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TESI DI DOTTORATO DI RICERCA
# FOOD AND FEED SAFETY IN THE CIRCULAR ECONOMY

CHEM-07/B – AGRI-09/B

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# TABLE OF CONTENTS

# ABSTRACT

This thesis addresses the valorization of agri food co/by-products and former food products (FFPs) for their introduction into animal feed within a circular economy framework. Chapter 1 validated an HPLC-UV method for quantifying theobromine and caffeine, and assessed cocoa hulls (CHs) compliance with EU feed limits, while also characterising polyphenols and antioxidant potential; Chapter 2 profiled FFPs for nutrient composition and methylxanthine levels to estimate methylxanthines exposure in target animals and provide a safety assessment for FFP inclusion in animal diets; Chapter 3 evaluated hemp (*Cannabis sativa* L.) co-products for total phenolic content (TPC), antioxidant activity following green solvent extraction and ex-vivo digestion, and identified main phenolic compounds by HPTLC; and Chapter 4 produced a proposal for a harmonized feed-consumption database built on a standardized feed classification, including a model data structure and two applied case studies (genetically modified feed and contaminants). Results show that CHs exhibit positive polyphenol-linked antioxidant activity, but also contain theobromine at levels that constrain safe inclusion rates in complete feeds. FFPs were confirmed as nutritionally valuable and generally compliant with EU maximum limits when included at practical levels in livestock diets. However, species and stage-specific adjustments are required to avoid methylxanthine overexposure. Hemp co-products showed appreciable TPC and in vitro/ex vivo antioxidant activity, with extraction solvent and digestion affecting bioaccessibility. The database proposal demonstrates feasibility and practical value for harmonised exposure assessments. Overall, the findings support targeted and regulated reuse of agri food co/by-products and FFPs, but also highlight toxicological monitoring needs. A recommendation for the development of a harmonised animal consumption database is also provided for a potentially more harmonised risk assessment, which could also facilitate the safety assessment of circular feed.

# RIASSUNTO

Questo lavoro di tesi affronta la valorizzazione dei co-prodotti/sottoprodotti agroalimentari e degli ex-prodotti alimentari (FFPs), per la loro potenziale introduzione nella mangimistica all'interno di un modello di economia circolare. Nel Capitolo 1 è stato convalidato un metodo HPLC-UV per la quantificazione di teobromina e caffeina e valutata la conformità delle bucce di cacao (CHs) ai limiti previsti dalla normativa europea per i mangimi, caratterizzandone al contempo il profilo polifenolico e il potenziale antiossidante; nel Capitolo 2 sono stati descritti il profilo nutrizionale dei FFPs e i livelli di metilxantine, stimando l'esposizione negli animali target e fornendo una valutazione di sicurezza in vista della loro inclusione nelle diete animali; nel Capitolo 3 sono stati analizzati i co-prodotti della canapa (*Cannabis sativa* L.) in termini di contenuto fenolico totale (TPC) e attività antiossidante, valutata dopo estrazione con solventi green e a seguito di digestione ex vivo, con l'identificazione dei principali composti fenolici mediante HPTLC; nel Capitolo 4 è stata sviluppata una proposta per un database armonizzato sul consumo di mangimi, basato su una classificazione standardizzata, fornendo una struttura di data model e due casi studio applicativi (mangimi geneticamente modificati e contaminanti). I risultati evidenziano che i CHs presentano un'attività antiossidante legata ai polifenoli, ma anche contenuti di teobromina che ne limitano i livelli sicuri di inclusione nei mangimi. I FFPs sono risultati nutrizionalmente validi e, nella maggior parte dei casi, conformi ai limiti massimi previsti dall'UE, purché utilizzati a livelli controllati e con opportune considerazioni specifiche per specie e fase produttiva, al fine di prevenire rischi di sovraesposizione alle metilxantine. I co-prodotti della canapa hanno mostrato un contenuto fenolico e un'attività antiossidante apprezzabili, con bioaccessibilità influenzata dal tipo di solvente utilizzato per l'estrazione e dai processi digestivi ex-vivo. Infine, la proposta per lo sviluppo di un database armonizzato sul consumo di mangimi ha dimostrato la sua fattibilità e utilità pratica per migliorare le valutazioni di esposizione e sicurezza. Nel complesso, i risultati sostengono il riutilizzo mirato e regolamentato di co-/sottoprodotti agroalimentari ed ex-prodotti alimentari nella mangimistica, evidenziando al contempo la necessità di un accurato monitoraggio. Infine, viene fornita una raccomandazione per lo sviluppo di un database sul consumo dei mangimi, come supporto per valutazioni del rischio più accurate, con un potenziale applicativo anche al fine di facilitare la valutazione della sicurezza dei mangimi derivanti da pratiche circolari.

# GENERAL INTRODUCTION

## Transforming the global agrifood system for a sustainable future

The global agrifood systems are facing significant challenges. A growing population and the need for preservation of resources have led to a bigger need to transition from a linear and resource-intensive production model to a circular and sustainable alternative (Pomoni et al., 2024).

In 2015, the General Assembly of the United Nations (UN) adopted resolution 70/1, named "Transforming our world: the 2030 Agenda for Sustainable Development". In this historic resolution, the UN General Assembly adopted a comprehensive set of universal and transformative Goals and targets and committed to the full implementation of the Agenda by 2030 (United Nations, 2015). The Agenda comprises 17 Sustainable Development Goals (SDGs) with 169 associated targets (Table 1).

Table 1. Sustainable Development Goals (adapted from United Nations, 2015)

| | SDGs | | |
|---|---|---|---|
| 1 NO POVERTY | End poverty in all its forms everywhere | 10 REDUCED INEQUALITIES | Reduce inequality within and among countries |
| 2 ZERO HUNGER | End hunger, achieve food security and improved nutrition and promote sustainable agriculture | 11 SUSTAINABLE CITIES AND COMMUNITIES | Make cities and human settlements inclusive, safe, resilient and sustainable |
| 3 GOOD HEALTH AND WELL-BEING | Ensure healthy lives and promote well-being for all at all ages | 12 RESPONSIBLE CONSUMPTION AND PRODUCTION | Ensure sustainable consumption and production patterns |
| 4 QUALITY EDUCATION | Ensure inclusive and equitable quality education and promote lifelong learning opportunities for all | 13 CLIMATE ACTION | Take urgent action to combat climate change and its impacts |
| 5 GENDER EQUALITY | Achieve gender equality and empower all women and girls | 14 LIFE BELOW WATER | Conserve and sustainably use the oceans, seas and marine resources for sustainable development |

| | | | |
|---|---|---|---|
| 6 CLEAN WATER AND SANITATION | Ensure availability and sustainable management of water and sanitation for all | 15 LIFE ON LAND | Protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, and halt and reverse land degradation and halt biodiversity loss |
| 7 AFFORDABLE AND CLEAN ENERGY | Ensure access to affordable, reliable, sustainable and modern energy for all | 16 PEACE, JUSTICE AND STRONG INSTITUTIONS | Promote peaceful and inclusive societies for sustainable development, provide access to justice for all and build effective, accountable and inclusive institutions at all levels |
| 8 DECENT WORK AND ECONOMIC GROWTH | Promote sustained, inclusive and sustainable economic growth, full and productive employment and decent work for all | 17 PARTNERSHIPS FOR THE GOALS | Strengthen the means of implementation and revitalize the Global Partnership for Sustainable Development |
| 9 INDUSTRY, INNOVATION AND INFRASTRUCTURE | Build resilient infrastructure, promote inclusive and sustainable industrialization and foster innovation | | |

Within these Goals, Goals 2, 12 and 13 are particularly relevant to the agrifood sector. Goal 2 aims to end hunger, achieve food security, improve nutrition and promote sustainable agriculture. Goal 12 emphasizes the need for sustainable consumption and production patterns. Under Goal 12, reducing food loss and waste (FLW) is a key element in enhancing the sustainability of agrifood systems and supports the principles and actions that drive circular economy initiatives (Pearson et al., 2024). In addition, Goal 13 calls for urgent action to combat climate change, which both affects and is affected by agricultural practices, making climate resilience a critical component of food system transformation. Together, these goals underline the essential role of the agrifood sector in achieving global sustainability and highlight the urgent need to rethink how food is produced, consumed and, unfortunately, wasted. Since 2015, yearly reports have been published, monitoring global progress. The latest one of 2025 showed that only 35 percent of SDG targets are on track or making moderate progress. Nearly half are moving too slowly and, alarmingly, 18 per cent are in reverse (United Nations, 2025). Regarding Goal 2, the report notes that global hunger and food insecurity have remained high following a sharp increase during the COVID-19 pandemic (Figure 1). The report underlined that achieving zero hunger will require intensified efforts to transform food systems and the foreseen UN Food Systems Summit and the World Health Assembly will help to align global efforts with nationally defined priorities. In relation to Goal 12, the report also underlines that global food waste reached 1.05 billion metric tons (132 kilograms per person) in 2022, revealing a significant gap in connecting this issue with the objective of halving food waste and reducing losses by 2030 (Figure 1).


Figure 1. SDG progress of SDGs 2 and 12 by target (United Nations, 2025)

However, there is a general increase in interest and implementations of supporting policies by governments and even by private companies, which are expanding their sustainability reporting (United Nations, 2025). The FAO (Food and Agriculture Organization) Voluntary Code of Conduct for Food Loss and Waste Reduction outlines key principles and recommended actions for countries to tackle the root causes of food loss and waste (FAO, 2022), emphasizing that all measures must consider food safety (FAO, 2017). These recommendations include promoting research and development (R&D) to identify innovations to reduce FLW, education and awareness to stimulate behaviour change; investments for the valorisation of non-edible parts, by-products and material that leave the food supply chain as FLW (FAO, 2022).

At the European level, the EU introduced a specific action plan aimed at reducing food loss and waste, incorporating both regulatory and non-regulatory measures, originally launched under the 2015 Circular Economy Action Plan (European Commission, 2015). In December 2019, the European Commission launched the European Green Deal, which aims to *transform the EU into a fair and prosperous society, with a modern, resource-efficient and competitive economy where there are no net emissions of greenhouse gases in 2050 and where economic growth is decoupled from resource use* (European Commission, 2019). To accomplish these goals, it is crucial to enhance the importance placed on protecting and restoring natural ecosystems, sustainably using resources, and improving human health. Focusing on the agrifood sector, the European Commission presented the Farm to Fork (F2F) strategy, which is at the heart of the EU Green Deal and aims to contribute to achieving a circular economy (European Commission, 2019) and is also central to the Commission's agenda to achieve the UN SDGs (European Commission, 2020). The F2F strategy includes different points (Figure 2), including sustainable food production, sustainable food processing and distribution, sustainable food consumption and food loss and waste prevention.

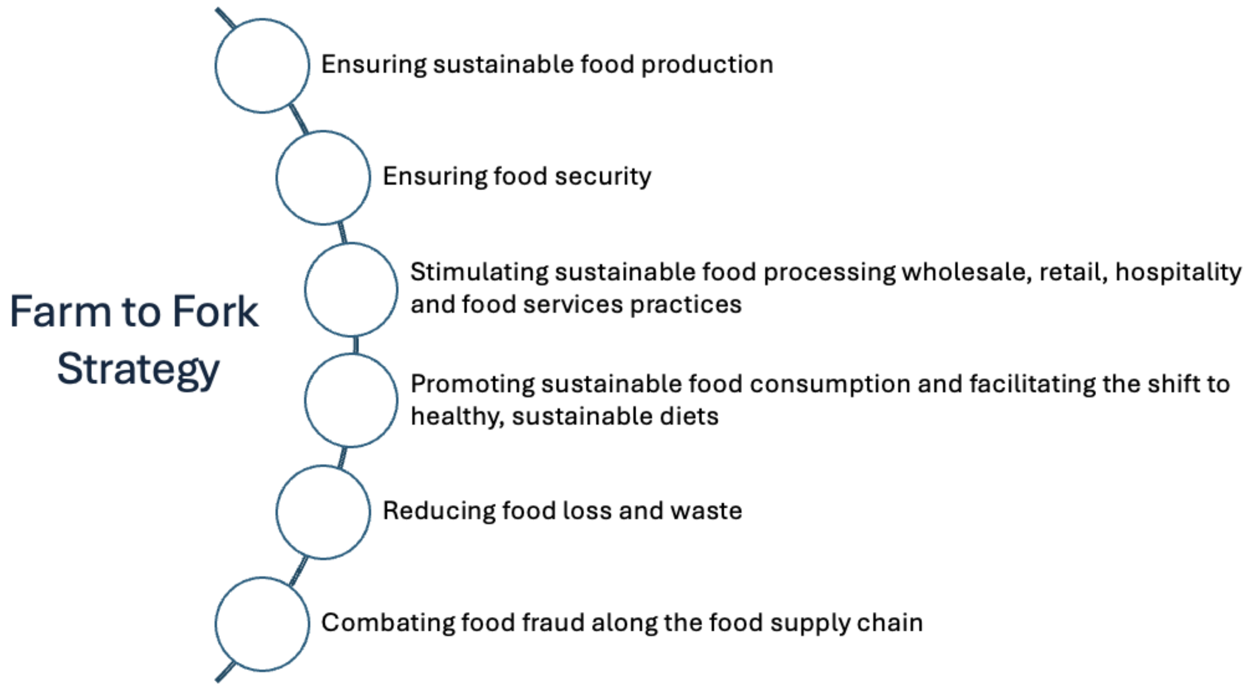


Figure 2. Farm to Fork Strategy: building the food chain that works for consumers, producers, climate and the environment (adapted from European Commission, 2020)

Crucially, the F2F strategy supports the reduction of the environmental and climate impact of animal production and the reduction of the dependency on critical feed materials (e.g. soya grown on deforested land) by fostering EU-grown plant proteins as well as alternative feed materials such as insects, marine feed stocks (e.g. algae) and by-products from the bio-economy (European Commission, 2020).

Alongside the F2F strategy, the European Commission is proposing to amend the Waste Framework Directive (European Parliament and the Council, 2008) for legally binding food waste reduction targets to be achieved by Member States by 2030. The specific objectives of the proposal (European Commission, 2023) are to clearly assign responsibility to Member States for accelerating the reduction of food waste throughout the food supply chain and in households within their territories; and second, to ensure that all Member States respond adequately and consistently to food waste reduction, aligning their efforts with those of the leading countries.

## Circular feed: food co/by-products and former food products

In efforts to promote circular economy principles, both food co/by-products and former food products (FFPs) are gaining attention as valuable resources for feed systems.

Food co/by-products consist of residual materials generated during primary food processing, which may still be rich in proteins, fibers, lipids, and bioactive compounds (Darko et al., 2024; Vastolo et al., 2022; Verni & Casanova, 2022) and used for farm animal nutrition (Figure 3). Different co/by-products are already used in the animals' diets (e.g., wheat bran, molasses, rapeseed meal, olive oil cake, etc.) (FEFAC, 2019), while others need a more careful consideration before their introduction into feed diets. While safety should always come first, the reuse of food co/by-products and FFPs would help to address key challenges in the "One Nutrition" approach, which seeks to integrate crop production, animal feed, and human nutrition sustainably and efficiently, reducing resource competition (Pinotti et al., 2025). By safely reusing co/by-products and FFPs, the animal feed industry can optimize resource utilization, reduce waste, and maintain feed safety and nutritional quality (FEFAC, 2022).

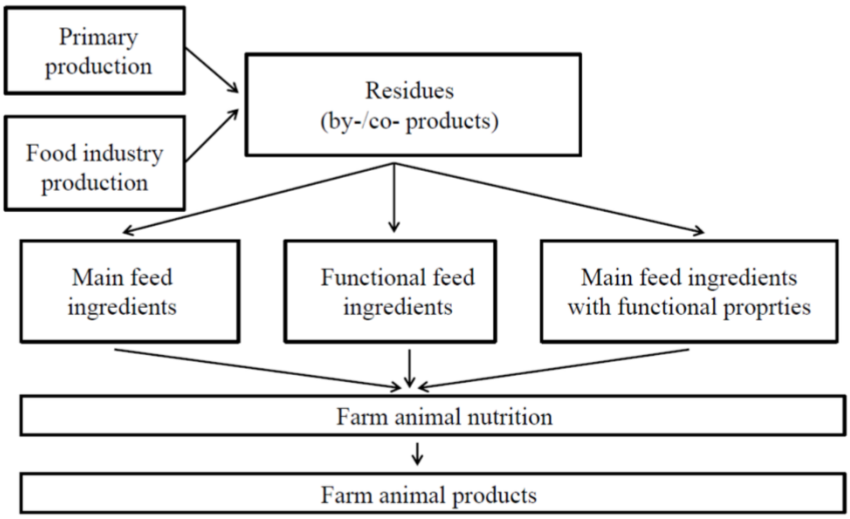


Figure 3. Representation of food-feed-food system (Kasapidou et al., 2015)

Cocoa Hulls (CHs)

Cocoa hulls (CHs) are the outer shells of cocoa beans (*Theobroma cacao* L.), produced as a byproduct during the dehulling step in the extraction of cocoa butter (INRA CIRAD AFZ Tables). The scientific name *Theobroma* means "food of the gods" (theos= gods, broma=food) highlights the historical and cultural significance of cacao (Rusconi & Conti, 2010). It is commonly known as cacao or cocoa, and it's a tropical evergreen tree native to the deep tropical regions of Central and South America. Cacao trees produce large pods containing seeds or beans, surrounded by the pulp and protected by a shell or hull (Porto de Souza Vandenberghe et al., 2022). These beans are the basis for chocolate production. The beans can then be processed into various forms, including cocoa powder and cocoa butter, used in a wide range of confectionery, desserts, and beverages.

In the year 2020-2021 (from the 1st of October to the 30th of September), 5245 thousand tonnes of cocoa beans were produced worldwide (ICCO—International Cocoa Organization, 2023), leading to a substantial amount of waste generated from their processing. During the pre-processing and processing steps for the transformation of cocoa fruit to cocoa mass, several by-products are produced. After the cocoa fruit is harvested and broken open, the first by-product, cocoa husk, is produced. During the fermentation stage, cocoa sweating is released. Cocoa beans are then obtained and the processing phase starts. Dried and fermented cocoa beans are either thermally pre-treated or roasted, then peeled, and at this stage the cocoa bean shell, also called CH is separated from the bean (Figure 4).

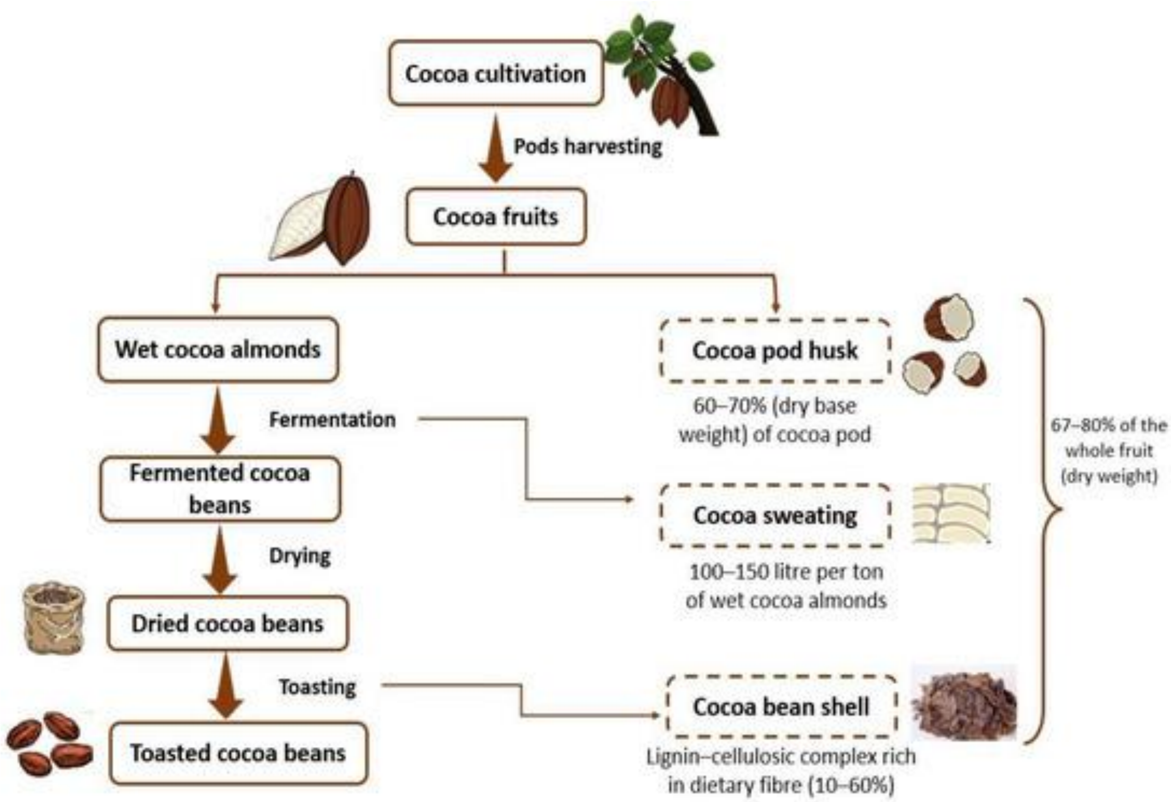


Figure 4. Cocoa processing chain and derived wastes/by-products (Sánchez et al., 2023)

While the cocoa bean is used for the production of the cocoa mass, the end product, used for the food industry, the CH is often discarded and wasted. All this residue, not being contaminated with animal products, can undergo the feed classification rather than the waste classification (Figure 5).

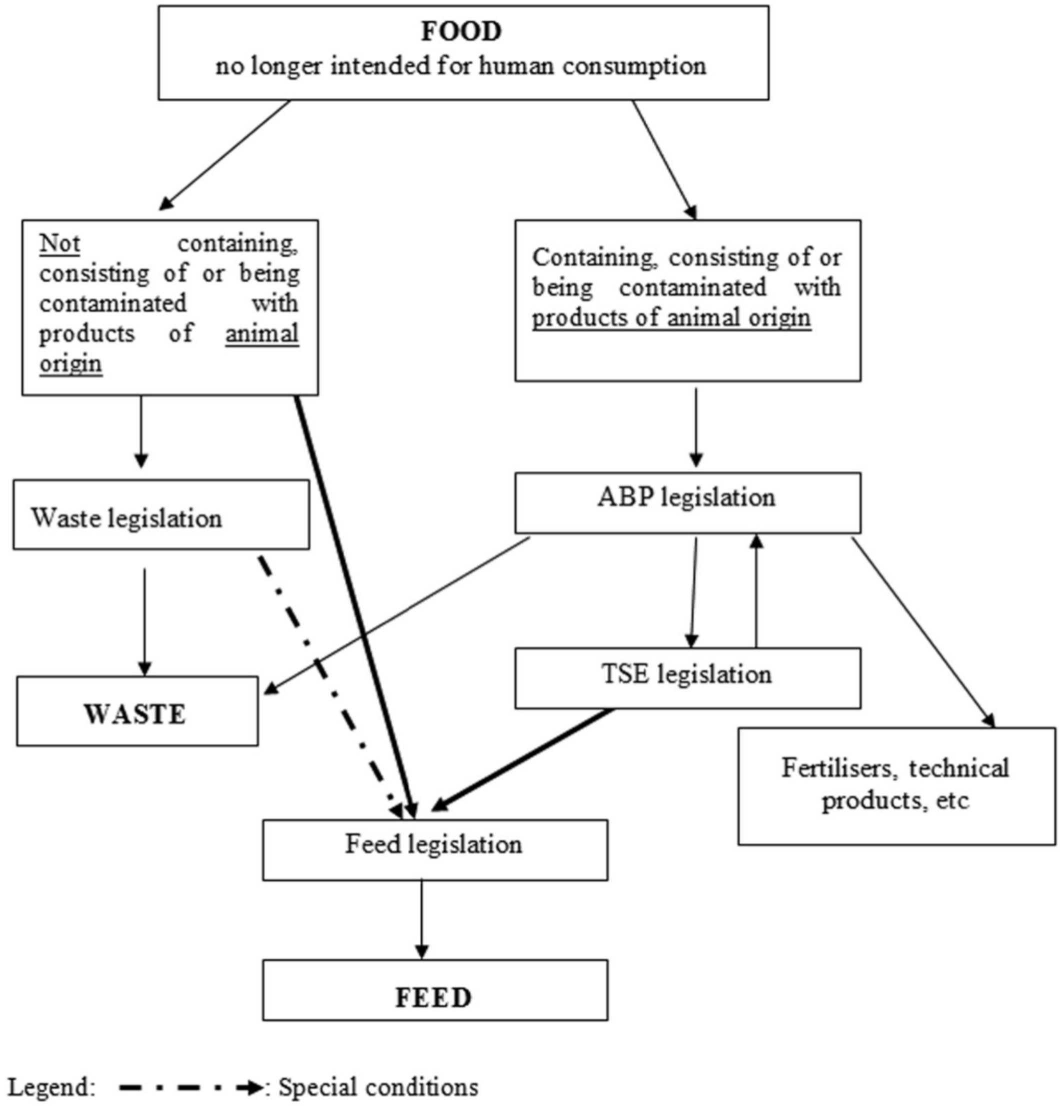


Figure 5. Guidelines for the feed use of food no longer intended for human consumption (2018/C 133/02 Official Journal of the European Union)

CHs have considerable amounts of proteins, minerals, and vitamins (Table 2), which make them an interesting and inexpensive material for livestock feed (Rojo-Poveda et al., 2020).

