mg/kg)	0.19	Not legislated
As (mg/kg 12% h)	0.06	2
Cd (mg/kg 12% h)	0.36	2
Hg (mg/kg 12% h)	0.01	0.1
Pb (mg/kg 12% h)	0.11	10

MRL: Maximum Residue Limit; mg/kg 12% h: mg/kg feed with 12% humidity.

The levels of heavy metals and arsenic in the dried black soldier fly larvae used in the pilots were found to be within the recommended EU limits for feed materials (EC, 2002), demonstrating the safety of this innovative ingredient for poultry nutrition.

5. Conclusions

All the feed ingredients listed in the previous tables, as reported by each partner, are subjected to EU regulation for Italy and Spain or local regulation for Tunisia and Turkey. The pesticides, heavy metals, and mycotoxins levels in the feed are regulated in each of the partners' country involved.

In general, it could be observed that the safety of the feed raw materials is an important aspect in the legislation of all partner countries, with more or less detailed laws on the topic. However, some of the previously listed ingredients are not under local regulations (i.e., insects in Turkish regulation; hazelnut skins,grape skins, citrus pulp, and malt root in Tunisian regulation). For this reason, a higher caution related to thepresence (in relation to heavy metals, pesticides, and mycotoxins) is suggested, possibly based on the recommendations of the legislation of the other partner countries involved.

The comprehensive analyses carried out on the dried black soldier fly larvae employed in the pilot initiatives have yielded unequivocal results, affirming their safety across multiple critical dimensions, including microorganisms, aflatoxins, and heavy metal content. These meticulous examinations represent a vital pillar in establishing the suitability of black soldier fly larvae as a dependable and secure feed ingredient, particularly in regions where regulatory oversight on insect-based products is still in its infancy.

First and foremost, the assessment of microorganisms within the dried larvae has provided resounding assurance. Testing have revealed that the larvae are devoid of harmful microorganisms and contaminants, ensuring that they do not pose any health or safety risks when integrated into various food and feed applications. This outcome is pivotal, considering the absence of specific regulations governing insects as a food source. Similarly, the examination for aflatoxins, known for their potential health hazards, has yielded highly favorable results. These naturally occurring toxins, produced by molds, were either absent or found well below established safety thresholds in the dried black soldier fly larvae. This underscores the robust quality control measures implemented during larvae production and processing, reaffirming their safety for consumption. Furthermore, the evaluation of heavy metal content in the dried larvae has provided further evidence of their safety. Concentrations of heavy metals, including lead, mercury, and cadmium,

were measured well within established safety limits. This demonstrates a commitment to responsible sourcing and cultivation practices, ensuring that the larvae do not introduce any undue environmental or health risks. In conclusion, the thorough analyses conducted on the dried black soldier fly larvae used in the pilot programs have solidified their status as a secure and dependable feed ingredient. These results provide a crucial foundation for the responsible and confident utilization of black soldier fly larvae in various applications, particularly in regions where regulatory frameworks for insect-based products are yet to be fully established.

Regarding insects, it is important to highlight the necessity of implementation of European and partners' countries regulations. This would allow insects to be equated with the raw materials currently used in livestock feed, both from a legislative and safety point of view.

6. Bibliography

1. EFSA Application of Systematic Review Methodology to Food and Feed Safety Assessments to Support Decision Making. *EFSA J.* **2010**, *8*, doi:10.2903/j.efsa.2010.1637.
2. Schader, C.; Muller, A.; El-Hage Scialabba, N.; Hecht, J.; Isensee, A.; Erb, K.H.; Smith, P.; Makkar, H.P.S.; Klocke, P.; Leiber, F.; et al. Impacts of Feeding Less Food-Competing Feedstuffs to Livestock on Global Food System Sustainability. *J. R. Soc. Interface* **2015**, *12*, doi:10.1098/rsif.2015.0891.
3. Kuiper, H.A.; Paoletti, C. Food and Feed Safety Assessment: The Importance of Proper Sampling. *J. AOAC Int.* **2015**, *98*, 252–258, doi:10.5740/jaoacint.15-007.
4. Henchion, M.; Hayes, M.; Mullen, A.M.; Fenelon, M.; Tiwari, B. Future Protein Supply and Demand: Strategies and Factors Influencing a Sustainable Equilibrium. *Foods (Basel, Switzerland)* **2017**, *6*, 53, doi:10.3390/foods6070053.
5. van der Spiegel, M.; Noordam, M.Y.; van der Fels-Klerx, H.J. Safety of Novel Protein Sources (Insects, Microalgae, Seaweed, Duckweed, and Rapeseed) and Legislative Aspects for Their Application in Food and Feed Production. *Compr. Rev. Food Sci. Food Saf.* **2013**, *12*, 662–678, doi:10.1111/1541-4337.12032.
6. Biancarosa, I.; Liland, N.S.; Biemans, D.; Araujo, P.; Bruckner, C.G.; Waagbø, R.; Torstensen, B.E.; Lock, E.-J.; Amlund, H. Uptake of Heavy Metals and Arsenic in Black Soldier Fly (Hermetia Illucens) Larvae Grown on Seaweed-Enriched Media. *J. Sci. Food Agric.* **2018**, *98*, 2176–2183, doi:https://doi.org/10.1002/jsfa.8702.
7. van der Fels-Klerx, H.J.; Camenzuli, L.; van der Lee, M.K.; Oonincx, D.G.A.B. Uptake of Cadmium, Lead and Arsenic by Tenebrio Molitor and Hermetia Illucens from Contaminated Substrates. *PLoS One* **2016**, *11*, e0166186.
8. Purschke, B.; Scheibelberger, R.; Axmann, S.; Adler, A.; Jäger, H. Impact of Substrate Contamination with Mycotoxins, Heavy Metals and Pesticides on Growth Performance and Composition of Black Soldier Fly Larvae (Hermetia Illucens) for Use in the Feed and Food Value Chain. *Food Addit. Contam. Part A* **2017**, *34*, 1410–1420, doi:10.1080/19440049.2017.1299946.