Table 2. Nutritional and chemical composition of CHs, (adapted from Rojo-Poveda et al., 2020)

| Parameter | Amount[a] |
|---|---|
| Energy (kcal/100 g) | 122.00 |
| Moisture (%) | 3.60-13.13 |
| Ash (g/100 g) | 5.96-11.42 |
| Proteins (g/100 g) | 10.30-27.40 |
| Fats (g/100 g) | 1.50-8.49 |
| Carbohydrates (g/100 g) | 7.85-70.25 |
| - Starch (g/100 g) | 0-2.80 |
| - Soluble sugars (g/100 g) | 0.16-1.66 |
| Dietary fiber (g/100 g) | 39.25-66.33 |
| - Soluble fiber (g/100 g) | 7.03-16.91 |
| - Insoluble fiber (g/100 g) | 28.34-50.42 |
| Pectin (g/100 g) | 7.62-15.59 |
| **Minerals** | |
| Calcium (g/100 g) | 0.23-0.44 |
| Phosphorus (g/100 g) | 0.58-1.00 |
| Magnesium (g/100 g) | 0.48-1.29 |
| Potassium (g/100 g) | 1.25-1.82 |
| Sodium (mg/100 g) | 16.00-192.20 |
| Iron (mg/100 g) | 27.60-80.50 |
| Manganese (mg/100 g) | 4.53 |
| Copper (mg/100 g) | 2.35-6.62 |
| Selenium (mg/100 g) | 0.21 |
| Cobalt (mg/100 g) | 0.10 |
| Zinc (mg/100 g) | 2.75-19.00 |
| Chromium (mg/100 g) | 0.67-4.86 |
| **Vitamins** | |
| B1 (µg/g) | 0.70-3.10 |
| B2 (µg/g) | 0.90-3.10 |
| B6 (µg/g) | tr |
| D (µg/g) | tr-0.53 |
| E (µg total tocopherols/g CBS fat) | 1.02 |
| **Polyphenol content** | |
| Total phenolic content[b] | 3.12-94.95 |
| Total flavonoid content[c] | 1.65-40.72 |
| Total tannin content[c] | 1.70-25.30 |
| **Flavonols** | |

| | |
|---|---|
| Epicatechin (mg/g) | 0.21-34.97 |
| Catechin (mg/g) | 0.18-4.50 |
| Procyanidin B1 (mg/g) | 0.55-0.83 |
| Procyanidin B2 (mg/g) | 0.23-1.38 |

[a] Data are referred to a CH dry weight basis unless indicated differently. Intervals have been created, comprising all the values from the cited literature; [b] mg of gallic acid equivalents/g of dried CH; [c] mg of catechin equivalents/g of dried CH; tr: Traces.

CHs are present in the Catalogue of feed materials (Reg. EU 2022/1104), which ensures adherence to standardized quality and safety requirements, thus providing clarity and legal certainty for feed manufacturers and users across the European Union. Their inclusion in the Catalogue highlights the broader trend of integrating sustainable feed resources within the circular economy approach. Their inclusion in animal diets is motivated not only by waste valorization and sustainability goals but also by the nutritional and functional attributes such as polyphenols, minerals, vitamins, and fiber (Rojo-Poveda et al., 2020; Vásquez et al., 2019; Wollgast & Anklam, 2000). Although these nutritional advantages exist, Directive 2002/32/EC on Undesirable Substances in Animal Feed sets species-specific maximum levels for theobromine content.

## Former food products (FFPs)

The development of alternative ingredients for feed production is becoming increasingly important due to its significant environmental impact, the growing demand for animal products, and the escalating competition between food and feed for arable land. Currently, the livestock sector accounts for 45% of total greenhouse gas emissions and uses 33% of cultivated land for forage production (Lanzoni et al., 2024). During the transformation of agricultural raw materials and other products into food items intended for distribution and sale to consumers, agri-food companies inevitably incur unintentional food losses (Luciano et al., 2020). Among these, generated products can be referred to as 'ex-foods' or “former food products” (FFPs).

FFPs are, by definition, *foodstuffs, other than catering reflux, which were manufactured for human consumption in full compliance with the EU food law but which are no longer intended for human consumption for practical or logistical reasons or due to problems of manufacturing or packaging defects or other defects and which do not present any health risks when used as feed* (Regulation (EU) No 2017/1017). FFPs convert food industry losses into feed ingredients and have been shown to support livestock performance (Luciano et al., 2020).

There are several reasons why these products are no longer considered suitable for human consumption, often due to quality defects or economic factors. Common causes include production errors, seasonal overproduction, the discontinuation of certain product lines, and logistical or delivery issues can also contribute to the accumulation of these materials (Pinotti et al., 2021). As a result, these products (Figure 6) no longer meet commercial standards; however, they still have significant nutritional and energy content and can be reintegrated into the food chain through their conversion into ingredients suitable for animal nutrition (Tretola et al., 2025).


Figure 6. Examples of packaged and unpackaged FFPs prepared for processing into FFP ingredients for feed production (Pinotti et al., 2021)

The main difference between FFPs and traditional cereals is their significantly higher fat content, from 3 to 6 times greater than wheat or barley (Luciano et al., 2020). Using FFPs also offers technological advantages in feed production by reducing the need to add fats separately, improving processing efficiency (Giromini et al., 2017). Furthermore, FFPs have higher digestibility than common cereals because heat processing gelatinizes starch and increases free sugars, facilitating carbohydrate absorption, especially important for young animals with limited digestion (Ottoboni et al., 2019; Tretola et al., 2019). Indeed, in vitro studies showed that FFPs generally have higher glycemic and hydrolysis indices than raw or heat-treated cereals, reflecting improved starch digestibility (Ottoboni et al., 2019).

The use of FFPs in feed production foresees different processing steps, including unpacking, grinding, mixing and drying. If not done correctly, they may compromise the quality and safety of the final product. Key concerns involve the risk of bacteria or chemical contamination, potential digestive issues in animals, and the presence of unwanted materials such as leftover packaging. FFPs, being low in moisture or heat-treated, are generally microbiologically stable; nevertheless, microbial analysis must confirm their hygienic quality, as inadequate handling can lead to contamination (Tretola et al., 2017). More studies demonstrated negligible microbiological risks and no adverse effects on gastrointestinal health, microbiota, growth performance and meat quality from post-weaning to finishing pigs fed diets partially replacing common feed materials with FFPs (Mazzoleni et al., 2023; Tretola et al., 2024). Additionally, FFPs did not impair the metabolic health or ruminal fermentation of early-lactating cows (Reiche et al., 2025). Visual inspection methods have also confirmed that the residual content of packaging materials in processed FFPs remains below national regulatory tolerance thresholds (Tretola et al., 2017; Van Raamsdonk et al., 2011). FFPs may also contain compounds naturally present in the original food matrix, which can represent another area of concern, particularly when FFPs include chocolate and cocoa-based confectionery, which are well-documented sources of methylxanthines such as caffeine and theobromine.

Theobromine and caffeine in alternative feed ingredients

Theobromine and caffeine may limit the inclusion of CHs and FFPs into animal diets. Indeed, the presence of alkaloids, particularly methylxanthines such as theobromine and caffeine, can be toxic to certain animals if consumed in high amounts (EFSA CONTAM Panel, 2008). Caffeine and theobromine are naturally present in cocoa and cocoa co/by-products, while they may be present in FFPs if these contain ingredients such as chocolate or bakery goods containing chocolate, coffee or their derivatives. The concentration of these alkaloids can, therefore, vary depending on the type and proportion of such ingredients in the FFPs.

Theobromine (3,7-dimethylxanthine) (Figure 7a) belongs to a class of alkaloid molecules known as methylxanthines and it's the primary methylxanthine found in products of the cacao tree, beans and CHs (Sugimoto et al., 2014). Theobromine is less active as a central nervous stimulant than caffeine (M. Zhang et al., 2024). Caffeine (1,3,7-trimethylxanthine) (Figure 7b) is also a methylxanthine which occurs naturally from plant sources and it's the main methylxanthine that occurs in coffee (Heckman et al., 2010).

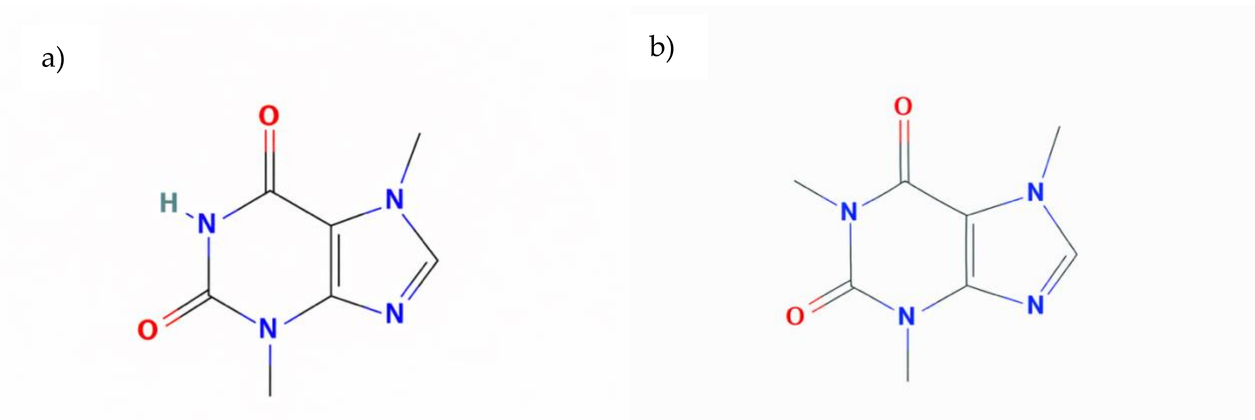


Figure 7. a: Theobromine (3,7-dimethylxanthine); b: Caffeine (1,3,7-trimethylxanthine), (adapted from Martínez-Pinilla et al., 2015)

They are heart stimulants, and although humans efficiently metabolize them, some animals, particularly dogs and horses, metabolize them much more slowly, resulting in prolonged effects and a higher risk of poisoning (Finlay & Guiton, 2005). Their main pharmacological effects, due to their structural similarity to adenosine, include adenosine receptor antagonism, phosphodiesterase inhibition, and regulation of intracellular calcium. They can also promote smooth muscle relaxation, diuresis, and cardiac stimulation (Martínez-Pinilla et al., 2015).

While Cocoa absolute CoE 452 is an authorized feed additive in the EU (Directive 70/524/EEC) and *Theobroma cacao* L. by-products (i.e., cocoa husks, hulls and bean meal) are in the Catalogue of feed materials (Reg. EU 2022/1104), it is essential to obtain proper formulation. This is necessary to comply with Directive 2002/32/EC on undesirable substances in animal feed, establishing species-specific maximum levels of theobromine in complete feedingstuffs (Table 3), when incorporating cocoa or its by-products in animal feed, for ensuring the safety and well-being of animals.

Table 3. Species-specific maximum levels of theobromine in various animal feeds (Directive 2002/32/EC)

| **Undesirable Substance** | **Product Intended for Animal Feed** | **Maximum Content in mg/kg (ppm) relative to a feed with a 12% moisture Content** |
|---|---|---|
| Theobromine | Complete feed | 300 |
| | Complete feed for pigs | 200 |
| | Complete feed for dogs, rabbits, horses, and fur animals | 50 |

Hemp co/by-products

Hemp (*Cannabis sativa* L.) is an herbaceous plant with a long and wide history of uses, ranging from industrial to nutritional and to medicinal applications (Bernstein, 2024; Rizzo et al., 2023; Vasantha Rupasinghe et al., 2020). Indeed, this highly versatile plant can be used to produce a wide range of industrially valuable products, including fiber and shives; bio-based construction and thermal insulation materials; seeds, flour, and oil that offer significant nutritional and functional benefits; as well as bioactive compounds with pharmacological potential (Farinon et al., 2020) (Figure 8).

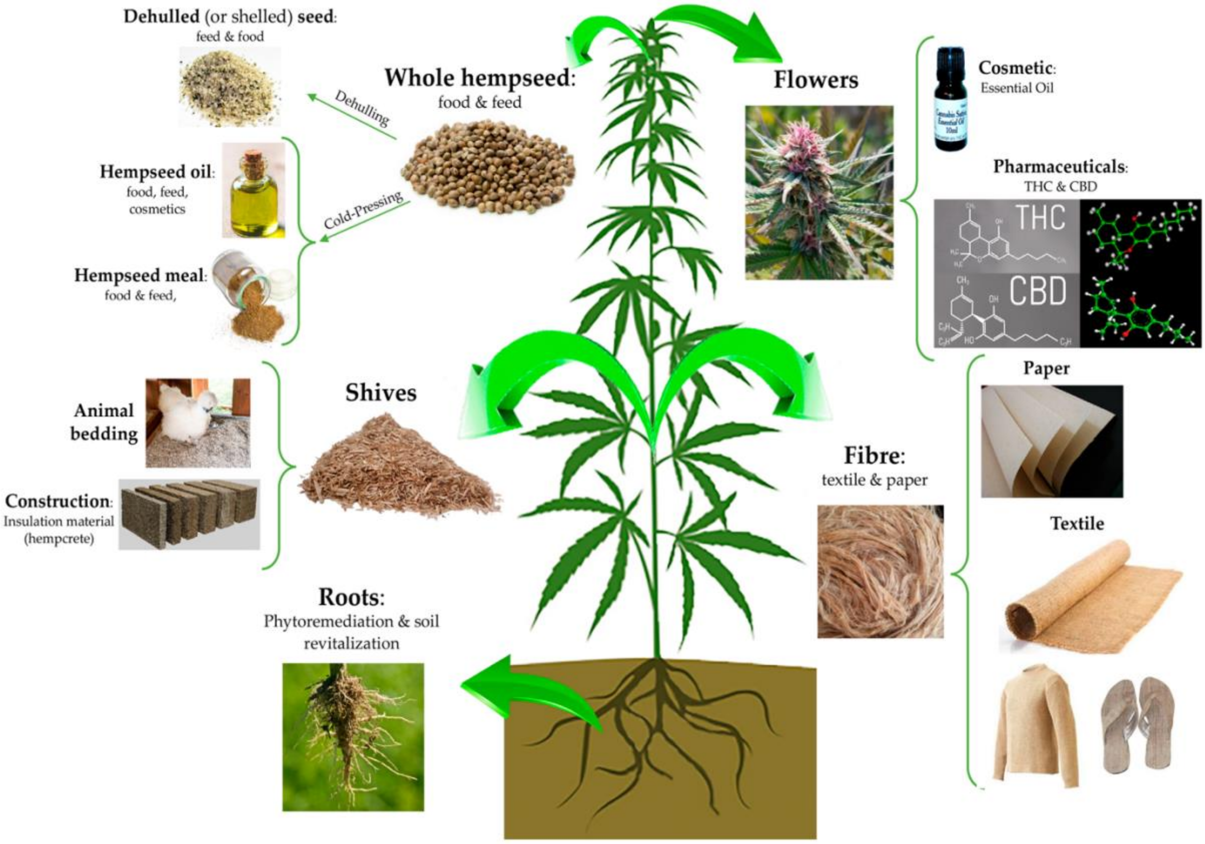


Figure 8: The different uses of hemp in various industries: Its seeds, whether whole or dehulled, are used in food, animal feed, and cosmetics, and can also be cold-pressed to extract oil for culinary and cosmetic products. The stem provides both shives and fiber, making it valuable for applications in animal care, construction, papermaking, and textiles. Hemp's extensive root system, more developed than that of many other herbaceous plants, makes it particularly effective for phytoremediation, helping remove heavy metals from soil. Additionally, hemp flowers have uses in ornamental gardening and are a source of cosmetic and pharmaceutical products, including essential oils and purified extracts of delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD). (Farinon et al., 2020).

Hemp is considered to contribute to the Europen Green Deal by it several environmental benefits (Table 4), including carbon storage, breaking disease cycles, soil erosion prevention, biodiversity and low or no pesticide use.

Table 4. Hemp environmental benefits (adapted from European Commission, n.d.)

| Environmental benefit | Description |
|---|---|
| Carbon storage | One hectare of hemp sequesters 9 to 15 tonnes of $CO_2$ in five months, comparable to a young forest but faster growth. |
| Breaking disease cycles | Hemp disrupts disease cycles in crop rotation and suppresses weed growth through fast growth and shading. |
| Soil erosion prevention | Dense hemp leaves act as natural soil cover, reducing water loss and protecting against erosion; ground coverage occurs within three weeks of germination. |
| Biodiversity | Flowers from July to September, providing abundant pollen during a seasonal pollen gap; offers shelter for birds and seeds as wildlife food. |
| Low or no pesticide use | Hemp is resistant to many pests due to lack of natural predators, minimizing the need for insecticides, herbicides, and fungicides. |

Traditionally, the primary product of hemp has been its fiber, obtained from the stalks, used mainly for textiles, construction and paper production (Vasantha Rupasinghe et al., 2020). Another primary product are hemp seeds, which are high in nutritional value and used in foods, for oil production and food supplements (Callaway, 2004). In addition, hemp flowers and leaves are important products for phytocannabinoids, extracted for nutraceutical and medicinal products (AL Ubeed et al., 2022).

Different anatomic parts of the hemp plant have different compositional profiles, influencing their suitability for distinct nutritional and industrial applications (Table 5).

Table 5. Nutritional facts of different anatomic parts of the hemp plant (Audu et al., 2014; House et al., 2010; Xu et al., 2022)

| Hemp part | Fat (%) | Protein (%) | Fiber (%) | Ash (%) | Moisture (%) |
|---|---|---|---|---|---|
| Seed | 25-35 | 20-25 | 20-30 | 7 | 6 |
| Dehulled hempseed | 47 | 36 | 8 | 6 | 5 |
| Hemp hulls | 10 | 13 | 65 | 4 | 5 |
| Leaf | 19.97 | 23.78 | 18.95 | 11.18 | 6.87 |
| Stem | 8.02 | 17.20 | 23.13 | 6.78 | 5.16 |

When hemp is processed, depending on its use, a wide range of co/by-products are generated, including leaves, hulls (HLs) and hempseed cakes (HSC), which are less studied and characterized (Lanzoni et al., 2024). The EU Catalogue for Feed Materials (Commission Regulation (EU) 2022/1104 of 1 July 2022 Amending Regulation (EU) No 68/2013 on the Catalogue of Feed Materials) includes hemp by-coproducts like HSC, also called hemp expeller and other hemp products i.e., hemp seed, hemp seed oil, hemp flour (ground from stems) and hemp fibre (obtained from hemp stems), all from varieties of *Cannabis sativa* L. with a tetrahydrocannabinol content $< 0.2\%$. HLs and hemp leaves are not yet included, although research shows that hemp HLs and leaves have high rumen digestibility and could be used (where permitted) as a high-quality animal feed ingredient, particularly for ruminants (Lopez et al., 2025; Terry et al., 2023), even though leaves showed higher concentrations of cannabinoids, posing regulatory challenges (Terry et al., 2023).

Polyphenols and antioxidant activity in animal nutrition

Polyphenols are a wide range of compounds found primarily in fruits, vegetables, and other plant-based foods like teas and chocolate (de Araújo et al., 2021). They can be classified into flavonoid compounds (flavonols, flavanols, flavones, flavanones, isoflavones, and anthocyanins), characterized by a C6-C3-C6 structure, and non-flavonoid compounds (phenolic acids, hydroxycinnamic acids, lignans, stilbenes, and tannins) (Figure 9) (Di Lorenzo et al., 2021).

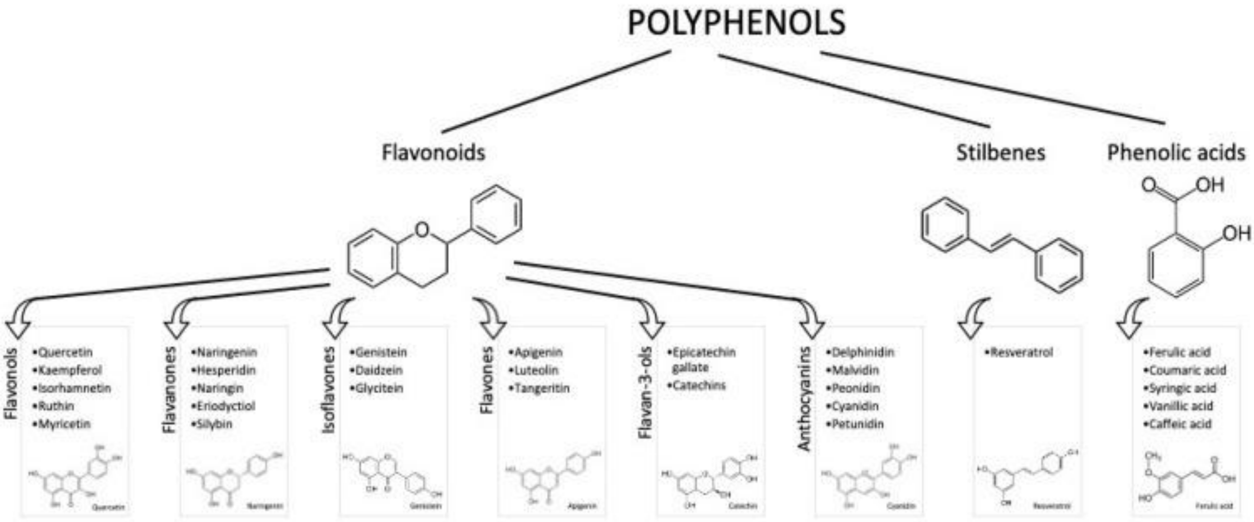


Figure 9. Polyphenol classes and chemical structures of some of their main compounds (Di Lorenzo et al., 2021)

Dietary polyphenols and flavonoids are thought to possess strong antioxidant properties that help counteract reactive oxygen species (ROS) and cellular oxidative stress (OS), thereby contributing to the prevention of oxidative stress-related disorders and diseases. The consumption of polyphenols has been increasingly associated with several health benefits, including anti-inflammatory and antioxidant activities, making them valuable in the context of nutrition (McDougall et al., 2017; Di Lorenzo et al., 2021).

In livestock, oxidative stress is considered a key factor in the onset of various pathological conditions that compromise animal health, welfare, and performance. During certain physiological stages, such as lactation, or under challenging environmental conditions, including weaning and heat stress, animals are exposed to multiple stressors that can diminish their antioxidant status. In addition, maintaining an optimal antioxidant balance has been shown to improve meat quality traits, notably by enhancing vitamin E concentrations and limiting lipid peroxidation (Corino et al., 2021).

Recent advancements have highlighted the significant potential of food co/by-products as sustainable sources of polyphenols, offering an environmentally friendly opportunity for the valorization of agricultural waste (Bernini et al., 2024; Machado et al., 2021). For example, co/by-products from the food industry, such as fruit peels, pomaces, and bran, contain considerable quantities of polyphenolic compounds and are already used in animal feed (FEFAC, 2019). When diets are based on low-quality forages and high grain content, it is necessary to supplement with n-3 polyunsaturated fatty acids (PUFAs) and antioxidants to increase their levels in meat and extend its shelf life. It is then suggested that combining nutrient antioxidants like vitamin E with non-nutrient antioxidants such as flavonoids and carotenoids can work together to effectively stabilize the high PUFA content in ruminant meat (Ponnampalam et al., 2024).

In particular, dietary supplementation with olive oil co/by-products rich in polyphenols in monogastric animals has been shown to enhance the antioxidant status (reductions in lipid peroxidation markers in meat and overall animal wellness) of these animals (Ferlisi et al., 2023). Dietary inclusion of grape pomace concentrate and mulberry leaves to poultry showed increased antioxidant activity and reduced lipid peroxidation markers (Turcu et al., 2020; B. Zhang et al., 2022). Similarly, dietary supplementation with grape pomace and mulberry leaves to pigs significantly enhanced antioxidant function by increasing superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) activities, reducing malondialdehyde (MDA) levels, and improving gut health and immunity in finishing pigs (Ma et al., 2023; Tian et al., 2023). In ruminants, co-by products rich in polyphenols (i.e. grape pomace, olive co/by-products, and pomegranate pomace) showed to increase antioxidant enzyme activities (e.g., CAT), total antioxidative capacity and to reduce lipid peroxidation (lower MDA) (Ferlisi et al., 2023; Guerra-Rivas et al., 2016; Li et al., 2024; Natalello et al., 2020). Moreover, supplementing dairy cows' diets with citrus pulp showed a significant increase in the concentrations of total polyphenols and flavonoids even in milk, improving its antioxidant capacity and nutritional quality (Hartinger et al., 2024; Santos et al., 2014). For what concerns hemp, the use of its products in the feed sector is nowadays limited mainly due to the current regulatory status, different processes on matrices, and limited produced volumes, although hemp co-products showed valuable results in terms of total polyphenol content and antioxidant activity, besides an interesting nutritional profile, especially in terms of protein and lipids (Lanzoni et al., 2024 ). Future research focusing on data about the safety of products from hemp-fed animals will enable faster advancement of studies needed for hemp to be fully accepted as a feed ingredient (Altman et al., 2024).

**Animal dietary exposure in the risk analysis framework and in feed risk assessment**

Animal dietary exposure plays a crucial role in the comprehensive risk analysis of feed and feed ingredients. Within the Codex risk analysis framework, risk analysis consists of three closely interlinked components: risk assessment, risk management, and risk communication (Figure 10). Together, they address the potential risks to human health associated with the presence of hazards in the feed of food-producing animals and the subsequent transfer of hazards to edible products (Codex Alimentarius, 2013).

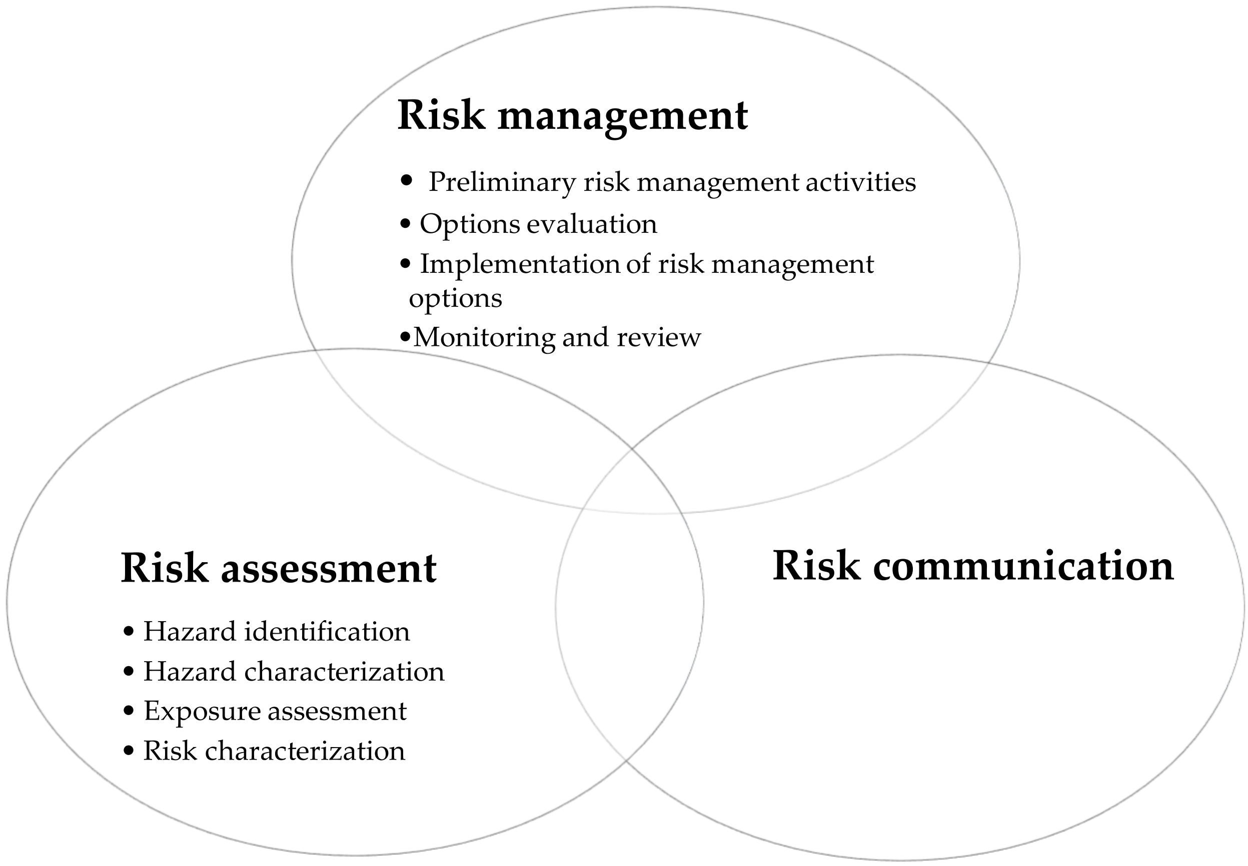


Figure 10. Codex risk analysis framework (adapted from Codex Alimentarius CXG 80-2013, 2013).

The risk assessment procedure is composed of I) hazard identification II) hazard characterization III) exposure assessment IV) risk characterization (Codex Alimentarius, 2013).

Animal dietary exposure assessment is integral to the first step of the exposure assessment. In evaluating the potential exposure of animals to a specific hazard, the Codex Alimentarius Guidelines CAC/GL 80-2013 recommends first recognizing which feedstuffs could act as sources of that hazard. Once these feeds are identified, analytical methods are applied to determine the concentration of the hazard in each feed component. This data should then be integrated with information on the quantity of feed consumed, inclusion rate of feedstuffs in the diet and animal body weight to estimate the dietary exposure of the hazard by the animal population under study. (EFSA, 2019).

To date, animal dietary exposure relies on default values based on guidance documents and recommendations issued by international regulatory bodies and organizations involved in feed safety assessment and agricultural development, including EFSA, the Food and Agriculture Organization of the United Nations (FAO), and the Organisation for Economic Co-operation and Development (OECD) given the absence of a comprehensive databases for feed intake (EFSA, 2019). The absence of a feed consumption database was identified as one of the main contributors to the uncertainty and limitations influencing animal dietary exposure assessment by the EFSA Panels on Contaminants (CONTAM), Additives and Products or Substances used in Animal Feed (FEEDAP) and Genetically Modified Organisms (GMO) (EFSA FEEDAP Panel, 2024; EFSA GMO Panel, 2023).

In March 2025, the FAO and the RIVM (Dutch National Institute for Public Health and the Environment) organised the International Workshop on Feed Risk Assessment - Chemical Safety, held in Utrecht, The Netherlands. During this workshop, an FAO manual on feed risk assessment was presented. This manual is developed to support the implementation of the Codex Guidelines on risk assessment of feed, to support hormonization of global assessment practices and to assist professionals who may be less familiar with feed risk assessment processes (RIVM, 2025). Even though the scarcity of animal health toxicity studies makes it difficult to perform comprehensive risk assessment, the manual aims to provide clarity and highlights the urgent need for more structured research and information exchange (RIVM, 2025).

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## AIM

The aim of this thesis is to contribute to the development of sustainable and innovative strategies in the agri-food and feed sectors by valorizing food supply chains co/by-products and FFPs within a circular economy framework. The research focuses on assessing the toxicological, nutritional and functional properties of alternative feed ingredients, such as cocoa hulls, FFPs, and hemp co-products, while also addressing methodological challenges related to feed exposure assessment through the proposal of a harmonised feed consumption database.

The work was conducted in collaboration across disciplines and sectors, involving the Department of Pharmacological and Biomolecular Sciences, the Department of Veterinary Medicine and Animal Sciences, and the Department of Computer Science of the University of Milan, as well as ToxHub Srl (Rome). This interdisciplinary and intersectoral synergy enabled the integration of complementary expertise in feed safety, toxicology, nutrition, computer science, and regulatory science.

In line with the objectives of the Intersectoral Innovation doctoral programme, this thesis not only aims to generate new scientific knowledge but also aims to foster the transfer of innovation between academia, industry, and regulatory stakeholders, promoting practical applications for a more sustainable, safe, and resource-efficient agri-food system.

The objective of the individual chapters is briefly reported in the following list:

- Chapter 1: This study aims to validate a high-performance liquid chromatography method with ultraviolet detection (HPLC-UV) for quantifying theobromine and caffeine, and to assess the compliance of cocoa hulls (CHs) with EU safety limits for animal feed. Additionally, this study investigates the presence of polyphenols and antioxidant activity to support the valorization of CHs as a sustainable ingredient within a circular economy framework.
- Chapter 2: This study aims to characterize the nutritional profile and determine the levels of theobromine and caffeine in FFPs, in order to estimate alkaloid dietary exposure in target animals and draw safety considerations regarding their inclusion in animal diets, thereby ensuring animal health and feed safety while supporting feed practices that reduce the food–feed competition.
- Chapter 3: This study aims to investigate the nutritional and functional profile of hemp co-products to assess their potential for livestock feed. In particular, total phenolic content and antioxidant activity were monitored following a green chemical extraction, also identifying the main phenolic compounds by High-Performance Thin-Layer Chromatography (HPTLC). In parallel, total phenolic content and antioxidant activity were also evaluated following an ex vivo digestion protocol.
- Chapter 4: This chapter aims to develop a proposal for a feed consumption database by mapping existing systems and methodologies for feed classification, proposing updates to FoodEx2, building a model database and data structure and performing two case studies focusing on genetically modified feed and on contaminants.

# CHAPTER 1

## - Preliminary Assessment of Alkaloid Content in Cocoa (*Theobroma cacao* L.) Hulls for Safe Consumption as a Feed Ingredient

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# Preliminary Assessment of Alkaloid Content in Cocoa (*Theobroma cacao* L.) Hulls for Safe Consumption as a Feed Ingredient

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**Abstract:** The European Circular Economy Action Plan outlines a forward-looking strategy that emphasizes waste reduction and the acquisition of high-quality secondary resources. Previous research has shown that cocoa processing by-products contain compounds of interest for various industrial areas, making them an attractive matrix for reuse. However, a gap remains in our understanding of the safety of these by-products intended for feed. In this study, theobromine and caffeine were quantified by High-Performance Liquid Chromatography (HPLC-UV) in cocoa hulls for safety considerations, evaluating theobromine compliance with toxicological and safety levels, and considering their potential application as an ingredient in animal feed. In addition, the identification of phenolic components and associated antioxidant activity was conducted through High-Performance Thin-Layer Chromatography (HPTLC). This preliminary study indicates that theobromine content is a limiting factor for the inclusion of cocoa hulls in animal diets, as it restricts inclusion levels to remain within current regulatory limits. Examples of general estimates of dietary theobromine exposure at inclusion levels based on regulatory limits for dairy cows and veal calves confirmed a low risk for animal health. Furthermore, the detection of antioxidant activity linked to the presence of polyphenols highlights the potential of cocoa hulls as a sustainable food by-product for feed formulation.

**Keywords**: cocoa by-products; circular economy; theobromine; caffeine; animal dietary exposure

**Key Contribution:** Theobromine content is a limiting factor for the inclusion of cocoa hulls into animal diets; while the presence of polyphenols with antioxidant activity and the potential for circular use support their utilization

## 1. Introduction

The food value chain is responsible for significant resource and environmental pressures, and it is estimated that in Europe, around 20% of the total food produced is lost or wasted [1]. The European Circular Economy Action Plan provides a future-oriented agenda for achieving a cleaner and more competitive Europe. In this context, it is essential to reduce waste and ensure high-quality secondary raw materials [1–5]. The valorization of cocoa (*Theobroma cacao* L.) processing waste has generated great interest from both nutritional and functional points of view; notably, about 80% of processed cocoa fruit is discarded [6], highlighting the substantial volume of generated waste. This extensive production of residual biomass poses a significant environmental issue for countries involved in cocoa cultivation and production [6,7], but also in the EU, where chocolate production occurs and therefore processing waste is produced.

On the other hand, cocoa by-products (e.g., cocoa pod shells, mucilages, and hulls) contain compounds of interest for various economic areas, such as food and the cosmetic and chemical industries [8]. In fact, cocoa bean by-products contain an interesting profile of polyphenols (flavonols, phenolic acids) [9,10], methylxanthines, dietary fibers, and lipids [11]. In the year 2020–2021 (from the 1st of October to the 30th of September), 5245 thousand tonnes of cocoa beans were produced worldwide [7], leading to a substantial amount of waste generated from their processing. From this production, each year, an estimated 700 to 900 thousand tons of cocoa hulls (CHs) are produced worldwide, with approximately 300 thousand tons of waste generated in Europe [11]. CHs, also called cocoa bean shells, are the by-product of the dehulling step in the extraction of cocoa butter; they are removed from the beans and often discarded as waste. Interest has therefore been raised in healthy ingredients that can perform positive functions, such as antioxidant protection and anti-inflammatory action, but also for ingredients that would decrease feed–food competition. There are some studies evaluating alternative uses for food by-products, for example, the production of prebiotic and functional ingredients [12], and the most common applications include uses as biofuel, activated carbon preparation, mulch and fertilizer, and feedstuff for livestock [13]. CHs, being a rich source of carbohydrates, dietary fiber, protein, ash, and polyphenols such as quercetin, epicatechin, and catechin [14,15], are an interesting matrix for reuse, as in the case of animal feed, in line with the circular economy aim of optimizing available resources and reducing food waste [16]. Indeed, modern animal husbandry includes, among its objectives, the study of new dietetic formulations in order to improve animal welfare [17].

According to INRAE-CIRAD-AFZ (French National Institute for Agricultural Research–French Agricultural Research and Cooperation Organization–French Association for Animal Production) feed tables [18], CHs contain approximately 17.8% of crude protein on dry matter (DM), 5.9% of ether extract and 7.8% of starch, leading to a gross energy of 4490 kcal/kg on DM. CHs also contain a high insoluble fiber content, around 46.5%, and minerals including phosphorus and magnesium.

This chemical composition makes CHs particularly interesting as ruminant feed ingredients [19]. In addition, the presence of tannins (1.70–25.30 mg/g) [13] contributes both functional benefits and challenges; at appropriate levels, tannins can improve protein utilization by reducing excessive ruminal protein degradation and help control bloat and parasites, while excessive amounts may negatively affect nutrient availability and animal health [20].

While CHs offer potential for reuse in animal feed due to their nutritional value, the presence of naturally occurring methylxanthines, particularly theobromine and caffeine, poses a significant toxicological concern that may limit the use of CHs in animal nutrition. Toxic effects of theobromine have been documented in several species. In an EFSA Scientific Opinion [21], the Contaminants in the Food Chain Panel reported toxicological studies where theobromine showed toxicity in rodents, with target organs the testes and thymus; the study allowed for the definition of a No Observed Adverse Effect Level (NOAEL) for testicular toxicity of 150 mg/kg b.w. in rats. Theobromine also showed adverse effects in skeletal development of rabbit offspring, leading to a NOAEL of 21 mg/kg b.w. Adverse effects were also reported in target animals. For example, pigs showed growth retardation, diarrhea, and lethargy [22]. Horses and dogs, which are especially sensitive to theobromine, showed liver and thyroid damage and fatal intoxications, respectively. The mutagenic and clastogenic effects of theobromine were generally reported as equivocal; no long-term carcinogenicity studies are available, and no ADI (acceptable daily intake) has been established [21]. So overall, the EFSA Panel stated that there is a general lack of data regarding the feed theobromine levels able to determine a negative effect on animals and underlined the need for further information on the use of various feed materials containing cocoa.

The European Union (EU) has recognized these risks, and Directive 2002/32/EC of the European Parliament and of the Council lists several compounds that are considered undesirable, including theobromine, in animal feeds and prescribes their ML for different feed commodities. The current theobromine EU ML for complete feed with a moisture content of 12% is 300 mg/kg, apart from that for pigs, for which the maximum content is set at 200 mg/kg, and for dogs, rabbits, horses, and fur animals, for which it is set at 50 mg/kg of feed, as last amended in 2019.

Differently for theobromine, the Directive (2002/32/EC) does not regulate caffeine, which also demonstrates toxicological relevance. In a 90-day toxicity study in rats and mice, caffeine led to a slight body weight decrease with no toxicity signs [23]. Numerous genotoxicity data are available showing no evidence of mutagenic effects for this molecule; reproductive effects were observed alongside general toxicity in parental mice with a NOAEL of 22 mg/kg b.w. for F0 parental and F1 offspring, and 88 mg/kg b.w. for F1 parental and F2 offspring [23].

Despite these known risks, various *Theobroma cacao* L. by-products (i.e., cocoa husks, hulls, and bean meal) are on the Catalogue of Feed Materials (Reg. EU 2022/1104) [24] and Cocoa absolute CoE 452 is authorized as a feed additive in the European Union (EU) (Council Directive 70/524/EEC) [25].

In response to this dual context of nutritional potential and toxicological risk, the present study aims to contribute to the development of evidence-based strategies for the safe and sustainable incorporation of cocoa by-products into animal feed formulations. This study aims to validate a High-Performance Liquid Chromatography method with ultraviolet detection (HPLC-UV) for the quantification of theobromine and caffeine in cocoa hulls (CHs) and to evaluate their compliance with EU safety limits for animal feed. Additionally, this study investigates the presence of polyphenols and antioxidant activity to support the valorization of CHs as a sustainable ingredient within a circular economy framework.

## 2. Results and Discussion

### 2.1. Development and Validation of the High-Performance Liquid Chromatography (HPLC) Method

The HPLC-UV method was developed to quantify alkaloids in CHs. The method was validated, according to FDA recommendations [26], to quantify theobromine and caffeine. Validation parameters are illustrated in Table 1, and chromatographic patterns are reported in Figure 1.

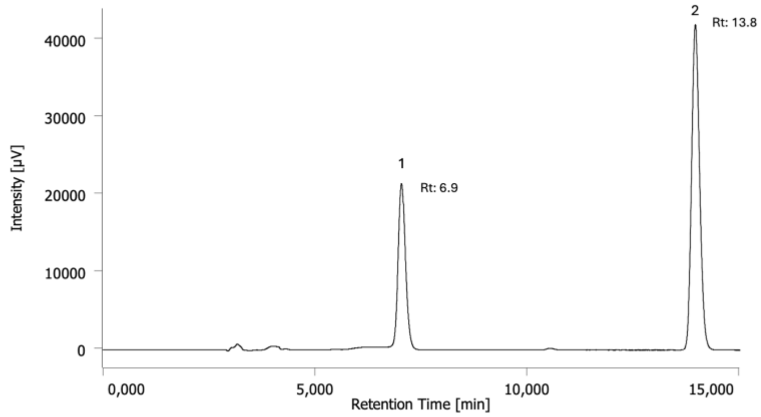


Figure 1. Chromatographic profiles of a mix of the standards at a concentration of 10 µg/mL and their retention times (Rt) (min). Peak 1: theobromine; peak 2: caffeine.

Table 1. HPLC-UV validation parameters for the theobromine (TB) and caffeine (CAF).

| | Linearity | | Sensitivity | | | | Recovery | Stability | | Precision | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Linear range | $R^2$ | LOD | | LOQ | | % | Variation% | | Intraday | Interday |
| | (µg/mL) | | (ng/mL) | (ng/g) | (ng/mL) | (ng/g) | | 24 h | 20 days | (CV%) | (CV%) |
| TB | 10–100 | 0.9820 | 3 | 30 | 10 | 100 | 95 | 1.43 | 7.74 | 3.11 | 6.93 |
| CAF | 10–100 | 0.9973 | 10 | 100 | 40 | 400 | 84 | 2.54 | 9.53 | 2.72 | 6.27 |

A correlation coefficient ($R^2$) greater than 0.98 (Table 1) was obtained for both standards and demonstrated a good linearity for the selected range of concentrations. The sensitivity of the method, defined as the lowest concentration of analyte that can be detected (LOD) or quantified (LOQ), is illustrated in Table 1. The recovery, which includes efficiency and reproducibility of the extraction, was confirmed within the range of 80–115% as required by the validation test [26]. The stability in the short term (after 24 h) and long term (after 20 days) showed a percentage of variation within

±10% and ±15%, respectively. Precision, assessed as intraday and interday variation, showed a coefficient of variation (CV%) of less than 15%, as required [26].

### 2.2. Measurement of Theobromine and Caffeine by HPLC and Safety Considerations

In all samples, theobromine was found at higher concentrations than caffeine. Sample 3 (S3), which is in pellet form, contained a higher concentration of analyzed analytes when compared to all samples in flake form (S1 and S2). This suggests that the pellet form may preserve or concentrate these compounds more effectively than the flake form [27]. These results agree with the results obtained by the HPTLC method (Section 2.3), where the comparison between the samples showed a higher presence of analytes in sample S3 than in samples S2 and S1. Figure 2 shows the HPLC profile of S1 (a), S2 (b), and S3 (c).

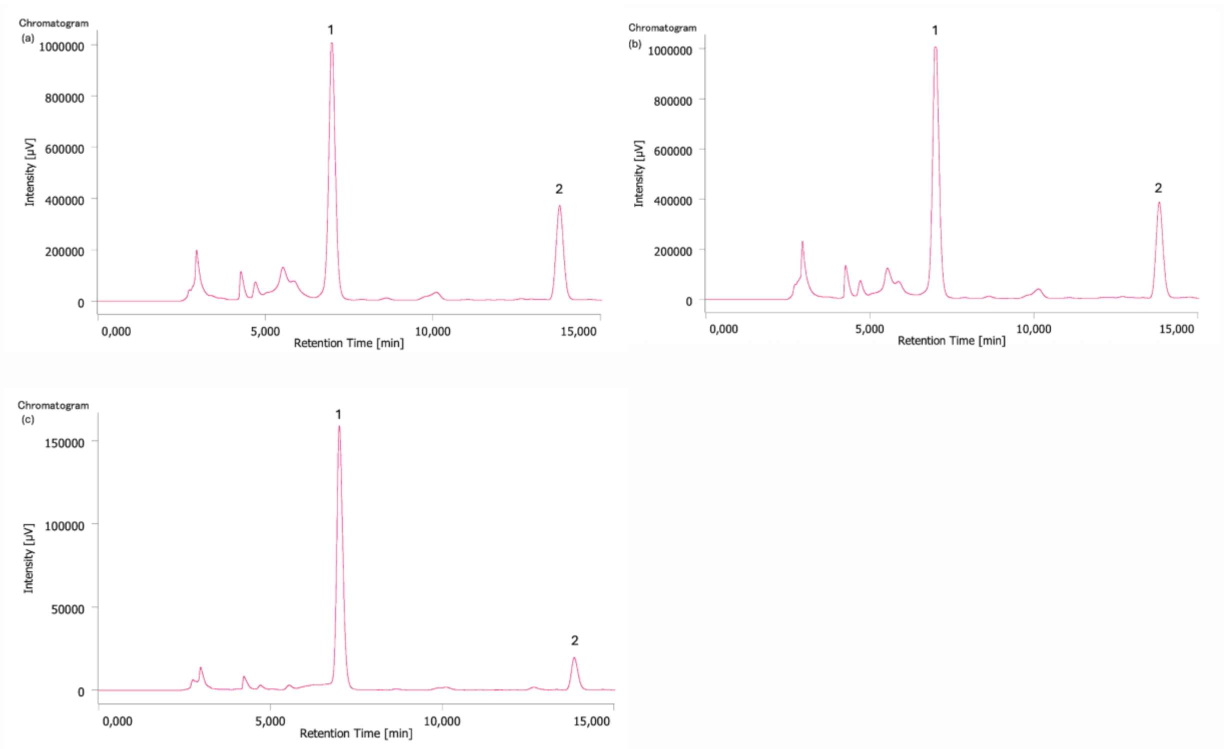


Figure 2. Chromatograms of samples S1 (a), concentrated 3:1; S2 (b), concentrated 3:1; and S3 (c), diluted 1:10. Peak 1: theobromine (Rt: 6.9); peak 2: caffeine (Rt: 13.8).

Peaks 1 and 2 (Figure 2) were identified as theobromine and caffeine, respectively; their quantification in µg/g is reported in Table 2.

Table 2. Content of theobromine (TB) and caffeine (CAF) in the CHs analyzed by HPLC-UV and expressed as mean ± SD (μg/g); $n$ = 3. Values with the same letter are not statistically different ($p$ > 0.05).

| Compound | S1 | S2 | S3 |
|---|---|---|---|
| TB | 4199.3 ± 86.97 $^{a}$ | 4036.65 ± 80.53 $^{a}$ | 5463.44 ± 109.84 $^{b}$ |
| CAF | 349.57 ± 13.19 $^{a}$ | 259.17 ± 6.46 $^{a}$ | 535.51 ± 16.84 $^{b}$ |

CHs contain CNS-stimulating alkaloids, primarily caffeine and theobromine, and constituents that could be classified as toxic or anti-nutritious, such as biogenic amines, tannin and trypsin inhibitor, even if poorly investigated [21]. It is therefore essential to propose a suitable formulation in case of the inclusion of cocoa or its by-products into animal feed.

The quantification by HPLC-UV performed in this study (Table 2) showed a theobromine concentration of approximately 0.400 g/100 g for samples in flakes form and 0.550 g/100 g for pellet form. In particular, theobromine concentration ranged between 4036.65 ± 80.53 μg/g for S2 and 5463.44 ± 109.84 μg/g in S3 (Table 2). These results are consistent with data previously published, where the theobromine content in CHs was in a range of 0.39–1.83 g/100 g [13]. To remain within the EU ML set for theobromine in complete feed (300 mg/kg, with exceptions for pigs, dogs, rabbits, horses, and fur animals, for which it is set at lower levels) [28], the CHs analyzed in this study could be used in a ruminant diet with an inclusion rate of a maximum of 7.14% for S1, 7.42% for S2, and 5.49% for S3. These values should be adjusted if other sources of theobromine (e.g., tea leaves, herbal feed supplements) are present in the proposed diet. To provide examples of potential theobromine exposure from CHs in ruminant diets, calculations are provided for dairy cows and veal calves. Using the calculated inclusion rates of CHs and assuming default values for live weight of 650 kg for dairy cows and 100 kg for veal calves [29], and default values for feed intake of 20.0 kg/day and 1.89 kg/day, respectively [29], the animal daily theobromine intake can be estimated at 9.23 mg/kg b.w. for dairy cow and 5.67 mg/kg b.w. for veal calves. These estimated exposure levels, based on inclusion rates aligned with the current regulatory limit of 300 mg/kg, fall below thresholds associated with observed effects in the literature. Specifically, previous studies showed no health effects in cows at around 23 mg/kg b.w. [30], with milk yield and composition affected only at higher intakes of 14 to 45 mg/kg b.w. [31]. Calves exhibited toxicity only at higher doses of 45 to 90 mg/kg b.w. [32]. These findings indicate that the current regulatory limit and calculated CH inclusion rates ensure low risk exposure to theobromine in both dairy cows and veal calves.

While this study provides valuable insights, it is important to recognize that the analysis was limited to three CHs batches. As such, the findings may not comprehensively reflect the full range of compositional variability that could be present across different sources or production lots. The theobromine content of the raw materials can vary significantly depending on origin, processing, and storage conditions. Furthermore, the effects of feed processing techniques, such as grinding, pelletization, or extrusion, on the stability and bioavailability of theobromine may also influence the actual risk of toxicity in practical feeding scenarios [33]. More research involving a larger sample size, diverse sources of CHs, and different production processes is needed to validate these findings and improve the robustness of animal dietary recommendations.

Previous studies on the influence of CHs on animals, with different inclusion rates, have demonstrated species-specific outcomes. In poultry, CHs affected performance based on inclusion level and treatment: untreated CHs reduced broiler weight and egg production at 4% and 6% inclusion levels [34,35], but hot water-treated CHs could be safely used up to 20% in layers' diets [36,37]. In rabbits, untreated CHs were tolerated at 100 g/kg, and hot water-treated CHs at 200 g/kg, supporting growth and profitability [38]. In pigs, CHs improved gut microbiota and health markers [37], with 20% being optimal as a maize substitute [39]. In aquaculture, cocoa by-products supported growth in Clarias gariepinus [40] and Nile tilapia [41], though bitterness limited the intake. For ruminants, the inclusion of 40% cocoa bean waste (mixture of CHs, cocoa pulp, and cocoa placenta) as a feed source improved daily weight gain and feed efficiency in cattle [42], while the inclusion of 12% of CHs enhanced milk quality in ewes without affecting yield [43]. Early-lactating dairy cows also benefited from CH inclusion without health or methane production [19]. Goats fed diets with CHs and lignocellulosic materials showed favorable weight gains and feed conversion [44], and similar benefits were observed in dairy goats without affecting milk yield [45]. In vitro digestibility studies further confirmed the nutritional value of CHs, particularly when ensiled with poultry manure, which improved nutrient availability, reduced anti-nutritional factors, and demonstrated high organic matter digestibility and metabolizable energy, making it a viable and eco-friendly feed option for ruminants [46]. While these findings highlight the potential of CHs as a sustainable livestock feed ingredient, especially for ruminants, species-specific sensitivities must be taken into account. Notably, dogs are particularly vulnerable, with reported clinical signs, including cardiovascular, neurological, and gastrointestinal effects, after chocolate ingestion [21]. Due to the documented sensitivity of dogs to theobromine toxicity, feed manufacturers exclude cocoa by-products from feeds for dogs [21].

In response to such concerns, several detoxification strategies have been developed to reduce alkaloid levels in cocoa by-products and enhance their safety for use in animal feed [47]. Various strategies have been explored, especially to reduce the theobromine content: physicochemical methods like 15 min boiling have shown a good balance between theobromine reduction and nutrient retention [36], while alkali treatments also reduce theobromine but at the cost of essential nutrients [48]. Biological treatments offer a more nutrient-preserving alternative. Fungi such as *Aspergillus niger*, *Talaromyces verruculosus*, and *T. marneffei* have demonstrated strong detheobromination potential [49,50]. *T. verruculosus* TvTD was also identified as a suitable bio-tool for cocoa by-product detoxification [51], with additional evidence suggesting it also degrades other methylxanthines like caffeine. However, many regulatory authorities require a thorough evaluation of the safety and efficacy of microbial detoxification products before they can be approved for use [52]. In parallel to detheobromination, supercritical $CO_2$ extraction has been used to selectively remove caffeine up to 80% from cocoa products while retaining theobromine, polyphenols, and antioxidant activity [53].

Besides considering safety, toxicological limits, and detoxification strategies, nutritional considerations and the potential variations in caffeine and theobromine content in different batches and sources of these by-products must also be considered during the diet formulation phase to ensure accurate and safe dietary practices. Moreover, it is crucial to acknowledge the challenges associated with variability in CHs sourced from complex supply chains, such as those of cocoa production. The process of acquiring cocoa from multiple suppliers in different countries worldwide creates a complex network for large production chains [54]. Cocoa production companies prioritize consistency in their products over time, which requires suppliers to deliver cocoa with uniform characteristics [55]. This commitment to product uniformity also extends to by-products like CHs, where consistent quality standards are maintained. Nevertheless, conducting quality assurance analyses alongside safety assessments is important to ensure that these standards are consistently met and to prevent the distribution of animal feed products that may contain undesirable substances (e.g., theobromine) with levels exceeding the EU's ML, as prohibited in Directive 2002/32/EC.

### 2.3. Screening of Other Constituents of Nutritional Interest by High-Performance Thin-Layer Chromatography (HPTLC)

HPTLC analysis allowed for the identification and semi-quantification of the phenolic compounds present in the samples by derivatization with Fast Blue B Salt, while the overall antioxidant activity was appreciated by derivatization with the DPPH solution.

The HPTLC patterns, detected at visible light, are shown in Figure 3.

The samples, illustrated in Figure 3a, have a significant content of phenolic compounds, which is higher for sample S3 when compared to S2 and S1. The epicatechin band is present in all the samples (Rf = 0.46). Considering Figure 3b, all the standards included in this study, except for caffeine, have antioxidant capacity, as shown by the discoloration of the corresponding bands. All samples (Figure 3b) show a considerable presence of antioxidant molecules and, in particular, of protocatechuic acid (Rf = 0.59) and epicatechin (Rf = 0.45). Epicatechin is a flavanol, a subgroup of flavonoids, present in several foods, among which the main source is cocoa [56]. In 2012, the EFSA Panel on Dietetic Products, Nutrition, and Allergies published a Scientific Opinion on the health claim related to cocoa flavanols, concluding that they help to maintain endothelium-dependent vasodilation, which contributes to normal blood flow [57]. This reinforces the hypothesis that polyphenolic compounds are integral to the antioxidant efficacy of cocoa by-products, thus underscoring their potential benefits. In the literature, the inclusion of polyphenols in feed rations showed different positive effects, such as enhancing the oxidative stability of meat and meat products and reducing the number of additives, like vitamin E and other synthetic antioxidants [58].

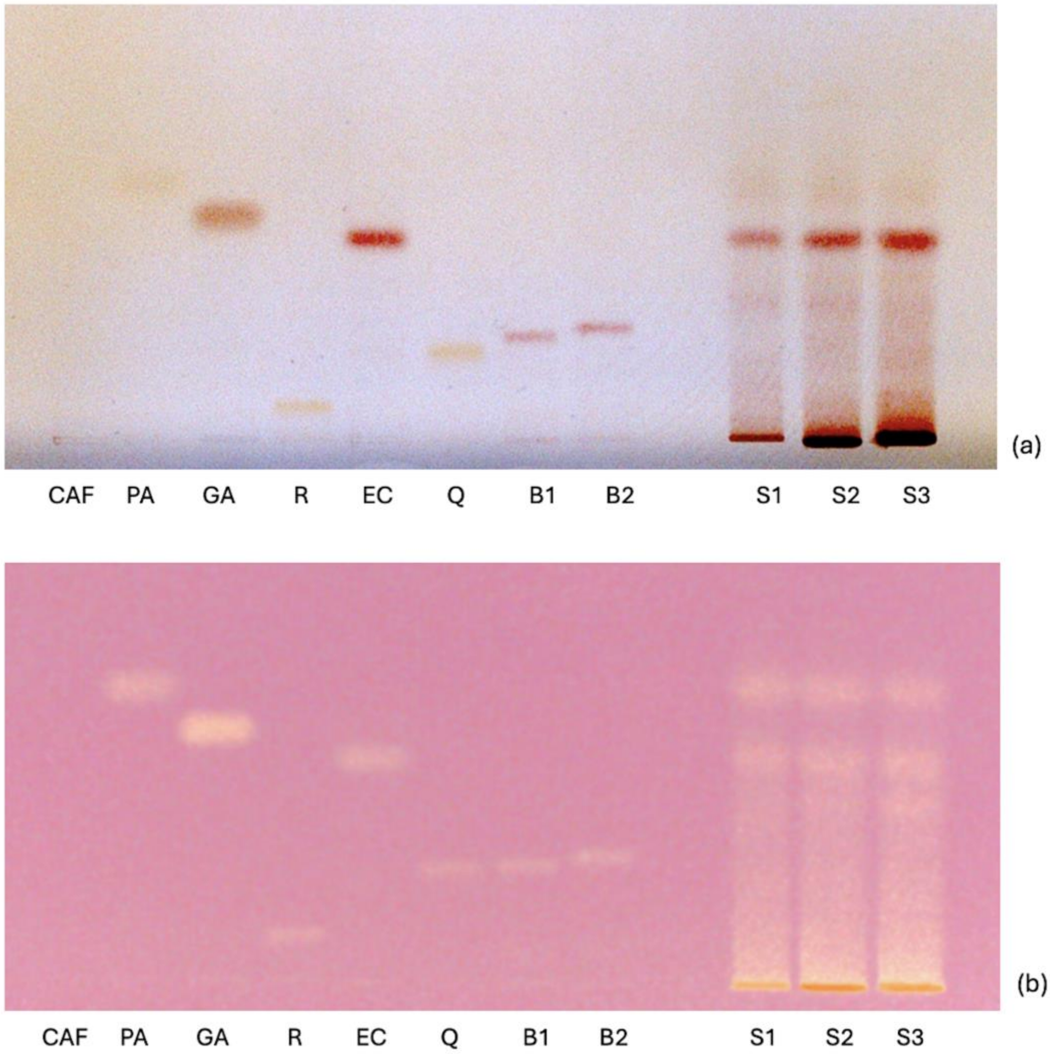


Figure 3. HPTLC plate after derivatization with Fast Blue B Salt (a) and DPPH (b), detected by visible light. CAF: caffeine; PA: protocatechuic acid; GA: gallic acid; R: rutin; EC: epicatechin; Q: quercetin; B1: procyanidin B1; B2: procyanidin B2; S1: sample 1; S2: sample S2; S3: sample S3.

**3. Conclusions**

Our study investigated the potential of CHs, a cocoa by-product, as a source of phenolic compounds and antioxidant activity, although the associated alkaloid content must be suitably controlled. An HPLC method for theobromine and caffeine quantification was validated, and the analysis confirmed theobromine as the predominant analyte. S3 contained the highest concentrations of all analyzed compounds compared to S1 and S2. The HPLC quantification of theobromine showed that theobromine is a limiting factor for including CHs in animal diets. The inclusion of CHs analyzed in this study should not exceed 7.14% for S1, 7.42% for S2, and 5.49% for S3 in the ruminant diet to meet the EU's maximum theobromine limits in complete feed. These inclusion values are specific to the limited sample size of CHs examined in this study and may not reflect the broader variability of theobromine content in CHs. Such variation may also arise from the different geographic origins of the raw materials and the influence of different processing methods. General estimates of dietary theobromine exposure for dairy cows and veal calves were performed, confirming a low risk to animal health when the CH inclusion levels are based on regulatory limits. In addition, the HPTLC analysis confirmed the presence of phenolic compounds and the associated antioxidant capacity in all samples. Further research is needed to support broader dietary recommendations, including CH inclusion rates in animal diets. Adjustments should also be made when other sources of theobromine, such as tea leaves or herbal feed supplements, are present in the diet. Additionally, this study suggests that establishing maximum caffeine limits in feed, as they exist for theobromine, would contribute to ensuring animal safety in response to evolving feed practices, such as the use of food by-products in feed.

## 4. Materials and Methods

### 4.1. Materials

Methanol, ethanol, HPLC-grade water, acetonitrile, acetone, toluene, n-hexane, reagents for analysis, and acids were purchased from VWR International (Fontenay-sous-Bois, France). 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH), Fast Blu B Salt, caffeine, theobromine, epicatechin, gallic acid, procyanidin B1, procyanidin B2, quercetin, and rutin were purchased from Sigma Aldrich (Merck, Steinheim, Germany).

### 4.2. Samples, Particle Size Determination, and Extraction Method

CHs included in this study were provided by a Swiss former foodstuff processor and consisted of sample 1 (S1) and sample 2 (S2), from different batches of flake-form product, and sample 3 (S3) in pellet form. All samples were maintained at −20 °C upon receipt and until use. The particle size of samples was determined upon arrival by dry sieving analysis for flake samples and wet sieving for pellet particle size, performed according to ISO 2591-1 standard [59], using laboratory sieves ranging from 125 to 4000 µm (Endecotts Ltd., London, UK). Sieving analysis of CHs (Table 3) showed the same particle size distribution in S1 and S2. This was expected since the different samples underwent the same flaking process, while S3 underwent a pelletization process.

Table 3. Cumulative particle size (µm) of samples S1, S2 and S3 expressed as mean (%) ± SD; $n = 3$. Values with the different letters are statistically different ($p < 0.001$).

| Sieve Openings (µm) | Cumulative Particle Size | | |
|---|---|---|---|
| | S1 | S2 | S3 |
| 4000 | 100 | 100 | 100 |
| 2000 | 85.15 ± 0.09 [a] | 85.47 ± 0.08 [a] | 98.89 ± 0.10 [b] |
| 1000 | 33.93 ± 0.11 [a] | 33.69 ± 0.30 [a] | 97.31 ± 0.14 [b] |
| 800 | 14.69 ± 0.16 [a] | 14.24 ± 0.13 [a] | 95.25 ± 0.04 [b] |
| 630 | 11.63 ± 0.26 [a] | 11.64 ± 0.06 [a] | 93.94 ± 0.06 [b] |
| 400 | 9.40 ± 0.20 [a] | 9.78 ± 0.01 [a] | 6.40 ± 0.41 [b] |
| 250 | 5.72 ± 0.13 [a] | 6.60 ± 0.07 [a] | 3.70 ± 0.33 [b] |
| 125 | 3.33 ± 0.10 | 4.26 ± 0.04 | 0.00 |
| 0 | 0.61 ± 0.09 | 0.71 ± 0.03 | 0.00 |

To ensure uniformity and accuracy in analytical testing, samples were homogenized. Each (0.5 g) sample was defatted 4 times with 5 mL of n-hexane by mechanical stirring. Five mL of 80:20 (*v*/*v*) methanol/water solution was added to the defatted samples, homogenized with Ultra-Turrax (IKA T-25; IKA. Staufen, Germany) at 17,000 rpm for 2 min, and centrifuged at 3000× *g* at 4 °C for 14 min (Centrifuge 5810R, Eppendorf, Hamburg, Germany). The extract was then filtered with a 0.45 µm PTFE filter (VWR, Fontenay-sous-Bois, France) and kept at −20 °C until analyzed. To evaluate the reproducibility of the extraction, the process was repeated three times. Each analysis was then performed in triplicate.

### 4.3. High-Performance Thin-Layer Chromatography (HPTLC)

HPTLC is a chromatographic technique that allows for the separation of compounds based on the different affinity of the analytes for the stationary phase (silica gel) and the mobile phase. Through HPTLC analysis, it is possible to carry out qualitative and semi-quantitative analyses regarding the content of phenolic compounds and the antioxidant capacity of the samples [60].

Aliquots of 5 µL of standard solutions (gallic acid, caffeine, epicatechin, procyanidin B1, procyanidin B2, quercetin, and rutin) at the concentration of 200 µg/mL were loaded onto HPTL silica gel plates 60 F254 (dimensions: 10 × 20 cm, manufacturer: Merck, Darmstadt, Germany) by a semi-automatic sample applicator (Linomat 4, CAMAG, Muttenz, Switzerland).

Sample volumes of 10 µL, prepared as described in Section 4.2, were also loaded onto the plates following the procedure published by Colombo and colleagues [61].

After the chromatographic run, where the mobile phase consisted of 10 mL of acetone/toluene/formic acid (in a ratio of 4.5:4.5:1 *v*/*v*/*v*), the plates were exposed to UV light at 254 and 366 nm. Subsequently, they were derivatized with a 0.05% DPPH methanolic solution, kept in the dark for 30 min, and then examined under visible light using VisionCats software v. 1.3.12236.2 (CAMAG, Muttenz, Switzerland). The same operating protocol was also performed for the plate derivatized with Fast Blue B Salt (dianisidine-bis-(diazotised)-zinc double salt).

### 4.4. High-Performance Liquid Chromatography Coupled with Ultraviolet Detection (HPLC-UV)

An HPLC method coupled with an ultraviolet (UV) detector was employed for the quantification of theobromine and caffeine. The gradient elution, set at a flow rate of 1 mL/min, was obtained by mixing the following mobile phases: A, formic acid/water (0.5% *v*/*v*); B, formic acid/acetonitrile (0.5% *v*/*v*). The gradient was set up as follows: 0–30 min: 90–75% A; 30–35 min: 75–0% A; 35–39 min: 0% A isocratic; 39–40 min: 0–90% A; 40–50 min: 90% isocratic A. The UV detector was set at 280 nm, and the column was kept at room temperature (≅25 °C). The method was validated according to the Food and Drug Administration (FDA) Guidelines on Bioanalytical Method Validation [26] by calculating their linearity, sensitivity, recovery, stability, and precision. Linearity was estimated by the correlation coefficient ($R^2$). Sensitivity was evaluated by determining the limit of detection (LOD) and quantification (LOQ) at a signal-to-noise ratio of 3 and 10, respectively. The recovery was assessed as extraction efficiency by adding different concentrations of the standard solutions to sample S2. The stability of standards was evaluated in the aliquot extracted and maintained at −20 °C after different storage times (24 h and 20 days). Precision was assessed by calculating the intraday and interday precision expressed as a coefficient of variation (CV%).

Standard stock solutions were prepared at a concentration of 500 μg/mL in methanol/water 80:20 (*v*/*v*). Each standard was suitably diluted to its final concentration range of 10–100 μg/mL in methanol/water 80:20 (*v*/*v*). All solutions were stored at −20 °C until use. Samples were prepared as described in Section 4.2, analyzed in triplicate ($n$ = 3) as such, suitably concentrated 3:1 or diluted 1:10, and added with methanol/water 80:20 (*v*/*v*) before the analysis.

The chromatographic separations were carried out on a reversed-phase YMC-Triart C18 column (250 mm, particle size 3.0 μm). The HPLC equipment (Jasco, Tokyo, Japan) consisted of two pumps (model PU-1580), an interface (LC-NETII/ADC), an autosampler (model AS-2059 plus), a degasser (DG-2080-54), a UV detector (model UV-975), and an injection valve (Rheodyne, Cotati, CA, USA) with a 100 mL loop. ChromNAV software v.1.18.03 (Jasco, Tokyo, Japan) was used for data acquisition and processing.

The following formula was used to compute the permissible percentage of samples to be included in feed to comply with the EU ML set for theobromine of 300 mg/kg; exceptions exist for pigs, dogs, rabbits, horses and fur animals, for which lower levels are set (Directive 2002/32/EC) [28]:

$$\text{\% permissible CHs in feed: ML/TC} \times 100 \tag{1}$$

where ML: EU maximum level for theobromine (mg/kg); TC: theobromine content (mg/kg).

The following formula was used to estimate animal dietary exposure [29]:

$$\text{Estimated daily animal dietary exposure: (TCF} \times \text{FI)/b.w.} \tag{2}$$

where TCF: theobromine content in feed (mg/kg); FI: feed intake (kg/day); b.w.: body weight (kg).

### 4.5. Statistical Analysis

Descriptive statistical analyses for calculating the mean values, the standard error of the mean and the correlation coefficient ($R^2$) were performed using IBM SPSS Statistics for Macintosh software v. 29.0.2.0 (IBM Corp, Armonk, NY, USA). In addition, data were analyzed with one-way ANOVA when normality and homogeneity of variances were met. Normality was assessed using the Shapiro–Wilk test, while homogeneity of variances was evaluated with Levene's test. For data that did not meet these assumptions, the Kruskal–Wallis test was used. Significant differences were determined by post hoc Tukey test for ANOVA and Bonferroni for the Kruskal–Wallis test, with significance set at $p < 0.05$.

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**Abbreviations:**

| | |
|---|---|
| ADI | Acceptable daily intake |
| b.w. | Body weight |
| CAF | Caffeine |
| CH | Cocoa hull |
| CSN | Central nervous system |
| CV | Coefficient of variation |
| DPPH | 1,1-diphenyl-2-picryl-hydrazyl free radical |
| EC | Epicatechin |

| | |
|---|---|
| EU | European Union |
| EFSA | European Food Safety Authority |
| FDA | Food and Drug Administration |
| HBA | p-hydroxybenzoic acid |
| HPLC | High-Performance Liquid Chromatography |
| HPTLC | High-Performance Thin-Layer Chromatography |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| ML | Maximum level |
| NOAEL | No Observed Adverse Effect Level |
| *Rf* | Ratio frontis |
| Rt | Retention time |
| SD | Standard deviation |
| TB | Theobromine |
| TC | Theobromine content |

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# CHAPTER 2

## - Animal dietary exposure to methylxanthines through the inclusion of Former Food Products (FFPs) in feed

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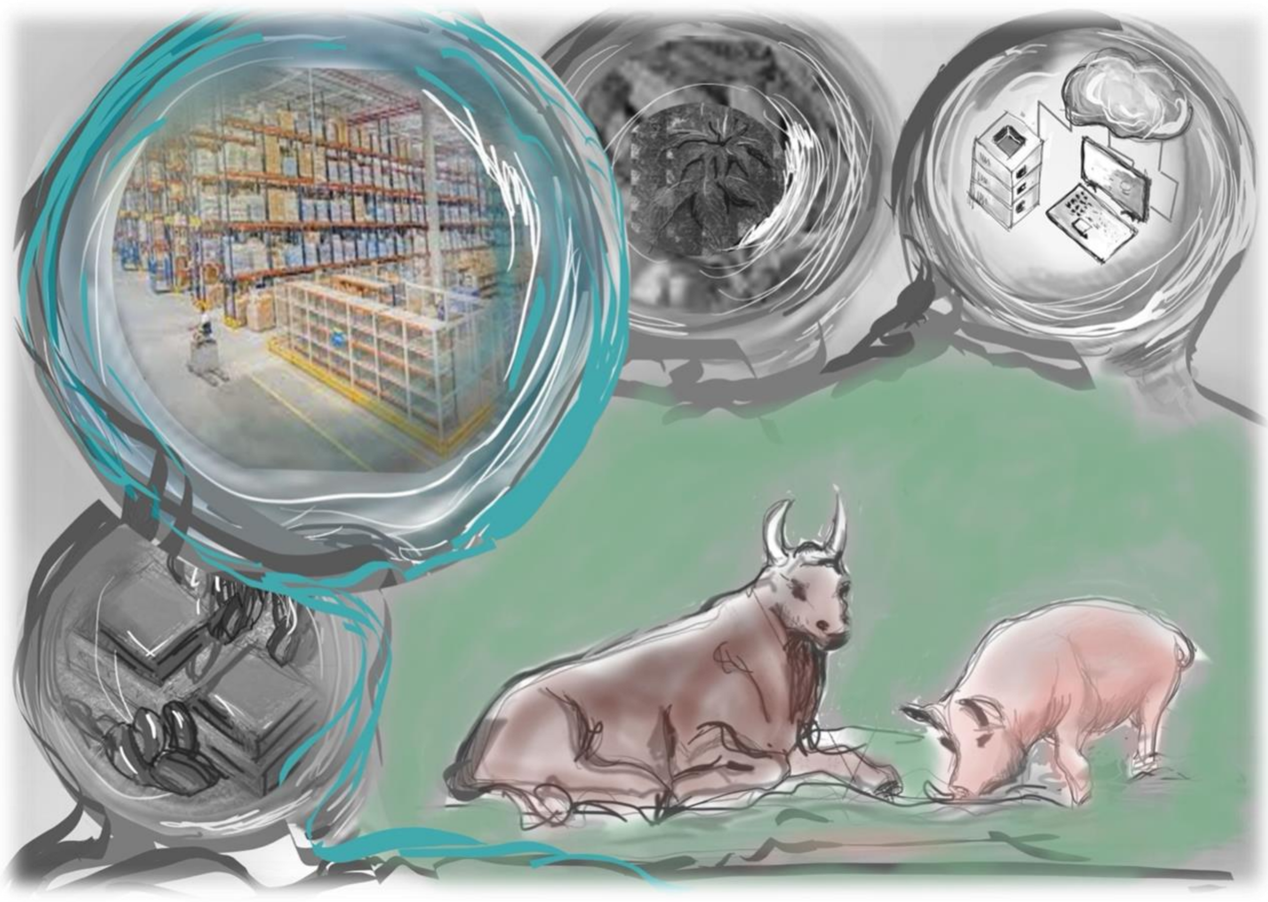

# Animal dietary exposure to methylxanthines through the inclusion of Former Food Products (FFPs) in feed

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**Abstract:** Former food products (FFPs) are increasingly recognized as sustainable feed ingredients. While nutritionally valuable, FFPs may contain cocoa-based confectionery, which is a source of methylxanthines such as theobromine (TB) and caffeine (CF) and can impact animal health. This study quantified TB and CF concentrations in 12 FFPs using HPLC-UV, evaluated FFP inclusion rates in animals' diets against European Union (EU) maximum levels (MLs), and dietary exposure against toxicological thresholds. TB levels ranged from 59.6 to 1147.1 $\mu$g/g and CF from 9.3 to 118.1 $\mu$g/g. All products, except one, complied with EU MLs when included at 30% in the diet (on a dry basis). Modeled animal dietary exposure (ADE) indicated that, in most proposed species, TB intake was below safety thresholds; however, the maximum exposure scenarios in piglets exceeded reported no-observed adverse effect levels (NOAEL). These findings highlight the need for species-specific and production-stage-specific evaluations and accurate quantification of methylxanthines when formulating diets with FFPs.

**Keywords**: ex-food, theobromine, alkaloids, food sustainability, food losses and food waste.

## Introduction

In recent years, the global agri-food system has faced growing pressure to adopt more sustainable and circular production models in response to food waste, environmental degradation, and resource scarcity.[1] In September 2015, as part of the 2030 Sustainable Development Goals, the United Nations General Assembly adopted a target of halving per capita food waste at the retail and consumer level and reducing food losses along production and supply chains.[2] One of the central promoted strategies is the valorization of food losses and food waste (FLW) through their reintegration into productive chains. In this context, former food products (FFPs) refer to foodstuffs originally intended for human consumption but, due to various nonsafety-related reasons such as packaging errors, aesthetic flaws, or logistical oversupply, are no longer marketable for humans (consolidated text of Commission Regulation (EU) No. 68/2013).[3] Despite their removal from the retail food chain, FFPs often retain a high nutritional value, including significant levels of starch, sugars, and fats, making them promising alternative feed ingredients.[4] FFPs may include different processed food items such as bread, pasta, biscuits, snacks, and confectionery products.[5]

These materials, although technically safe for consumption, are often discarded by the food industry due to visual or packaging defects, as the costs associated with repackaging or reprocessing exceed their commercial value. While these items may still be safe for consumption, they often fail to reach consumers due to logistical challenges or regulatory constraints.[6] In 2018, the European waste legislation was amended and announced new measures for waste prevention, including the exclusion of materials of nonanimal origin destined as feed from its scope.[7,8] The amendment reduced the regulatory burden on operators by removing the need to comply with waste legislation for food of nonanimal origin intended for feed.[9] This facilitates the reuse of such materials, supports circular economy practices, and eliminates legal inconsistencies among European Member States. Given this context, the integration of FFPs into animal feed has emerged as a promising circular economy strategy.

According to Commission Regulation (EU) No. 68/2013 on the Catalogue of Feed Materials, former foods (e.g., products from the bakery, pasta, pastry, and confectionery industry and breakfast cereal manufacture) are explicitly listed in the Catalogue of Feed Materials. However, FFPs may contain compounds naturally present in the original food matrix, which can represent an area of concern, particularly when FFPs include chocolate- and cocoa-based confectionery, which are well-documented sources of methylxanthines such as theobromine (TB) and caffeine (CF). These compounds can exert bioactive effects on animals, potentially affecting the cardiovascular, nervous, and gastrointestinal systems.[10–16] The primary mechanism of action for methylxanthines has long been recognized as the inhibition of adenosine receptors. Interestingly, theobromine exhibits significantly lower affinity for adenosine receptors compared to caffeine.[17] Nevertheless, caffeine and theobromine display varying affinities for different adenosine receptor subtypes, which may help explain why these two compounds produce distinct physiological effects.[12] In particular, TB has stronger coronary vasodilator and cardiac stimulation effects than CF, while CF is a stronger central nervous system, respiratory, and skeletal muscle stimulant and has mildly stronger diuresis effects than TB.[10]

In the European Union (EU), animal feed is considered noncompliant if the concentration of undesirable substances exceeds the maximum levels (MLs) specified in Annex I of Directive 2002/32/EC of the European Parliament and Council, as last amended in 2019 (Art. 3.2).[18] TB is classified among these undesirable substances (Annex I, Directive 2002/ 32/EC). When this Directive first entered into force in May 2022, it reported generally higher theobromine EU MLs than the MLs in force nowadays. The MLs were 300 mg/kg in complete feedstuffs, with the exception of feed intended for adult cattle, which had an even higher ML of 700 mg/kg. After the EFSA CONTAM Panel highlighted that existing TB MLs may be insufficient to safeguard certain species,[19] the Commission decided to lower TB MLs.[20] To date, the current ML is 300 mg/kg for complete feed with 12% moisture content, except for pigs, where the limit is 200 mg/kg, and for dogs, rabbits, horses, and fur animals, where the limit is set at 50 mg/kg. Unlike TB, Directive 2002/32/EC does not include caffeine in the list of undesirable substances in feed, although the ingestion of caffeine-rich coffee coproducts (e.g., coffee husks and coffee pulp) has shown undesirable effects, including a decrease in weight gain, feed conversion, and metabolized energy in poultry[21] and toxicosis cases given by coffee husks in horses.[22]

From a nutritional perspective, FFPs are highly variable, although former foodstuff processors have established methods to produce consistent commercial products.[23] FFPs are characterized by high energy density, abundant simple carbohydrates, and elevated fat content due to their processed nature. These properties make them particularly suitable for inclusion in livestock diets under controlled conditions.[4,24] Studies have shown no adverse effects on gastrointestinal health, microbiota, growth performance,[25] and meat quality,[26] and no undesirable effects on physiology and metabolism, especially considering the plasma metabolome[27] and liver proteome[28] from postweaning to finishing pigs fed diets partially replacing common feed materials with FFP. Additionally, FFPs, even in combination with cocoa shells, did not impair the metabolic health or ruminal fermentation of early lactating cows[29] and did not cause any significant difference in terms of feed intake, body condition score, milk yield, and milk quality in lactating buffaloes.[30] Overall, from a nutritional point of view, FFPs may help reduce food and feed competition without compromising animal productivity and health. However, their use requires careful regulation of composition and inclusion rate into the animal diet.[31] In addition to nutritional adequacy, the sustainability implications of using FFPs as feeds are significant. Life cycle assessments indicate that diverting FFPs to animal feed conserves water and reduces environmental burdens[32] compared to alternative disposal methods such as the production of electricity, heat, and digestate.[33] From a feed safety perspective, FFPs must comply with comprehensive feed regulatory frameworks. Specifically, Regulation (EC) No. 183/2005 mandates that all feed business operators, including former foodstuff processors, implement Hazard Analysis and Critical Control Point (HACCP) principles to ensure feed hygiene and safety throughout the production chain.[34] Additionally, FFPs must adhere to Regulation (EC) No. 178/2002, which establishes general principles of food law, including traceability and the obligation to withdraw unsafe products from the market. Moreover, Regulation (EC) No. 767/2009[35] on the placing on the market and use of feed also fully applies to former foodstuff processors, outlining essential requirements, including accurate and transparent labeling, claims, and conditions of use, ensuring that end users receive safe, appropriately described products.[36]

Regulatory frameworks, such as those established following the Bovine Spongiform Encephalopathy (BSE) crisis, restrict the use of certain waste-derived materials, especially those containing animal byproducts.[37] Additionally, the boundary between feed-eligible surplus and outright waste is tightly regulated, with postconsumer waste (e.g., leftovers from canteens or households) excluded from animal feed chains.[7,38] In this study, the aim was to investigate the presence of TB and CF in 12 FFPs, from FFP processors based in different EU countries, to derive dietary exposure to target animals and draw safety considerations on FFP implementation in animals' diets based on methylxanthine content. This assessment is particularly relevant in the context of Directive 2002/32/EC, which establishes species-specific thresholds and the maximum levels for undesirable substances in animal feed, including TB, to guarantee animal health and feed safety.

## Materials and Methods

### Chemicals

Caffeine and theobromine standards were purchased from SigmaAldrich (Merck, Steinheim, Germany). Methanol, ethanol, HPLC grade water, acetonitrile, acetone, toluene, *n*-hexane, ethyl acetate, reagents for analysis, and acids were purchased from VWR
International (Fontenay-sous-Bois, France).

### Samples

Twelve FFP samples (Figure 1) were collected within the framework of a voluntary campaign conducted across European former food processors to collect FFPs and contribute to the development of a realistic overview of FFPs currently available on the market for feed applications. FFP1, FFP2, and FFP3 consist of sweets, baked products, pastry items, and cereal products such as wheat flour and bran. FFP4 includes puffed and extruded rice and corn cakes, while FFP5 comprises different products from the food industry, including pasta, baked goods, sweets, and pastry products. FFP6 and FFP10 primarily contain bakery products, including biscuits. FFP7, FFP8, and FFP9 derive from confectionery items and food byproducts. FFP11 includes bread, pasta, and savory baked snacks, while FFP12 includes chocolate and biscuits, and it is formulated for piglets. In addition to the FFPs, a negative control (NC) containing no former food material and composed of a conventional postweaning piglet diet (see Mazzoleni et al.[25]), and a positive control (PC) composed of cocoa shells, as expected to contain TB and CF, were included in this study. All samples were milled using a 1 mm screen mill (Model 160D, Jacobsen Machine Works, Minneapolis, MN, USA) and stored at 4 °C until analysis.


Figure 1: FFP samples (FFP1-FFP12), positive control (PC) and negative control (NC)

**Chemical composition analysis**

Samples were analyzed for moisture, starch, sugars, crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), and ash using standardized procedures based on the Association of Official Analytical Chemists[39] and European Commission regulation 152/ 2009.[40] Samples FFP1-FFP6 were previously analyzed in the context of other studies.[41,42] Moisture content was determined by oven-drying at 130 °C for 2 h, following Commission Regulation No. 152/2009. Results were expressed on a dry matter (DM) basis. Starch was measured using a polarimetric method as per European Commission guidelines,[40] and sugar content was determined according to the official method outlined in the same regulation. Crude protein was analyzed using the Kjeldahl method 2001.11.[39] Ether extract was determined via the Soxhlet method after acid hydrolysis, following Commission Regulation No. 152/2009.[40] Neutral detergent fiber (NDF) was analyzed using an Ankom 220 fiber analyzer (ANKOM Technology, Fairport, NY, USA) according to AOAC method 2002.04, with the use of heat-stable amylase, and results were expressed without including residual ash (aNDFom). Ash content was measured by combustion in a muffle furnace at 550 °C[39] (method 942.05).

**Extraction method for the chromatographic techniques**

One gram of each ground sample was defatted by mechanical stirring with 5 mL of *n*-hexane, repeated four times until clear. After defatting, 10 mL of an 80:20 (v/v) methanol:water solution was added to the samples for overnight extraction under continuous stirring by a rotary plate (ASAL srl, Milano, Italia). The samples were centrifuged at 3000*g* at 4 °C for 15 min (Centrifuge 5810R, Eppendorf, Hamburg, Germany). The resulting extract was filtered through a 0.45 $\mu$m PTFE filter (VWR, Fontenay-sous-Bois, France) and stored at −20 °C until analysis.

### High Performance Thin Layer Chromatography analysis

High-performance thin-layer chromatography (HPTLC) is a chromatographic technique that allows a relatively fast, accurate, and cost-efficient detection of compounds.[43]

In this study, HPTLC was used for the initial screening of CF and TB in the FFP samples. Extracted samples were dried by nitrogen flow and suspended in methanol. Ten $\mu$L of samples (2.0 g/mL) and standards of CF and TB (0.5 mg/mL) were loaded onto HPTLC silica-gel plates 60 F254 (dimensions: 10 × 20 cm, manufacturer: Merck, Darmstadt, Germany) with a semiautomatic sample applicator (Linomat 4, CAMAG, Muttenz, Switzerland). The mobile phase consisted of 10 mL of water:methanol:ethyl acetate 0.7:1.1:8.2, *v/v/v*. The chromatographic run was revealed at 254 nm (Software VisionCats, CAMAG, Muttenz, Switzerland).[43]

### High-Performance Liquid Chromatography analysis

High-performance liquid chromatography coupled with ultraviolet detection (HPLC-UV) is a widely utilized analytical technique for quantifying compounds that absorb UV light. In this study, the quantification of TB and CF was conducted following the procedure described by Mercogliano and colleagues,[44] with minor adaptations for the present matrices. Chromatographic separations were performed using a reversed-phase YMC-Triart C18 column (250 mm length, 3.0 $\mu$m particle size). The HPLC system (Jasco, Tokyo, Japan) included two PU-1580 pumps, a DG-2080-54 degasser, an AS2059 Plus autosampler, a UV-975 UV detector, and an LC-NETII/ ADC interface coupled with a Rheodyne injection valve (Cotati, CA, USA) equipped with a 100 $\mu$L loop. Data acquisition and processing were carried out by using ChromNAV software (Jasco, Tokyo, Japan). The mobile phases consisted of (A) 0.5% (v/v) formic acid in water and (B) 0.5% (v/v) formic acid in acetonitrile. Gradient elution was applied at a flow rate of 1.0 mL/min as follows: 0−30 min: 90−75%, A; 30−35 min: 75−0% A; 35−39 min: 0% A isocratic; 39−40 min: 0−90% A; and 40−50 min: 90% isocratic A. The UV detector was set at 280 nm. Standard stock solutions were prepared at a concentration of 500 $\mu$g/mL in methanol:water (80:20, v/v). Working standard solutions were obtained by spiking the blank sample (showing no interferences at analyte retention time) to achieve final concentrations of 0.5−50 $\mu$g/mL for TB and 0.2−4 $\mu$g/mL for CF. Acceptance criteria for linearity were $R^2 \geq 0.99$. Solutions were stored at −20 °C until use. The limit of detection (LOD) and quantification (LOQ) were confirmed at a signal-to-noise ratio of 3 and 10, respectively.[45] Samples were prepared as described in the extraction section and analyzed in triplicate as such or diluted with methanol:water (80:20, v/v) prior to injection.

### Animal dietary exposure assessment

Animal dietary exposure (ADE) to TB and CF was calculated based on default values for animal weight and feed intake used by the EFSA (European Food Safety Authority) Panels on Additives and Products or Substances used in Animal Feed (FEEDAP) and by the Panel on Contaminants (CONTAM).[46,47]

After the quantification of TB and CF in the samples, the corresponding concentration per kilogram of DM feed was calculated as follows:

$$Cfeed, DM = \frac{Csample}{\left(1 - \frac{MoistureFFP}{100}\right)} \times \frac{IR}{100}$$

(1)

where $C_{feed}$, DM (mg/kg DM) is the concentration of TB or CF per kilogram of complete feed in a DM basis, $C_{sample}$ ($\mu$g/g) is the concentration of TB or CF measured in the FFP, $moisture_{FFP}$ (%) is the moisture in the FFP, and IR (%) is the inclusion rate of the FFP in feed on a DM basis.

According to the available literature, FFPs have been proposed as partial substitutes for conventional feed ingredients, with inclusion levels up to 30% (on DM basis) in the diets of ruminants and pigs, not representing a risk mainly in terms of growth performance,[25,29,48,49] product quality,[31] gut health and physiology,[26,50,51] and environmental impact.[31,32,50,51] This level of substitution was therefore considered for the ADE calculations in this study. Ruminants[24,29–31,48,49] and pigs[25,52] were selected as target animal categories since the use of FFPs has been specifically proposed for these animal species, while the literature data on their inclusion in poultry diets remain very limited.[53] In addition, due to the established susceptibility of dogs and horses to theobromine toxicity, cocoa byproducts and confectionery residues are generally not incorporated into their feeds,[19] and therefore, these species were not considered as target animals in this study.

Four scenarios were then evaluated by using the lowest and highest measured TB or CF occurrence values and the median and the mean values across all FFP samples.

The ADE was calculated as follows:

$$ADE = \left(\frac{Cfeed, DM \times FI}{bw}\right)$$

(2)

where ADE (mg/kg of bw/day) is the animal dietary exposure, $C_{feed}$, DM (mg/kg DM) is the concentration of TB or CF per kilogram of complete feed on DM basis, FI (kg/day DM) is the feed intake on DM basis, and bw is the body weight.

The modeled ADE values, derived from the inclusion of FFPs in animals' diets, were compared with published and derived NOAEL and LOAEL (no-observed adverse effect; lowest-observed adverse effect) values to evaluate potential toxicological risks.

**Statistical analysis**

Descriptive statistical analyses of HPLC results (mean values, standard deviation), Pearson correlation coefficient ($R^2$), and Kruskal−Wallis were performed using IBM SPSS Statistics for Macintosh, software 29.0.2.0 (IBM Corp, Armonk, NY, USA). Graphs were generated using GraphPad Prism for MacOS, version 10.3.1. Normality was assessed using the Shapiro−Wilk test, and homogeneity of variances was evaluated with Levene's test. Given the nonnormality, the nonparametric Kruskal–Wallis was used and significant differences were determined by *post hoc* Bonferroni, with the significance set at $p < 0.05$.

## Results and Discussion

### Chemical composition

The nutritional composition of the FFPs (Table 1) demonstrated variability across the samples. Moisture content ranged from 6.2 to 8.7%. Starch concentrations varied markedly, with FFP4 containing mainly rice and corn cakes, reaching 73.4%, while FFP12, which contained biscuits, had the lowest value at 38.6%. Sugar content was generally high in FFPs, reaching a maximum of 27.3% in FFP6, consistent with the literature, which highlights high simple sugar levels in ex- foods.[50] Crude protein averaged approximately 10%, comparable to cereal grains, such as wheat, although FFP12 exhibited higher protein levels (up to 19.3%), in line with the PC (19.0%), both intentionally produced for piglets. Ether extract values ranged from 4.8 to 15.4%, confirming that FFPs are lipid-enriched compared to standard cereal-based feeds.[41] Fiber content was heterogeneous, consisting of crude fiber ranging between 0.6 and 5.5%, while NDF ranged from 5.4 to 32.1%, with FFP8 and FFP12 presenting the highest NDF concentrations. Crude ash content varied from 1.4 to 5.5%. These compositional profiles, particularly elevated starch, fat, and digestible carbohydrate, support the classification of FFPs as a "fortified version of common cereal grains".[52] Such characteristics make FFPs promising energy-dense feed ingredients, although variability across samples suggests careful nutritional considerations.

Table 1: Nutrient composition of FFPs (% DM basis)

| Sample | Moisture | Starch | Sugars | Crude protein | Ether extract | Crude fibre | Neutral detergent fibre | Ash |
|---|---|---|---|---|---|---|---|---|
| **FFP1** | 7.4 | 45.9 | 25.7 | 9.2 | 11.7 | 2.7 | 10.1 | 2.2 |
| **FFP2** | 8.7 | 42.3 | 22.1 | 11.5 | 11.0 | 4.4 | 15.7 | 3.2 |
| **FFP3** | 8.5 | 44.6 | 20.1 | 12.0 | 9.9 | 4.5 | 16.2 | 3.4 |
| **FFP4** | 8.1 | 73.4 | 12.4 | 9.1 | 4.8 | 1.9 | 5.4 | 1.4 |
| **FFP5** | 8.7 | 56.4 | 20.2 | 10.9 | 10.4 | 1.5 | 7.7 | 2.1 |
| **FFP6** | 6.2 | 51.5 | 27.3 | 7.3 | 12.9 | 1.3 | 5.6 | 1.8 |
| **FFP7** | 7.8 | 42.6 | 11.2 | 11.7 | 11.7 | 3.3 | 21.9 | 3.5 |
| **FFP8** | 8.7 | 40.6 | 12.3 | 12.9 | 8.0 | 5.5 | 32.1 | 3.7 |
| **FFP9** | 8.5 | 40.3 | 10.4 | 12.8 | 10.1 | 4.1 | 25.5 | 3.8 |
| **FFP10** | 6.9 | 49.0 | 20.0 | 9.4 | 15.4 | 0.6 | 8.5 | 1.9 |
| **FFP11** | 7.3 | 51.5 | 14.9 | 9.5 | 12.5 | 0.7 | 16.3 | 3.5 |
| **FFP12** | 8.7 | 38.6 | 11.7 | 19.3 | 6.1 | 3.1 | 28.7 | 5.5 |
| **PC** | 10.4 | <3.2 | 1.0 | 19.0 | 8.9 | 16.2 | 48.6 | 8.2 |
| **NC** | 9.9 | 39.9 | 4.69 | 19.1 | 3.9 | 3.1 | 11.2 | 6.1 |

**Screening for theobromine and caffeine by HPTLC**

HPTLC was employed for quick qualitative screening to identify the possible presence of TB and CF in FFPs. The chromatographic analysis (Figure 2) revealed distinct retention factor (Rf) values for both TB (Rf = 0.40) and CF (Rf = 0.46), enabling their identification based on comparison with known standards. In this preliminary screening, TB was identified in 11 FFP samples among the 12 analyzed and was not detected in FFP4 and the NC. CF was only clearly visible in the PC. Given these results, quantification of TB and CF was performed by HPLC for more sensitive and quantitative analysis.

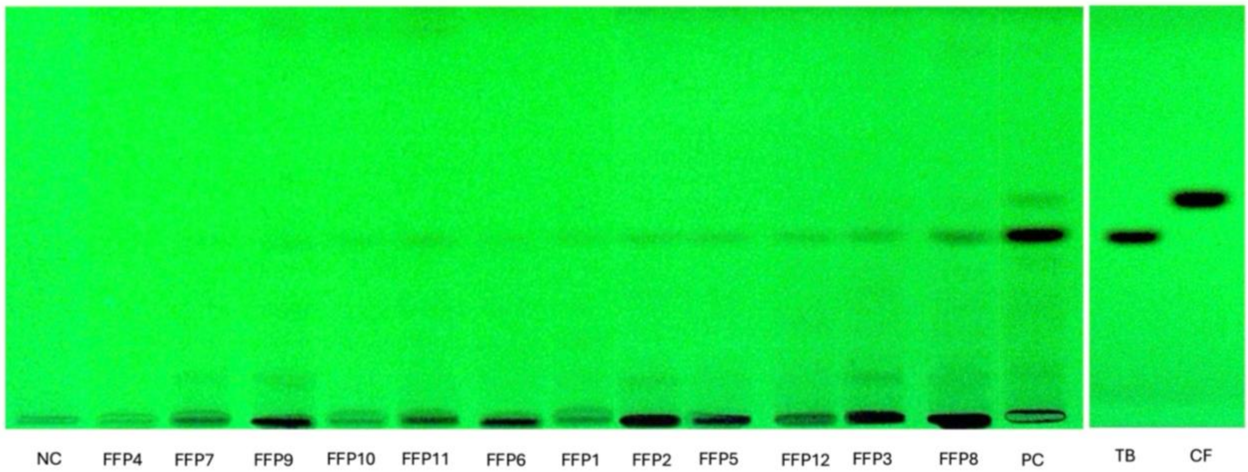


Figure 2: HPTLC plates detected at 254 nm. TB: theobromine; CF: caffeine.

**Quantification of theobromine and caffeine in FFPs and resulting concentrations in feed**

HPLC-UV analysis was carried out to quantify TB and CF across the FFP samples. The chromatographic profiles, shown in Figure 3, displayed a first integrated peak, corresponding to TB, at a retention time (Rt) of 6.8 min and a second integrated peak corresponding to CF with an Rt of 13.6 min in all FFP samples and PC, while no peaks were detectable in the NC.

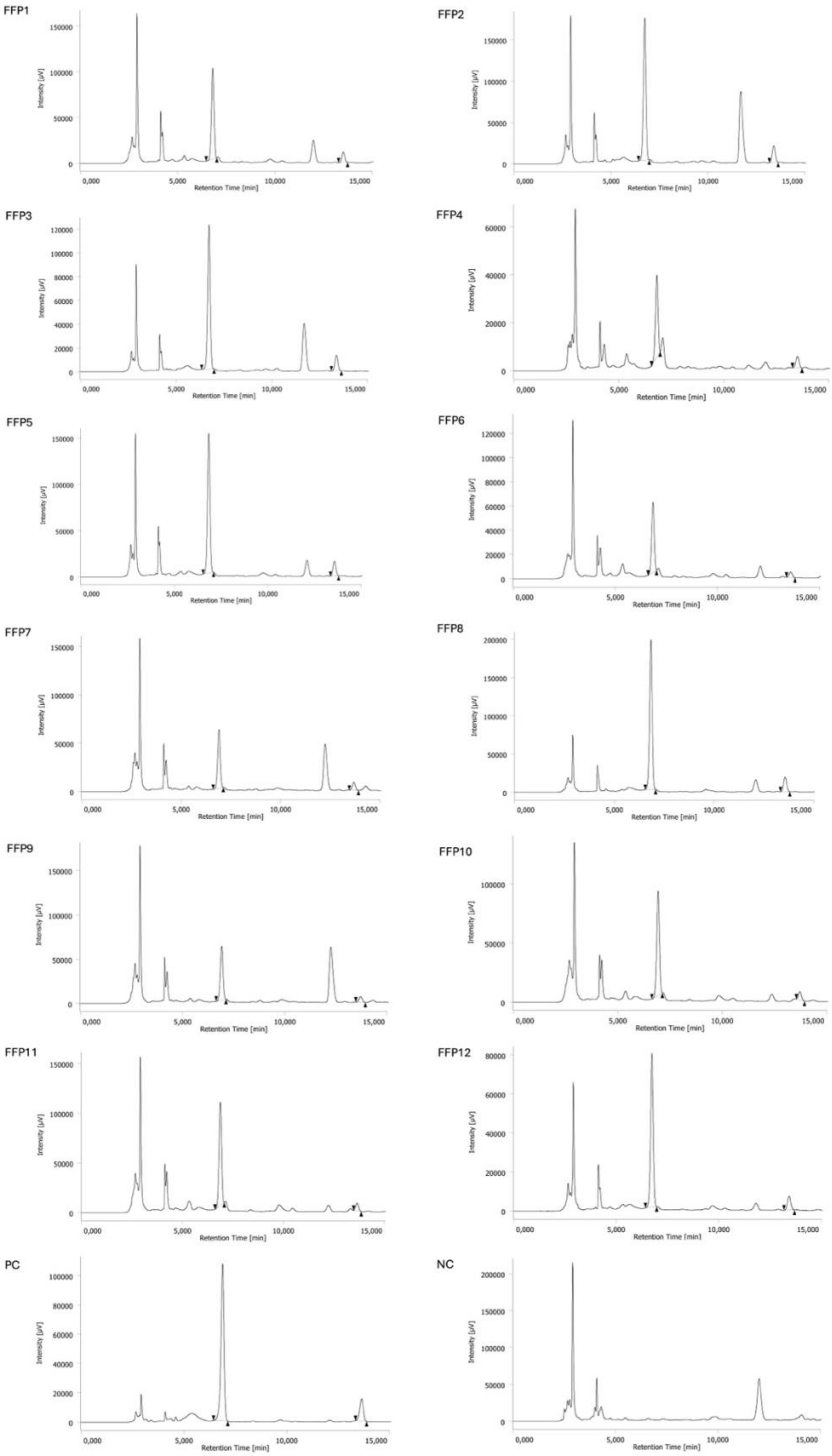


Figure 3. Chromatograms of FFP samples, PC and NC. The first integrated peak corresponds to TB, and the second integrated peak corresponds to CF.

Quantitative results of TB and CF, reported in Table 2, are expressed as the mean ± standard deviation ($\mu$g/g) and TB/ CF ratio.

Table 2. Content of TB and CF in FFPs expressed as mean ± SD (μg/g); $n$=3; values with the different letters are statistically significant ($p < 0.05$) within the FFP samples. LOD for TB: 0.03 μg/g; LOD for CF: 0.1 μg/g. IR%: selected inclusion rate; TB/CF: TB and CF ratio; TB and CF in feed: resulting mg of TB and CF concentrations per kilogram of feed with a moisture content of 12%.

| **Sample** | **TB (μg/g)** | **CF (μg/g)** | **TB/CF ratio** | **IR%** | **TB in feed (mg/Kg of feed 12% DM)** | **CF in feed (mg/Kg of feed 12% DM)** |
|---|---|---|---|---|---|---|
| **FFP1** | 187.45 ± 2.29 $^{abc}$ | 23.34 ± 0.74 $^{abc}$ | 8.0 | 30% | 53.44 | 7.00 |
| **FFP2** | 329.57 ± 4.02 $^{abc}$ | 40.91 ± 1.85 $^{abc}$ | 8.1 | 30% | 95.30 | 12.27 |
| **FFP3** | 458.11 ± 7.80 $^{bc}$ | 52.31 ± 2.22 $^{bc}$ | 8.8 | 30% | 132.18 | 15.69 |
| **FFP4** | 59.59 ± 1.46 $^{a}$ | 9.97 ± 0.49 $^{a}$ | 6.0 | 30% | 17.12 | 2.99 |
| **FFP5** | 293.52 ± 5.99 $^{abc}$ | 32.33 ± 1.14 $^{abc}$ | 9.1 | 30% | 84.87 | 9.70 |
| **FFP6** | 102.92 ± 4.37 $^{ab}$ | 9.28 ± 0.43 $^{a}$ | 11.1 | 30% | 28.97 | 2.78 |
| **FFP7** | 113.87 ± 4.87 $^{abc}$ | 17.25 ± 0.52 $^{abc}$ | 6.6 | 30% | 32.60 | 5.17 |
| **FFP8** | 1147.08 ± 21.95 $^{c}$ | 118.08 ± 2.39 $^{c}$ | 9.7 | 27.1%*<br>18.1%** | 300.00<br>200.00 | 30.88<br>20.59 |
| **FFP9** | 124.94 ± 6.66 $^{abc}$ | 13.32 ± 0.25 $^{abc}$ | 9.4 | 30% | 36.05 | 4.00 |
| **FFP10** | 162.87 ± 6.02 $^{abc}$ | 13.11 ± 0.43 $^{abc}$ | 12.4 | 30% | 46.18 | 3.93 |
| **FFP11** | 190.61 ± 10.19 $^{abc}$ | 15.14 ± 0.65 $^{abc}$ | 12.6 | 30% | 54.28 | 4.54 |
| **FFP12** | 298.71 ± 6.78 $^{abc}$ | 30.77 ± 0.77 $^{abc}$ | 9.7 | 30% | 86.38 | 9.23 |
| **PC** | 7115.48 ± 261.19 | 1105.66 ± 16.41 | 6.4 | - | - | - |
| **NC** | <LOD | <LOD | - | - | - | - |

*Resulting inclusion rate for not exceeding the EU ML (Directive 2002/32/EC) of TB in complete feed for ruminants (300 mg/kg)

**Resulting inclusion rate for not exceeding the EU ML (Directive 2002/32/EC) of TB in complete feed for pigs (200 mg/kg)

The detection of TB and CF in all tested FFPs suggests the presence of ingredients containing The detection of TB and CF in all tested FFPs suggests the presence of ingredients containing methylxanthines within these products. Among purine alkaloids, theobromine is the principal compound in cocoa and chocolate products.[54] The TB/CF ratios observed (6.0−12.6) are consistent with cocoa and/or chocolate-derived products[12,55] and clearly differ from those of other methylxantine-containing products like coffee, teas, and guarana, in which CF predominates over TB.[12,56] While minor contributions from these sources cannot be excluded, the alkaloid profile is most consistent with cocoaand chocolate-derived products and is therefore the likely principal source of these compounds in the samples.

Among FFPs, TB concentrations ranged from 59.59 ± 1.46 $\mu$g/g in FFP4 to 1147.08 ± 21.95 $\mu$g/g in FFP8, exhibiting substantial variability. CF concentrations ranged from 9.28 ± 0.43 $\mu$g/g in FFP6 to 118.08 ± 2.39 $\mu$g/g in FFP8. This variability may reflect both differences in raw material composition and the relative presence of chocolate or cocoa ingredients within each product category. FFP4 and FFP6 showed the lowest TB and CF contents, respectively. FFP4, composed of puffed and extruded rice and corn cakes, was not expected to contain cocoa-derived compounds, and the trace amounts observed may reflect cross-contamination during processing. Similarly, FFP6, derived from bakery and confectionery items, showed low levels of TB and CF, suggesting cross-contamination or limited inclusion of cocoabased ingredients. In contrast, FFP8, deriving from confectionery products and food byproducts, exhibited the highest concentrations of both alkaloids, indicating a potential high cocoa content in this product. Intermediate concentrations were observed in mixed-composition samples, including FFP1, FFP2, and FFP3, which included a combination of sweets, baked products, and cereal items, with TB ranging from 187.45 to 458.11 $\mu$g/g and CF from 23.34 to 52.31 $\mu$g/g. Declared chocolate-containing FFP12 showed moderately elevated levels (298.71 ± 6.78 $\mu$g/g TB, 30.77 ± 0.77 $\mu$g/g CF), comparable to those measured in FFP5, a product containing pasta, baked goods, sweets, and pastry products. FFP6 and FFP10, containing bakery products and biscuits clustered in lower ranges (102.92−162.87 $\mu$g/g for TB and 9.28−13.11 $\mu$g/g for CF). Other confectionery-derived samples (FFP7 and FFP9) displayed relatively low concentrations (113.87−124.94 $\mu$g/g TB, 13.32−17.25 $\mu$g/g CF) compared to FFP8, suggesting variability and heterogeneity of cocoa content across different confectionery products. This variability may also reflect a different inclusion of food byproducts containing TB and CF in the FFPs, such as cocoa hulls and husks. The negative control remained below detection limits for both compounds. To the authors' knowledge, this is the first study that quantifies TB and CF in FFPs. A previous study quantified plasma TB and CF in postweaning piglets after consumption of 30% salty or sugary FFPs in their diets. The results showed that the TB and CF concentrations were increased ($p < 0.05$) in both groups compared with the control group, which had TB levels below identification limits, suggesting the presence of cocoaderived products in both salty and sugary FFPs. [27]

Based on the selected inclusion rate of 30% for the target animal categories (ruminants and pigs), TB concentrations in feed containing FFP8 would exceed the EU MLs of 300 mg/kg for ruminants and 200 mg/kg for pigs, whereas feeds formulated with any other FFP remain below these thresholds (Figure 4), provided no additional theobromine sources are incorporated in the diet. An inclusion rate of 26.2% of FFP8 in ruminant feed and 17.4% in pig feed (Table 2) would already bring TB concentrations up to the EU MLs and were then selected for the maximum exposure scenario in ADE calculations.

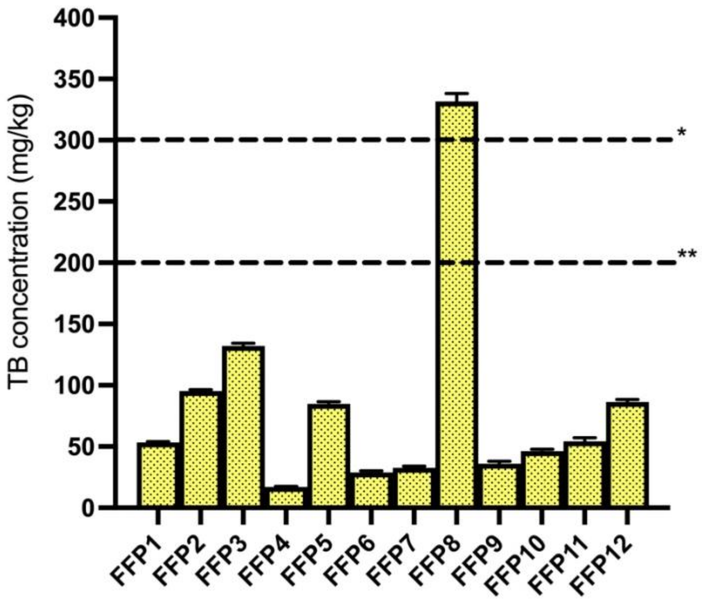


Figure 4. TB levels (mg/kg) in feed with a moisture content of 12%, assuming a 30% on DM basis inclusion rate of the FFPs. *: General TB EU ML (Directive 2002/32/EC) in complete feed, applicable for ruminants; **: TB EU ML (Directive 2002/32/EC) in complete feed for pigs.

**Animal dietary exposure assessment and safety considerations**

ADE was calculated for each of the four scenarios (minimum, maximum, median, and mean exposure) using the TB and CF concentrations measured in the FFPs and the calculated resulting concentrations per kilogram of DM feed (Table 2). The estimated ADE values for ruminants (dairy cows, cattle for fattening, veal calves, and dairy sheep/goat) and pigs (weaned piglets, pigs for fattening, and lactating sows) are reported in Table 3 for TB and in Table 4 for CF, expressed as mg/kg body weight (bw)/day.

At an inclusion rate of 30% (DM basis) of FFPs in the animals' diets and considering the minimum, median, and mean occurrence values, pigs exhibit the highest exposure to TB and CF. Specifically, weaned piglets exhibited the highest exposure levels, whereas lactating sows showed the lowest. TB exposure values ranged from 0.59 to 0.86 mg/kg bw/day (minimum), 1.85–2.69 mg/kg bw/day (median), and 2.48– 3.61 mg/kg bw/day (mean). As for TB, the same exposure patterns were observed for CF, with piglets showing the highest exposure to CF, approximately 1.5 times that of lactating sows, which showed the lowest.

Ruminants would generally be exposed to lower TB and CF concentrations. TB ranged from 0.30 to 0.60 mg/kg bw/day (minimum), 1.16–1.88 mg/kg bw/day (median), and 1.73– 2.82 mg/kg bw/day (mean). As for pigs, also for ruminants, TB and CF show a comparable pattern, with veal calves, dairy sheep/goat, and cattle for fattening showing similar exposure levels, while dairy cows exhibited approximately 1.5 times higher exposure.

In the maximum exposure scenario, the inclusion rate of FFP8 was reduced from the standard 30 to 27.1% for ruminants and to 18.1% for pigs to remain within the EU MLs for TB. In this case, animal exposure corresponds to the levels associated with feed containing TB at the EU ML of 300 ppm (12% DM) for ruminants and 200 ppm (12% DM) for pigs. In this scenario, ruminant TB exposure ranges from 6.44 mg/kg of body weight (bw)/day in veal calves to 10.49 mg/kg of bw/day in dairy cows. Similarly, pig TB exposure ranges from 6.86 to 10.00 mg/kg bw/day. By adjustment of the IR% so that TB concentrations align with the EU MLs, the calculated ADE for ruminants and pigs converged to a more comparable range. Nevertheless, within each species category, dairy cows and weaned piglets still exhibited exposures around 1.5 times higher than those of veal calves and lactating sows, respectively, reflecting differences in the production stage and physiological demands.

This can be explained by relative feed intake, although all animals in the same category receive feed containing the same TB concentration, those with higher daily intake per kilogram of body weight naturally consume higher levels of TB and CF. By dividing feed intake values by body weight values (Table 3), it is clear that dairy cows have high physiological demands[57] and ingest around 3.1% of their body weight in DM feed, also based on the need to support milk yield, whereas veal calves only consume around 1.9% of their body weight. This higher intake ratio in dairy cows directly translates into higher ADE values. In the case of weaned piglets, they are in a growth stage, demanding even higher intakes, around 4.4% of their body weight, compared to lactating sows that consume comparatively lower amounts (3.1%). Overall, these estimates are based on the assumption that FFPs are the only source of TB and CF in the diet and are based on default body weight and intake parameters; access to a comprehensive EU feed consumption database with real-world values of feed intake or model diets across developmental and physiological stages would enable even more accurate exposure assessments.[58,59]

When the derived ADE values for TB are compared with reported NOAEL or LOAEL values across the selected animal categories, ruminant exposure remains below these levels. In dairy cows, effects on milk production, namely, reduced milk yield and increased fat content, were observed at around 15 mg/kg bw/day of TB for an exposure period of 1 week.[19,60] No health effects were observed at approximately 23 mg/kg bw/ day for up to seven months.[61] These values remain above the predicted median and mean exposure scenarios (1.88−2.82 mg/kg of body weight (bw)/day), while the maximum exposure estimate (10.49 mg/kg of body weight (bw)/day) is closer to the value at which only changes in milk yield and fat are observed.

For young cattle, adverse effects levels were drawn from a suspected chocolate poisoning report of calves,[62] in which TB concentrations were estimated and clinical signs (i.e., hyperexcitability, sweating, and increased respiration and heart rate) were reported at 45−90 mg/kg bw/day over a period of several weeks. All modeled exposures for veal calves (0.37−6.44 mg/ kg bw/day) remained well below these levels, although a definitive no-effect dose has not been established. A more recent report of suspected poisoning in dairy cattle supplemented with various amounts of chocolate chips was published in 2021,[11] but chocolate chips and theobromine intake at the time of the incident were unknown. In goats, reduced voluntary intake and body weight gain occurred at the lowest theobromine tested level following the inclusion of cocoa shell or cocoa dust into goat feed for 56 days,[63] assumed to be approximately 300 mg/kg bw/day.[19] More recent findings confirmed reduced feed intake at TB intakes of 1.51 g/head per day from the inclusion of cocoa bean shell in the diet for 31 days,[64] corresponding approximately to 25.2 mg/kg bw/day, for a 60 kg lactating goat, without affecting milk yield, as well as milk fat, protein, and casein contents. In other studies in lactating

ewes, approximate exposure to TB at 9–10 mg/kg bw/day for 21 days[65] and 7.25 mg/kg bw/day for 35 days,[66] derived from cocoa bean shells and husk, respectively, did not have adverse effects on DM intake, milk yield, and fat and protein percentage. Overall, in the case of modeled ADE in sheep and goats (0.39–6.82 mg/kg bw/day), the ADE remained below these no-observed adverse effect levels.
In pigs, ingestion of cocoa meal corresponding to 24 mg/kg bw/day of TB for 126 days resulted in lethargy and growth retardation, most evident during the initial 4 to 5 weeks.[67] A NOAEL of 7 mg/kg bw/day for young growing pigs was identified by the EFSA Panel[19] based on reduced piglet growth performance,[14] when dried whey was substituted with a dried byproduct of milk chocolate, candy, and food industries, over a period of 35 days. TB concentrations in the substitute were not known and assumed to be 1.35 g/kg based on literature data. A study published in 2017 by Ogunsipe and colleagues studied the growth performance of weaning pigs on a dietary cocoa bean shell with declared TB content in an 84-day feeding trial. Animal feed intake was not affected, but live weight gain decreased when TB exposure exceeded approximately 29 mg/ kg bw/day, while feed conversion was poorer at approximately 14 mg/kg bw/day, which was the lowest tested dose.[68]
Older growing pigs appeared to tolerate somewhat higher TB exposures.[19] This is also supported by a more recent study[69] in which dietary inclusion of cocoa husks for 21 days in large white pigs did not affect feed intake, weight gain, or feed efficiency. Although TB concentrations in the cocoa husks were not reported, assuming concentration levels reported by Carta and colleagues,[66] the estimated TB exposure without adverse effects would be approximately 9 mg/kg bw/day.
In summary, modeled ADE scenarios including the minimum, median, and mean exposure (0.86–3.61 mg/kg bw/day) remained below the reported toxicological threshold values, whereas the maximum exposure scenario for piglets (10.49 mg/kg bw/day) exceeded the established NOAEL value of 7 mg/kg bw/day, suggesting higher susceptibility of this animal category when fed at the EU MLs.
These results underline the need for species-specific evaluation of diet formulation and TB quantification in FFPs before their inclusion in animal diets.
Furthermore, given the increasing interest in incorporating food co- and byproducts into animal feed, additional studies in which conventional feed is replaced by cocoa-derived or cocoacontaining ingredients, even when assessing different endpoints or aspects of animal production, would be extremely valuable if TB concentrations in both the original matrix and the feed, as well as actual feed intake and animal body weights, are consistently included. Such comprehensive reporting would facilitate more accurate safety assessments and support the refinement of toxicological thresholds.
Overall, the findings contribute to a growing body of evidence supporting the safe and sustainable use of FFPs in animal nutrition, reinforcing their role as a viable strategy for reducing food waste and enhancing the environmental efficiency of livestock production systems.

Table 3. Estimated TB ADE for each animal species under minimum (FFP4), maximum (FFP8), median and mean values (across FFP1-FFP12). For each species, body weight (kg) and daily DM feed intake (kg/day) are listed. TB concentration (mg/kg of DM feed), daily exposure (mg/day), and normalized exposure to body weight (mg/kg bw/day) are presented under each scenario.

| | | | **Minimum** | | | **Maximum** | | | **Median** | | | **Mean** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | Body weight* (kg) | Feed intake* DM (kg/day) | TB (mg/Kg of DM feed) | Exposure (mg/day) | Exposure (mg/kg bw/day) | TB (mg/Kg of feed) | Exposure (mg/day) | Exposure (mg/kg bw/day) | TB (mg/Kg of feed) | Exposure (mg/day) | Exposure (mg/kg bw/day) | TB (mg/Kg of feed) | Exposure (mg/day) | Exposure (mg/kg bw/day) |
| **Ruminants** | | | | | | | | | | | | | | |
| Dairy cows | 650 | 20 | 19.45 | 389.08 | 0.60 | 340.91 | 6818.18 | 10.49 | 61.21 | 1224.13 | 1.88 | 91.61 | 1832.14 | 2.82 |
| Cattle for fattening | 400 | 8 | 19.45 | 155.63 | 0.39 | 340.91 | 2727.27 | 6.82 | 61.21 | 489.65 | 1.22 | 91.61 | 732.85 | 1.83 |
| Veal calves | 100 | 1.89 | 19.45 | 36.77 | 0.37 | 340.91 | 644.32 | 6.44 | 61.21 | 115.68 | 1.16 | 91.61 | 173.14 | 1.73 |
| Dairy sheep/goat | 60 | 1.2 | 19.45 | 23.34 | 0.39 | 340.91 | 409.09 | 6.82 | 61.21 | 73.45 | 1.22 | 91.61 | 109.93 | 1.83 |
| **Pigs** | | | | | | | | | | | | | | |
| Piglets (weaned) | 20 | 0.88 | 19.45 | 17.12 | 0.86 | 227.27 | 200.00 | 10.00 | 61.21 | 53.86 | 2.69 | 82.14 | 72.28 | 3.61 |
| Pigs for fattening | 60 | 2.2 | 19.45 | 42.80 | 0.71 | 227.27 | 500.00 | 8.33 | 61.21 | 134.65 | 2.24 | 82.14 | 180.70 | 3.01 |
| Sows, lactating | 175 | 5.28 | 19.45 | 102.72 | 0.59 | 227.27 | 1200.00 | 6.86 | 61.21 | 323.17 | 1.85 | 82.14 | 433.68 | 2.48 |

* EFSA, 2017[46]; EFSA, 2024[47]

Table 4. Estimated CF ADE for each animal species under minimum (FFP6), maximum (FFP8), median and mean values (across FFP1-FFP12). For each species, body weight (kg) and daily DM feed intake (kg/day) are listed. CF concentration (mg/kg of DM feed), daily exposure (mg/day), and normalized exposure to body weight (mg/kg bw/day) are presented under each scenario.

| | | | **Minimum** | | | **Maximum** | | | **Median** | | | **Mean** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | Body weight* (kg) | Feed intake* DM (kg/day) | CF (mg/Kg of DM feed) | Exposure (mg/day) | Exposure (mg/kg bw/day) | CF (mg/Kg of feed) | Exposure (mg/day) | Exposure (mg/kg bw/day) | CF (mg/Kg of feed) | Exposure (mg/day) | Exposure (mg/kg bw/day) | CF (mg/Kg of feed) | Exposure (mg/day) | Exposure (mg/kg bw/day) |
| **Ruminants** | | | | | | | | | | | | | | |
| Dairy cows | 650 | 20 | 2.97 | 59.37 | 0.09 | 35.09 | 701.89 | 1.08 | 6.59 | 131.72 | 0.20 | 9.94 | 198.84 | 0.31 |
| Cattle for fattening | 400 | 8 | 2.97 | 23.75 | 0.06 | 35.09 | 280.76 | 0.70 | 6.59 | 52.69 | 0.13 | 9.94 | 79.54 | 0.20 |
| Veal calves | 100 | 1.89 | 2.97 | 5.61 | 0.06 | 35.09 | 66.33 | 0.66 | 6.59 | 12.45 | 0.12 | 9.94 | 18.79 | 0.19 |
| Dairy sheep/goat | 60 | 1.2 | 2.97 | 3.56 | 0.06 | 35.09 | 42.11 | 0.70 | 6.59 | 7.90 | 0.13 | 9.94 | 11.93 | 0.20 |
| **Pigs** | | | | | | | | | | | | | | |
| Piglets (weaned) | 20 | 0.88 | 2.97 | 2.61 | 0.13 | 23.40 | 20.59 | 1.03 | 6.59 | 5.80 | 0.29 | 8.97 | 7.89 | 0.39 |
| Pigs for fattening | 60 | 2.2 | 2.97 | 6.53 | 0.11 | 23.40 | 51.47 | 0.86 | 6.59 | 14.49 | 0.24 | 8.97 | 19.73 | 0.33 |
| Sows, lactating | 175 | 5.28 | 2.97 | 15.67 | 0.09 | 23.40 | 123.53 | 0.71 | 6.59 | 34.77 | 0.20 | 8.97 | 47.35 | 0.27 |

* EFSA, 2017[46]; EFSA, 2024[47]

**Abbreviations Used**

| | |
|---|---|
| ADE | animal dietary exposure |
| aNDFom | amylase Neutral Detergent Fiber on an organic matter basis |
| BSE | bovine Spongiform Encephalopathy |
| bw | body weight |
| CF | caffeine |
| CP | crude protein |
| DM | dry matter |
| EC | European Commission |
| EE | ether extract |
| EFSA | European Food Safety Authority |
| EU | Europe |
| FFP | former food product |
| FI | feed intake |
| FLW | Food losses and food waste |
| HACCP | Hazard Analysis and Critical Control Point |
| HPLC-UV | high-performance liquid chromatography coupled with ultraviolet detection |
| HPTLC | high performance thin layer chromatography |
| IR | inclusion rate |
| LOAEL | lowest-observed adverse effect |
| LOD | the limit of detection |
| LOQ | the limit of quantification |
| MLs | maximum levels |
| NC | negative control |
| NDF | neutral detergent fiber |
| NOAEL | no-observed adverse effect |
| PC | positive control |
| Rf | retention factor |
| Rt | retention time |
| SD | standard deviation |
| TB | theobromine |

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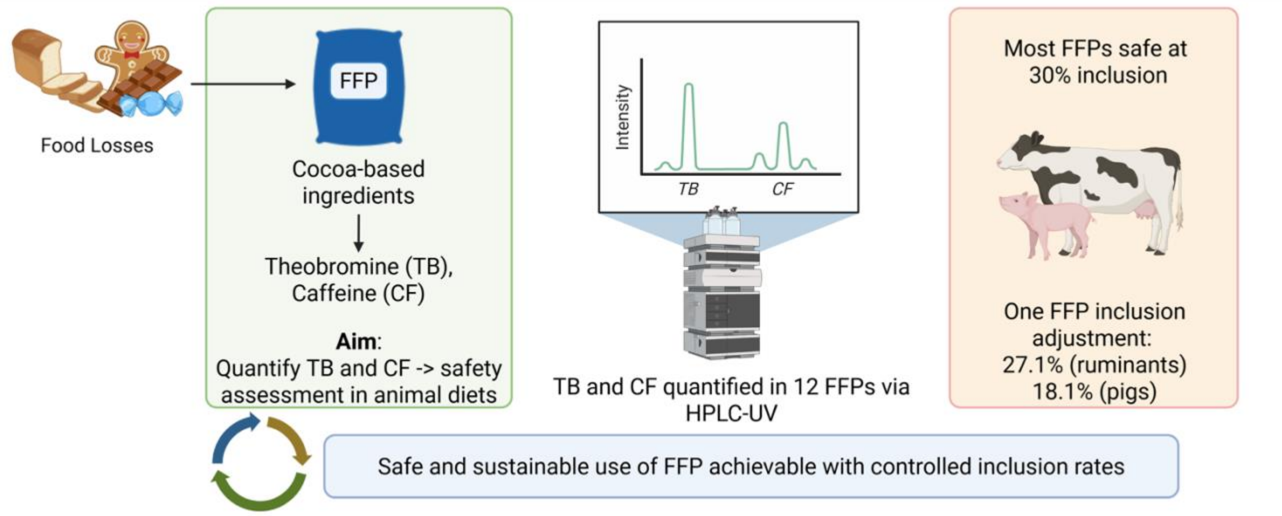
**Graphic for table of contents**



# CHAPTER 3

# - Phenolic profile and antioxidant activity of hemp co-products following green chemical extraction and ex vivo digestion

Davide Lanzoni, **Francesca Mercogliano**, Raffaella Rebucci, Corinne Bani, Luciano Pinotti, Chiara Di Lorenzo, Giovanni Savoini, Antonella Baldi & Carlotta Giromini

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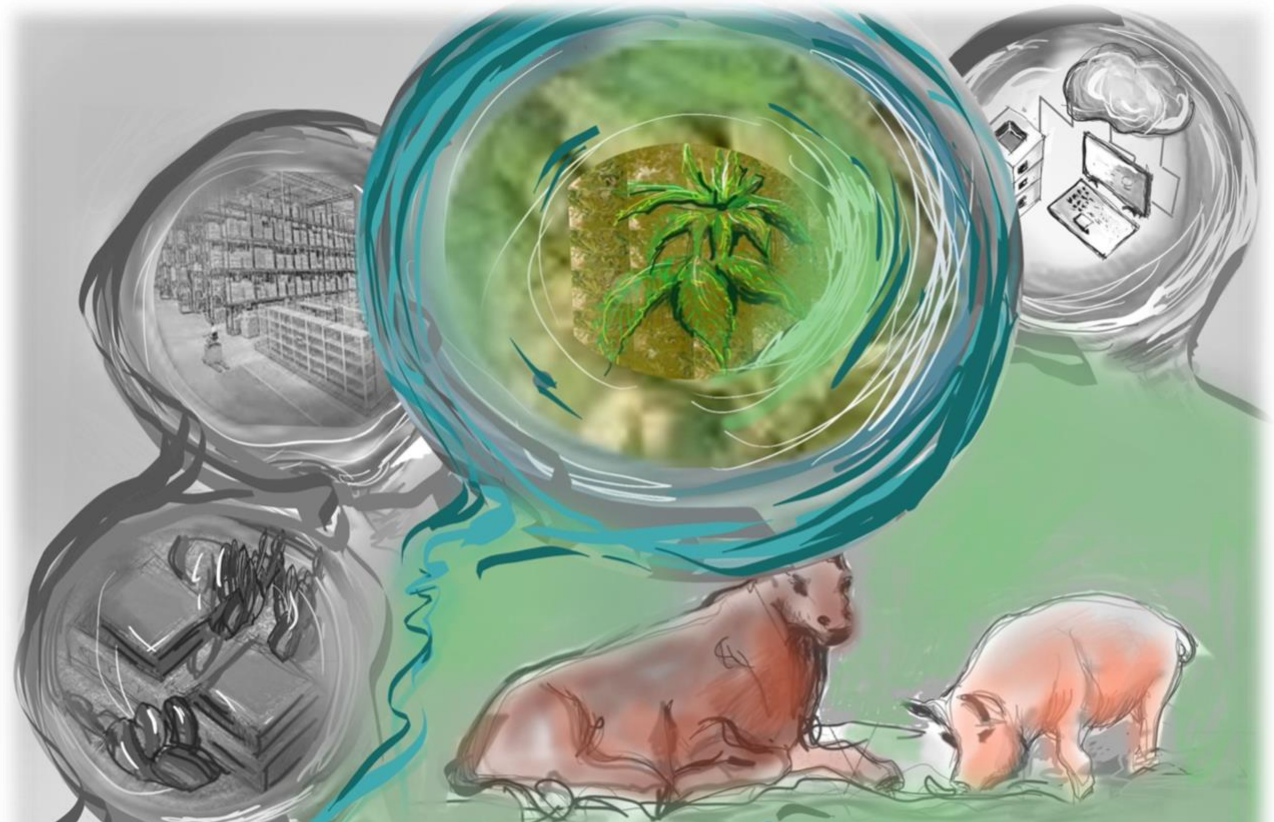

# Phenolic profile and antioxidant activity of hemp co-products following green chemical extraction and ex vivo digestion

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**Abstract:** The valorisation of biomass generated along the agri-food chain into co-products represents a valid approach to produce alternative and sustainable feed ingredients. Although to date, some co-products have already been included in the diets of livestock animals, the characterisation of hemp co-products (*Cannabis sativa* L.) is still at an early stage. For this, the aim of this this work was to investigate the nutritional and functional profile [total phenolic content (TPC) and antioxidant activity (ABTS, FRAP)] of hemp co-products [mix flowers and leaves (MFL), hulls (HLs) and hempseeds cake], after green chemical extraction (0% EtOH; 50% EtOH; 100% EtOH) and ex vivo digestion process. High-Performance Thin Layer Chromatography was performed on 50% EtOH chemical extracts to identify the main phenolic compounds. The results reported an interesting nutritional profile, although dependent on the processing to obtain the co-product. The functional aspect of the chemical extracts showed a higher TPC for MFL, especially after 50% EtOH extraction (3551.12±98.54 mg TAE/100 g) identifying Quercetin, Kaempferol, Rutin, Chlorogenic acid, Ferulic acid, Cannabigerol and $\alpha$-tocopherol as the main compounds present, a profile similar to that of HLs. This was also observed for ABTS (6950.10±546.82 mg TE/100 g) and FRAP (130.05±4.67 mg FeSO4/100 g). Ex vivo digestion confirmed the high functional profile of the two matrices. Despite this, it is important to emphasise that processing co-products are characterised by a high variability that may lead to different results and effects on the health and performance of monogastric animals.

**Keywords**: antioxidant activities; chemical extraction; digestion; hemp; phenols.

## Highlights

The high environmental impact of the food/feed sector has prompted scientific research to valorise the co-products of the food chain.

Hemp co-products are characterised by an interesting nutritional profile, particularly in terms of protein and fat content.

Hemp co-products, particularly hulls and mix flowers/leaves are characterised by high functional activity.

## Introduction

The high environmental impact of the food and especially feed sector is a highly debated topic in recent years, which is attracting increasing interest from the scientific community, particularly from the perspective of food-feed competition. Globally, the production, processing, and transport of feed for the livestock sector accounts for 45% of the total production of greenhouse gases (Makkar, 2018). Simultaneously, as reported by the same author, the area dedicated to feed-crop cultivation accounts for 33% of the total arable land, while about 30% is occupied by grazing, with more than 90% water consumption (Makkar, 2018). This scenario is bound to worsen if considering that the world's current population will exceed 9 billion people in the next 30 years, leading to an ever-increasing need for food and food production, which could have a further impact on climate change, as shown by projections for 2050 by the EAT-Lance commission (Willet et al., 2019). For this reason, the European Union (EU) is working to ensure a sustainable future through the implementation of strategies such as the Green Deal and One Health, the aim of which is the transformation of the EU into a prosperous society with a competitive, clean, and circular economy that recognises the close link between human, animal, and environmental health (Fetting, 2020; Sinclair, 2019; Pinotti et al., 2023). In this context, the Farm to Fork (F2F) strategy, the heart of the European Green Deal, plays a major role. F2F is a 10-year plan devised by the European Commission to guide the agricultural transition towards more equitable, healthy, and environmentally friendly food systems, making them more sustainable than they are today (European Union, 2020). To achieve these goals, one possible approach is to utilise the huge amount of non-edible biomass produced along the entire food chain (about 1.3 billion tonnes) as co-products for livestock (FAO, 2011; Rakita et al., 2021). As reported by Pinotti et al. (2020), the term co-product refers to any product obtained from different agro-industrial processes. To date, several co-products such as beet pulp, soybean meal, soybean molasses, sunflower meal, and grape marcs (GMs) have already been included in animal diets due to their interesting nutritional profile, but many others could be used (Vastolo et al., 2022; Mirzaei-Aghsaghali and Maheri-Sis, 2008). Among these, the co-products of industrial hemp (*Cannabis sativa* L.) are attracting increasing interest. Hemp is a dicotyledonous, annual, herbaceous, angiosperm plant that is widespread worldwide due to its easy adaptability (Lanzoni et al., 2023; Lanzoni et al., 2023a). It is considered a low environmental impact plant as it does not require large amounts of water, use of pesticides and acts as an antagonist to weeds (Rupasinghe et al., 2020). At the same time, as well as being able to grow rapidly in different agro-ecological conditions, it is an excellent candidate for carbon sequestration due to its height (it can reach up to four metres) (Farinon et al., 2020; Rehman et al., 2021; Rupasinghe et al., 2020).

Following EU Regulation No. 1307/2013, which allows the cultivation of hempseeds (HSs), on condition that they are registered in the European catalogue and have a Δ9-tetrahydrocannabinol (THC) content of less than 0.2%, this plant has been attracting the interest of several industries (EFSA, 2011; Lanzoni et al., 2023). In particular, the food and feed industry has studied the properties of HS, previously considered a waste product from the processing of this plant, recognising it as a feed material with important nutritional and functional properties (Lanzoni et al., 2023a; Farinon et al., 2020). In the F2F contest, hemp shows as a valuable opportunity to valorise the co-products of its processing. As reported by Ely and Fike (2022), Vastolo et al. (2021), and Lanzoni et al. (2023a), the hemp co-products can be summarised as follows: whole plant, stems, chaffs, stalks, hulls (HLs), HS cake (HSC), leaves (before and after cannabinoid extraction), and flowers (before and after cannabinoid extraction). As reported by the same authors, these co-products are characterised by an interesting nutritional profile, particularly regarding to protein content, which starts at 5% for stalks and reaches values of more than 20% for HSC, chaffs, flowers, and HLs. Interesting results are reported also for the lipid content (Ely and Fike, 2022; Vastolo et al., 2021). However, their nutritional and especially functional profile is still poorly characterised.

This is probably due to the fact that hemp cultivation is still small-scale, even though Europe, as reported by Horne (2020), is one of the main countries in terms of hemp production area. Furthermore, as reported by Ely and Fike (2022), this is because the various hemp industries are in their early stages, without significant markets capable of absorbing large quantities of hemp scraps and consequently generating large volumes of co-products. To date, the EU, through Regulation 2022/1104 of 1 July 2022, has registered only HS, HSC, HS oil, hemp flour and hemp fibre (both originating from stems) in the European Catalogue of feed materials (EU, 2022).

On this basis, the aim of this work was to investigate the nutritional and functional profile of hemp co-products (mix flowers/leaves (MFL), HLs and HSC), also in comparison to GMs, matrices highly described in the literature, to assess their potential for livestock feed. In particular, total phenolic content (TPC) and antioxidant activity [2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (*ABTS); Ferric Reducing Antioxidant Power (FRAP);* 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH)] were monitored following a green chemical extraction, also identifying the main phenolic compounds by High-Performance Thin-Layer Chromatography (HPTLC). In parallel, TPC, ABTS, and FRAP were also evaluated following an ex vivo digestion protocol.

## Material and Methods

## Material

Hulls: HLs are derived from the dehulling process of HSs using sieves and airflow (*C. sativa* L, variety Bialobrzeskie). The HSs were supplied by a Czech company (Chrastice, Czech Republic). Specifically, the HSs were sown in May 2022 and harvested in early October 2022.

Mix flowers/leaves: The MFL (*C. sativa* L, variety Carmagnola) was provided by a local company (CN, Italy). After hand-harvesting, the sample was dried slowly in the dark in a closed and ventilated environment in order to safeguard the functional compounds. Subsequently, the cannabidiol (CBD) content was recorded and resulted in a concentration of between 2-3%.

HSs cake: The HSC (*C. sativa* L, variety Carmagnola) was supplied by a local company (CN, Italy). Specifically, the seeds were sown in May 2022 and harvested in October 2022 by threshing. Following the harvesting process, the seeds were dried to stabilise the moisture content. Finally, they were cold-pressed for the production of HSs oil. The remaining fraction obtained (HSC) was harvested and stored in a dry and ventilated environment.

Grape marcs: The GMs (from red wine industry, also containing seeds) were provided by a local producer (PD, Italy).

## Methods

### Chemical analysis

Chemical analysis was performed following the Official Methods of Analysis (AOAC, 2005). In particular, dry matter (DM) and ashes were determined according to AOAC method 942.05. Protein content and ether extract were assessed with the AOAC method 2001.11 and DM 21/12/1998, respectively. Finally, the fibrous fractions were identified according to Van Soest et al. (1991).

### Green Chemical extractions

Green chemical extractions were performed using three different ratios of water:ethanol ($H_2O$:EtOH) (0% EtOH; 50% EtOH; 100% EtOH). More precisely, the protocol developed by Brighenti et al. (2017) was performed with minor modifications (Lanzoni et al., 2024). Following grinding (diameter of 1 mm, rotor mill Retsch Mod. zm 200, Hann, Germany) of the samples (0.150±0.05 g), 5 mL of solvent ($H_2O$:EtOH) were added and incubated for 1 h at room temperature (RT), under shaking conditions in the dark. At the end of the incubation, the samples were centrifuged at 4000 rpm for 5 min obtaining two fractions: (I) supernatant was recovered and stored at 4°C, (II) precipitate, corresponding to the residual fraction, was extracted twice, obtaining a final volume of extract equal to 15 mL per sample. The final extract obtained was stored at -20°C until further analysis (TPC and antioxidant activity). Each extraction was performed in triplicate (n=3). For each biological replicate, the technical duplicate was considered.

**Total phenolic content and Antioxidant Activity (ABTS and FRAP assays)**

The protocol of Attard (2013) with minor modifications (Lanzoni et al., 2023) was used to test TPC. Specifically, 200 µL of each sample were incubated with 1.0 mL of Folin-Ciocalteu reagent and 800 µL of sodium carbonate in the dark at RT for 20 min. At the end of the incubation, the samples were read with a spectrophotometer at a wavelength of 630 nm. Values were expressed in terms of Tannic acid equivalent (mg TAE/100 g of dried material).

At the same time, antioxidant activity was assessed using the ABTS (Re et al., 1999) and FRAP (Abdelaleem and Elbassiony, 2021) methods. For ABTS, 10 µL of sample were added to 1.0 mL of diluted $ABTS^{\circ+}$ solution ($A_{734nm}$ = 0.700±0.020), incubated for 6 min at RT in the dark, and read at 734 nm. Data were expressed as Trolox equivalent (mg TE/100 g of dried material).

For FRAP, the working solution (FRAP reagent) was prepared as follows: a) 25 mL of acetate buffer (300 mM; pH 3.6); b) 2.5 mL of 2,4,6-tripyridyl-s- triazine (10 mM); c) 2.5 mL of $FeCl_3$ (20 mM). Next, 10 µL of each sample were added to 300 µL of FRAP reagent and incubated at RT for 10 min in the dark and read at 595 nm. Results were expressed as mg $FeSO_4$/100 g of dried material.

**High-Performance Thin Layer Chromatography and DPPH of green chemical extractions**

In this study, the HPTLC technique was used to analyse flavonoids, phenolic acids, Cannabigerol (CBG) and $\alpha$-tocopherol after chemical extraction with 50% EtOH, as a high TPC was obtained with this ratio. Gallic acid, Chlorogenic acid, Caffeic acid, Ferulic acid, Ruthin, Epicatechin, and $\alpha$-tocopherol standards were purchased from Sigma Aldrich (Steinheim, Germany), while Quercetin 3-O-glucoside, Kaempferol, and Catechin were purchased from Extrasynthese (Genay, France). Cannabigerol by Linnea Natural Pharma Solutions (Riazzino, Switzerland).

In particular, following the extraction process, 5 mL of each sample were centrifuged at 3000 rcf at 4°C for 10 min (5810 R, Eppendorf, Hamburg, Germany). After centrifugation, the samples were filtered using 0.45 µm PTFE filters (VWE) and dried with nitrogen flow to concentrate the compounds and solubilise them in 200 µL of methanol (VWR International, Fontenay-sous-Bois, France), resulting in a final concentration of 250 mg/mL. Subsequently, 10 µL of each sample were loaded onto an HPTLC silica-gel plate 60 F254 (10×20 cm, Merck, Darmstadt, Germany) with 5 µL of Quercetin 3-O-glucoside, Rutin, Catechin, Epicatechin, Gallic acid, Caffeic acid, Chlorogenic acid, Ferulic acid, CBG and $\alpha$-tocopherol standard solution, by a semi-automatic sample applicator

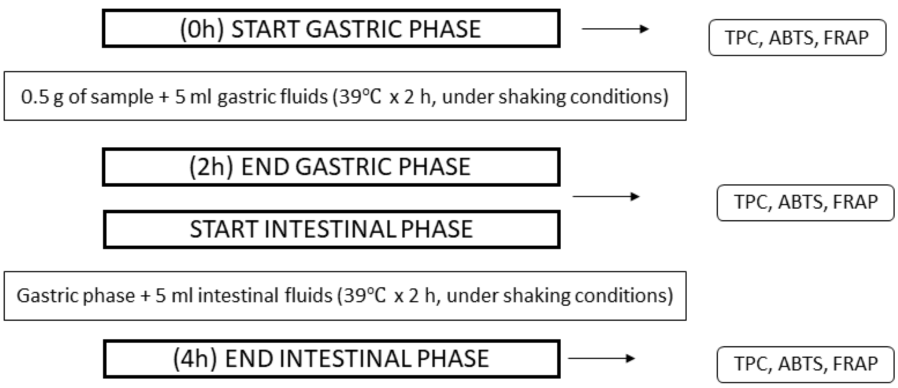
(Linomat 4, CAMAG, Muttenz, Switzerland). For all standards used, the concentration was 200 μg/mL, with the exception of CBG (1 mg/mL). After the chromatographic run, where the mobile phase consisted of 10 mL of acetone:toluene:formic acid (4.5:4.5:1 v/v/v) (VWR International, Fontenay-sous-Bois, France), the plate was exposed to UV light at 254 and 366 nm.

Subsequently, the plate was derivatized with a 0.05% DPPH methanolic solution (Sigma Aldrich Steinheim, Germany), kept in the dark for 30 min, and then examined under visible light using VisionCats software (CAMAG, Muttenz, Switzerland).

**Ex vivo digestion process**

The digestion process was adapted from Devle et al. (2014) and performed as reported by Lanzoni et al. (2024). More precisely, gastric and intestinal fluids were collected from pigs (n=20) at slaughter between 50 and 110 days of age. Then, in order to remove the undigested fraction, the fluids were centrifuged for 10 min at 4000 rcf and used to pool gastric and intestinal fluids to reduce variability. The resulting fluids were frozen at -20°C for up to 48h. Before digestion, pH and enzyme activity were checked and adjusted if necessary to match gastric and intestinal conditions. As reported in Figure 1, at the beginning of the gastric phase (0h), at the end of the gastric phase (2h), and at the end of the intestinal phase (4h), aliquots (1 mL) were taken to measure TPC and antioxidant activity (ABTS and FRAP) during ex vivo digestion. The digestion process was replicated three times (n=3) taking two technical replicates for each digestion step.

Figure 1. Workflow of ex vivo digestion protocol (Devle et al., 2014; Lanzoni et al., 2024). TPC: Total Phenolic Content.




**Statistical Analysis**

Total phenolic content and antioxidant activities of the green chemical extracts and ex vivo digestion were analysed by two-way Anova (*EtOH concentration x Co-products*; *Time x Co-products*, respectively) followed by Tukey's multiple comparison test, using GraphPad Prism 9 9.3.1

(GraphPad Software Inc., San Diego, CA, USA). All data are reported as means ± standard error of the mean (SEM) of at least three independent experiments. Values are considered statistically significant for a 95% confidence interval (*p-value* = 0.05).

## Results and Discussion

### Chemical Analysis

The nutritional profile of HLs, MFL, HSC, and GMs is shown in Table 1.

Table 1. Chemical composition of MFLs (mix flowers/leaves), HLs (hulls), HSC (HS cake) and GMs (grape marcs). Data are presented as mean ± standard error of mean (SEM). (n = 3). DM = dry matter; CP = crude protein; EE = ether extract; NDF=neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin. Ashes, CP, EE, NDF, ADF and ADL are expressed in % w/w on DM basis

| Sample | DM | ASHES | CP | EE | NDF | ADF | ADL |
|---|---|---|---|---|---|---|---|
| **MFL** | 94.58±0.43 | 17.40±0.19 | 15.79±0.02 | 12.02±0.37 | 42.06±0.31 | 18.05±0.72 | 5.34±0.15 |
| **HLs** | 91.28±2.11 | 12.03±0.31 | 14.49±0.75 | 9.85±0.04 | 44.70±1.47 | 29.30±0.50 | 10.67±0.11 |
| **HSC** | 94.46±0.05 | 8.27±0.85 | 32.54±0.71 | 7.69±0.05 | 52.59±1.84 | 29.00±1.59 | 12.14±0.95 |
| **GMs** | 94.02±0.02 | 7.57±0.14 | 10.69±0.27 | 8.33±0.62 | 55.49±0.66 | 47.54±0.12 | 35.47±0.47 |

As reported in Table 1, hemp co-products showed an interesting nutritional profile, even when compared to GMs, a co-product of the wine industry, which is highly used in the feed sector. In general, the results obtained in this study are partially confirmed in the literature (Ely and Fike, 2022; Vastolo et al; 2022; Kleinhenz et al; 2020). As reported by Vastolo et al. (2022), these differences are probably due, not only to the genotype of the plant, but also to harvesting, agrological, and environmental conditions. In more detail, MFL showed a high ash content. Kleinhenz et al. (2020) demonstrated that hemp leaves are characterised by an ash content of 21.2% while flowers at 14.1%, suggesting that a combination of them results in a reduction of the final percentage (intermediate in our case). The high ashes values are due to the presence of minerals such as calcium, phosphorus, magnesium, potassium, and sulphur, which are known to be high in hemp leaves and flowers (Kleinhenz et al., 2020) and which, in turn, may influence the digestibility of hemp, as shown by Lanzoni et al. (2023). As reported in Table 1, HSC showed a high protein content (32.54±0.71%). This feature is related to the treatment adopted for obtaining the specific co-product. In this case, the removal of oil (one of the main components of whole HSs) led to an increase in the total protein content (from 30% to 50%), as demonstrated by House et al. (2010) and Ely and Fike (2022). In parallel, HLs and MFL were characterised by a highly comparable protein content (14.49±0.75%; 15.79±0.02%, respectively), however lower than reported by Ely and Fike (2022). This difference, as previously reported, is due to the variability factors listed by Vastolo et al. (2022). It is important to emphasise that, although HLs are a matrix obtained following the removal of the fibrous outer layer of HSs (dehulling process), they showed a high protein content. As reported by House (2021), this could be due to the fact that, during the dehulling process, part of the HSs endosperm (rich in protein and lipids) is also inevitably removed, thus increasing the final protein content.

This also explains the high lipid content observed in HLs (9.85±0.04%), highly comparable with GMs (8.33±0.62%), confirming the values reported by House et al. (2010) and Baumgärtel et al. (2007). Among the matrices studied, the MFL showed the highest lipid content (12.02±0.37%), confirming the values observed by Ely and Fike (2022). This result could be justified by the presence of essential oil in the hemp flowers, which is highly used in the cosmetic and pharmaceutical sectors for its important functional properties (Farinon et al., 2020).

All analysed matrices presented high fibre content, expressed as Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF), and Acid Detergent Lignin (ADL). Highest values for all analysed fractions were obtained for HSC. In particular, NDF measured in HSC was close to GMs, confirming, at least partially, what was observed by Halle and Shone (2013). More precisely, the authors reported a percentage of NDF, ADF, and ADL of 45%, 30% and 12% (expressed on DM basis) respectively, values comparable with those reported in Table 1. In parallel, among the tested samples, MFL showed the lowest values for NDF (42.06±0.31%), ADF (18.05±0.72%), and ADL (5.34±0.15%). Although there is no data in the literature on the nutritional composition of the MFL, it is evident that the values observed in the present study are a combination of the results obtained from the analysis of the matrices tested individually, as reported by Kleinhenz et al. (2020) and Ely and Fike (2022). Finally, the HLs showed a lower content of NDF, ADF, ADL than observed by House et al. (2010), Kim and Nyachoti (2017), and Kim et al. (2018). More precisely, the authors showed that NDF in these matrices can reach 50-65% and 38-50% of ADF (on DM basis). Again, the observed differences can be attributed to genotype, collection, agrological and environmental differences.

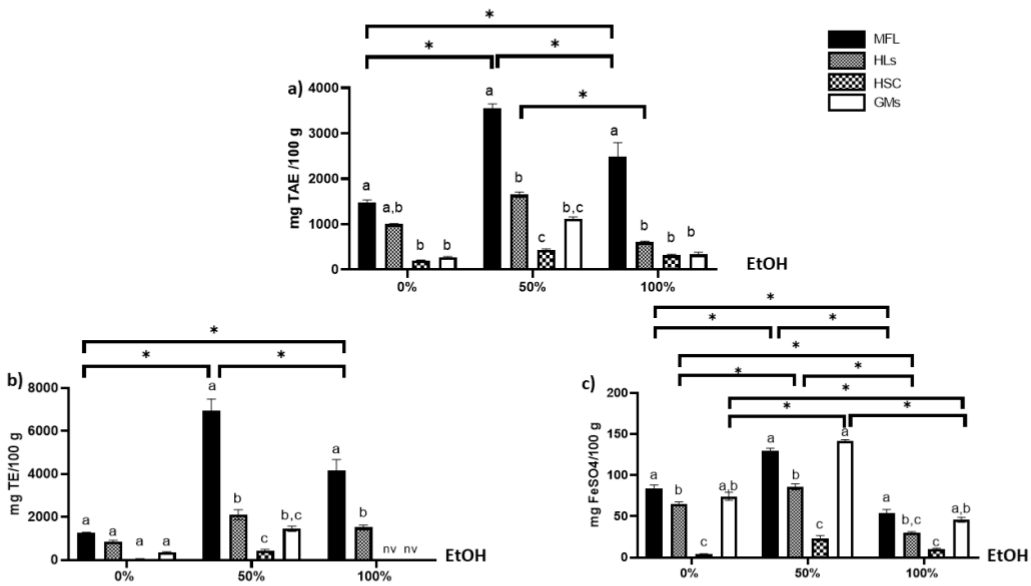
Although high levels of fibre may affect the total digestibility, it is important to emphasise that its adequate consumption may have a functional, especially probiotic, role, as reported by Farinon et al. (2020).

**Total phenolic content and Antioxidant activity (ABTS and FRAP) of green chemical extracts**

Scientific research is increasingly investigating the properties of phenolic compounds due to their associated benefits, which can be exploited in the formulation of functional foods and nutraceuticals (Abdalla et al. 2007). The properties of these compounds can be influenced by the type of extraction. To date, solvent-based extraction is one of the most widely used on a laboratory scale due to its speed and cost-effectiveness. The efficacy of this type of extraction depends mainly on the solvent used, identified by Rotta et al. (2017) as one of the main critical parameters due to the different chemical characteristics and polarity of the phenolic compounds. For this reason, as shown in Figure

2a, we decided to study the TPC of hemp co-products, using three different ratios of $H_2O$:EtOH, as previously reported.

Figure 2. Total phenolic content and Antioxidant activity (ABTS and FRAP) of green chemical extracts. TAE: Tannic Acid Equivalent, TE: Trolox Equivalent. Data are presented as mean ± standard error of mean (SEM), (n = 3). Different superscript letters in columns indicate significant differences ($p<0.05$) between the samples within each extraction. Bars indicate significant differences ($p<0.05$) between the same samples in different extractions. (**a**) Total Phenolic content; (**b**) ABTS assay; (**c**) FRAP assay.




As shown, 50% EtOH extraction (3551.12±98.54 mg TAE/100g) resulted in significantly higher TPC ($p<0.05$) for MFL. This was also observed for HLs (1647.11±60.53 mg TAE/100g) compared to 100% EtOH (600.53±23.55 mg TAE/100g). This trend, although not statistically significant compared to other ratios, was also reported for HSCs and GMs, partially confirming what has been reported in the literature (Prasad et al., 2009; Ozbek et al., 2020). More specifically, Prasad and colleagues (2009) showed that the extraction rate of longan fruit pericarp increased with increasing EtOH concentration from 25% to 50% and decreased for concentrations higher than 75%. A similar result was obtained by Ozbek et al. (2020) on pistachio HLs. Most probably, this trend can be explained by the "*like dissolves like principle*", where $H_2O$ allows polar compounds to dissolve, while EtOH, an organic solvent, dissolves less polar ones, resulting in a higher extraction rate (Lim et al. 2019).

Although Ozbek et al. (2020) reported a lower extraction rate with 100% EtOH than with 100% $H_2O$, it is evident in Figure 2a, how this depends on the matrix analysed. Indeed, as reported by Robbins (2003), in addition to the solvent used, sample-solvent ratio, pH, temperature, and extraction time, the nature of the compounds are important factors to consider, as they can influence the extraction rate.

Indeed, considering the individual matrices (Figure 2a), MFL showed significantly higher values (*$p<0.05$*) in each concentration tested, except in comparison with HLs for 0% EtOH extraction. Although, the phenolic profile of the leaves is little explored in the literature, that of the flowers has been characterised by Izzo et al. (2020). The authors reported how hemp flowers can be used as new resources for nutraceutical formulations, thanks to the high presence of phenols, in particular hydroxycinnamic acids (chlorogenic, p-coumaric, ferulic acids) and especially flavonoids. More precisely, Izzo and colleagues (2020) demonstrated how these compounds are 10 to 100 times more present in flowers than in any other part of hemp, thus explaining the high TPC levels, expressed as mg TAE/100 g, shown in Figure 2a.

At the same time, the results obtained for HLs in each extraction (999.65±13.58 mg TAE/100g; 1647.1±50.53 mg TAE/100g; 600.53±24.55 mg TAE/100g, for 0%, 50% and 100% EtOH, respectively), highly comparable with those of GMs, showed higher values than those obtained in a previous our publication for HSs (550.3±28.27 mg TAE/100g) extracted in pure methanol, a stronger solvent than EtOH (Lanzoni et al., 2023). This result is most probably due to the fact that phenolic compounds are more concentrated in the hull than in the kernel fraction of HSs, and the dehulling process leads to a higher concentration of phenols (Chen et al., 2012).

Finally, as shown in Figure 2a, HSC showed no significant difference in each ratio tested. These results partially confirmed what was observed by Teh et al. (2014). More specifically, the authors reported a TPC for HSC, extracted with 100% EtOH, of 351.33±2.08 mg GAE/100 g, highly comparable to that observed in this work (315.67±22.42 mg TAE/100 g). With regard to GMs, on the other hand, binary extraction was not significantly different between 0% EtOH and 100% EtOH, although an upward trend was observed, partially confirming what was reported by Spigno et al. (2007).

Rotta et al. (2017) reported the importance of measuring the antioxidant property using at least two methods based on different reaction mechanisms. Therefore, as shown in Figures 2b and 2c, the antioxidant capacity was evaluated using the ABTS and FRAP assays. More precisely, Figures 2b and 2c show that the antioxidant activity presents the same trend as the TPCs, confirming the active role of phenolic compounds in antioxidant properties, as reported by Ozbek et al. (2020) on pistachio HLs. At the same time, the authors demonstrated how concentrations between 70% and 100% EtOH reduced antioxidant capacity. In our study, this effect only occurred for MFLs in the ABTS assay, whereas for FRAP in all matrices except HSC. This distinction indicates that the type of bioactive compounds and the relative amounts recovered are influenced by the ratios of $H_2O$ and EtOH. Indeed, with the polarity of the solvent varying from very polar (100% $H_2O$) to less polar (0% $H_2O$), the ability of the solvent to dissolve selected groups of antioxidants also varies and this has an implication on the subsequent antioxidant activity (Lim et al., 2019).

Thus, as reported, solvent extraction is the most common means of recovering phenolic compounds from plant-derived materials due to the simplicity of the technique, its efficiency, and the wide range of possible applications. Although, as reported in the literature, there are several solvents that can be used (methanol, hexane, acetone, ethyl acetate) (Sun et al., 2015), the possibility of combining EtOH with $H_2O$ in any ratio, the different polarity of both solvents, and their acceptability for human consumption, make their mixtures the most suitable solvents to obtain phenolic extracts from plants, without leaving toxic residues (Conde-Hernandez and Guerrero-Beltran, 2014).

**High-Performance Thin Layer Chromatography and DPPH of green chemical extracts**

Because of the interesting results obtained with the 50% EtOH extraction, the next step was to characterise the phenolic composition by HPTLC analysis.

In this study, HPTLC was used to semi-quantitatively characterize flavonoids, phenolic acids, CBG, and $\alpha$-tocopherol content in the samples. The HPTLC analysis was also useful for assessing the contribution of various types of phenolic compounds (such as flavonols, flavan-3-ols and phenolic acids) to the overall antioxidant activity, which can be directly related to specific molecules in the samples.

As shown in Figure 3a, hemp co-products showed a complex and rich profile.

More specifically, the band patterns in the chromatographic run of the MFL and HLs samples showed a greater degree of similarity than those observed in the HSC and GMs samples. In particular, as shown in Figure 3b, MFL and HLs showed similar bands with same Ratio frontis (*Rf*) to those used for the standards, namely Quercetin (*Rf*=0.79), Kaempferol (*Rf*=0.83), Rutin (*Rf*=0.16), Chlorogenic acid (*Rf*=0.29), and Ferulic acid (*Rf*=0.78) (*Rf* are shown in Table 2). These phenolic compounds are characterised by high antioxidant activity, as shown in Figure 3b, confirming the results reported by Izzo et al. (2020a), Nagy et al. (2019), and Aloo et al. (2023). At the same time, the MFL revealed matches for the $\alpha$-tocopherol band, an isoform of vitamin E (*Rf*=0.96), supporting the results shown by Beleggia et al. (2023). In particular, the authors, characterising the phytochemical profile of hemp flowers, detected the high presence of tocopherol. Of this, the main isoform present was $\alpha$- (86.6-89.4 %), followed by $\beta$+$\gamma$- (8.1-11.2 %) and $\delta$-tocopherol (1.5-2.3 %) (Beleggia et al., 2023).

The presence of $\alpha$-tocopherol was also found in HLs. As reported by Izzo et al. (2020b) and Engin (2009), this molecule is highly present in HSs oil where it covers a key role in preserving the oxidative stability of oils, acting as a chain-breaker, and consequently slowing down the lipoperoxidation process, confirming the results reported in Figure 3b. What has just been observed plays an important role in that, even following the dehulling process, the lipid profile in the endosperm of the HSs is not altered, thus improving the nutritional and functional profile of the HLs.

In parallel, MFL showed a match for CBG, an important phytocannabinoid, confirming the reports of Farinon et al. (2020) and Nagy et al. (2019). Phytocannabinoids are produced, collected, and stored at the level of pedunculated glandular trichomes, small specialised secretory epidermal glands present and abundant on flowers, in fewer numbers on leaves and stems while they are absent at the level of seeds, so the latter organs should not contain cannabinoids (Farinon et al., 2020). However, HLs also showed bands for CBG. Most probably, this is caused by contamination with other parts of the plant during harvesting. In fact, as reported by Farinon et al. (2020), the presence of cannabinoids in HSs represents an accidental contamination due to both the considered cultivar and the seed cleaning process. Most probably, the confirmed presence of CBG, a highly antioxidant molecule as shown in Figure 3b, influenced the results previously reported with the ABTS and FRAP assays following chemical extraction for HLs, resulting in an increased antioxidant profile.

For HSC, bands were observed for Quercetin, Ferulic acid, Caffeic acid (*Rf*=0.75), and Catechin (*Rf*=0.57), partially confirming the results reported by Teh et al. (2014). More specifically, the authors reported the presence of Caffeic acid and Quercetin in HSC, but the absence of Ferulic acid and Catechin, although the latter, as shown in Figure 3b, is more evident following derivatisation with DPPH. As reported by Ingallina et al. (2019), the presence/absence and relative concentrations of phenolic compounds depend on environmental conditions and seasonality, resulting in different profiles even when considering the same matrix. These compounds are characterised by important antioxidant activity, as shown in Figure 3b, particularly Caffeic acid and Catechin, confirming the results reported by Gülçin (2006) and Grzesik et al. (2018).

Figure 3. HPTLC plate of standards and samples detected 254 nm (a) and after derivatization with DPPH detected at visible light (vis) (b). K: Kaempferol, Q: Quercetin 3-O-glucoside, R: Ruthin; C: Catechin; EC: Epicatechin; GA: Gallic acid; CA: Caffeic acid; CLA: Chlorogenic acid; FA: Ferulic acid; CBG: Cannabigerol; TP: $\alpha$-tocopherol; MFL: Mix Flowers/leaves; HLs: Hulls; HSC: Hemp Seed Cake; GMs: Grape Marcs.

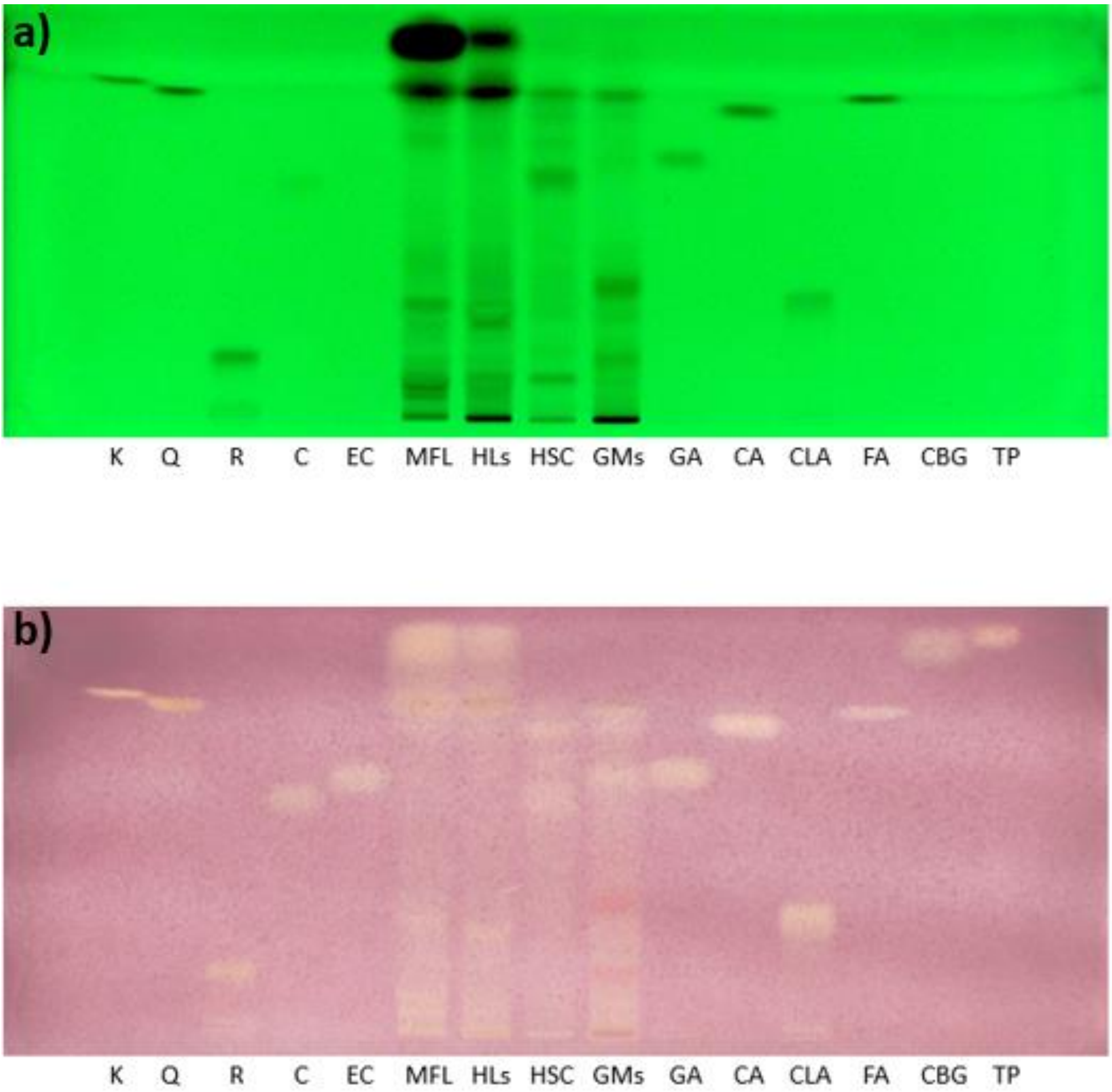



Table 2. HTPLC standards with their correspondent Ratio frontis (Rf) and $\gamma$ (nm) values. vis: visible light (380-700 nm).

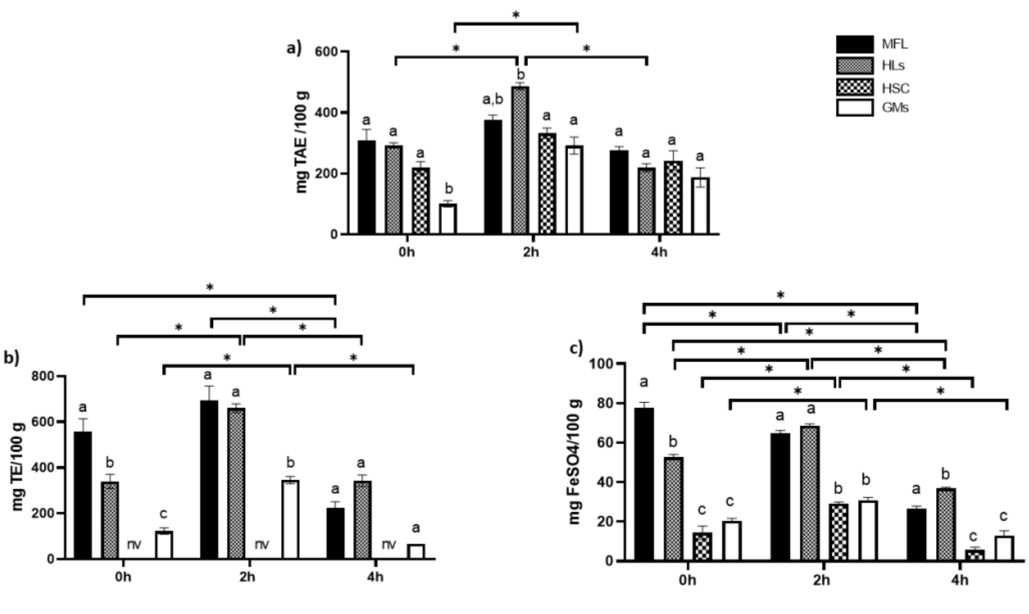
| **Standard** | **Abbreviations** | **Ratio frontis (*Rf*)** | **$\gamma$ (nm)** |
|---|---|---|---|
| Kaempferol | K | 0.83 | 254, vis |
| Quercetin3-O-glucoside | Q | 0.79 | 254, vis |
| Rutin | R | 0.16 | 254, vis |

| | | | |
|---|---|---|---|
| Catechin | C | 0.57 | vis |
| Epicatechin | EC | 0.62 | vis |
| Gallic acid | GA | 0.63 | 254, vis |
| Caffeic acid | CA | 0.75 | 254, vis |
| Chlorogenic acid | CLA | 0.29 | 254, vis |
| Ferulic acid | FA | 0.78 | 254, vis |
| Cannabigerol | CBG | 0.94 | vis |
| $\alpha$-tocopherol | TP | 0.96 | vis |

**Total phenolic content and antioxidant activity of hemp co-products ex vivo digested**

Figure 4 shows graphs of the TPC, ABTS, and FRAP of hemp co-products following ex vivo digestion.

Figure 4. Total Phenolic Content and Antioxidant activity (ABTS and FRAP) of ex vivo digestion of hemp co-products. TAE: Tannic Acid Equivalent; TE: Trolox Equivalent. Data are presented as mean ± standard error of mean (SEM), (n = 3). Different superscript letters in columns indicate significant differences (*p<0.05*) between the samples within each digestion phase. Bars indicate significant differences (*p<0.05*) between the same samples in different digestion phase. (a) Total Phenolic content; (b) ABTS assay; (c) FRAP assay. 0h: start of gastric phase; 2h: end of gastric phase; 4h end of intestinal phase.




As reported in Figure 4a, all the matrices analysed showed the same trend, characterised by an increase in TPC after the end of the gastric phase (2h), although it was only statistically significant (*p<0.05*) for HLs and GMs. This trend is confirmed on matrices digested with in vitro digestion protocols (Lanzoni et al., 2023; Ma et al., 2020; Goulas and Hadjisolomou, 2019). In general, as reported in the literature, the behaviour of phenols during the digestive process is highly influenced by both the food matrix and the digestion conditions (Olivas-Aguirre et al., 2017). More specifically, in the stomach, the acidic pH (2.0-3.0) allows the release of phenolic compounds from the food matrix following the breaking of protein and polysaccharide (fibre) bonds (Goulaas and Hadjisolomou, 2019), increasing their availability, thus confirming the results obtained in this study. In parallel, as shown by Pineda-Vadillo et al. (2016), the acid pH protects phenols during gastric digestion, not altering their quantification. As shown in Figure 4a, at the end of the intestinal phase (4h), there was a decrease in TPC compared to the gastric phase, although statistically significant ($p<0.05$) only for HLs. This trend is caused by the instability of phenolic compounds at the alkaline pH (>7.0), typical of the intestinal environment, especially during pancreatic action, which leads to the transformation of phenols into unknown structural forms with different chemical properties, different bioaccessibility, bioavailability, and biological activity (Wojtunik-Kulesza et al., 2020). Considering the individual matrices, interesting results are reported for HLs (486.57±11.56 mg TAE/100 g) at the end of the gastric phase, with statistically ($p<0.05$) higher differences compared to HSC (332.28±17.10 mg TE/100 g) and GMs (291.68±27.86). Although, dietary fibres are the main carriers of phenolic compounds, thus influencing their bioaccessibility, as reported by Wojtunik-Kulesza et al. (2020), it is also true that in HSs, as previously observed, the phenols are mainly localised in the HL, and following the gastric phase, the disintegration of the food matrix results in a high release, leading to a greater concentration than in other samples (House, 2021).

In parallel, as shown in Figures 4b and 4c, the ABTS and FRAP (with the exception of MFL) assays showed the same trend as the TPC, confirming that phenolic compounds are actively involved in antioxidant activity, as reported above. As phenolic compounds, the antioxidant capacity is also influenced by digestion conditions. Wojtunik-Kulesza et al. (2020) reported that the low pH, typical of the gastric phase, increases the antioxidant power of the compounds due to an increase in their ability to donate electrons. In parallel, the decrease observed for each sample at the end of the intestinal phase could be attributed either to a structural reorganisation of some phenolic compounds due to their sensitivity to alkaline pH or to the fact that, during digestion, some of them are able to bind other constituents of the food matrix, forming complexes that inhibit their antioxidant power (Wojtunik-Kulesza et al., 2020). Considering the individual matrices, MFL and HLs showed statistically significant differences from HSC and GMS, with the exception of the ABTS assay at the end of the intestinal phase, confirming what was observed following the green chemical extraction. Although these results can be attributed to the high concentration of phenols, compounds such as $\alpha$-tocopherol and CBG play a major role in functional activity, as shown before. At the same time, multiple biological functions are correlated with CBG, including anti-inflammatory, antibacterial, antifungal, redox balance regulation, and as a neuromodulator (Jastrzab et al., 2022). However, the presence of phytocannabinoids, particularly THC, despite their positive effects, raises concerns about possible bioaccumulation in animal products with a resulting health risk for the consumer. For this reason, it should be emphasised that the supplementation of hemp-based products in the diet of broilers did not result in traces of THC in the breast, liver, lungs, and thighs, confirming the safety of these compounds (Jing et al., 2017). As shown in Figure 4c, MFL showed a different trend at 0h, suggesting a ready availability of compounds with antioxidant capacity to chelate metals, the principle of action of the FRAP assay.

In Figure 4b, the values for HSC were not shown, as the ABTS assay obtained non-detectable values at each stage of the digestive process. This result could most likely be related to the behaviour of the phenols during digestion. As reported above, HSC is mainly characterised by the presence of caffeic acid, ferulic acid, quercetin and catechin. However, as reported by Wojtunik-Kulesza et al. (2020), not all phenols are able to perform the same antioxidant activity. More precisely, as shown by Khokhar and Apenten (2003), some conditions such as alkaline pH or the presence of oxygen can inhibit the antioxidant action and induce a pro-oxidant behaviour of some phenols, especially to the damages of small compounds, among them caffeic acid. In parallel, ferulic acid appears to be one of the main phenolic compounds linked to lignin and non-starch polysaccharides in HSs; factors that could inhibit its antioxidant action (Wojtunik-Kulesza et al., 2020). However, results obtained by FRAP assay showed highly comparable values with GMs at every stage of the digestive process. This result could be related to the fact that Ferulic acid, Caffeic acid, Quercetin, and Catechin are phenolic compounds also characterised by metal chelating antioxidant activity (Zdunska et al., 2018; Gülçin, 2006; Leopoldini et al., 2006; Kashima, 1999).

Hemp co-products were characterised by an adequate nutritional profile and a high functional potential. However, to date, the use of hemp-based products in the feed sector is limited, due both to the current regulatory status, the different processes on matrices, the cost and limited volumes produced (Salami et al., 2019; Vastolo et al., 2022). More precisely, as shown by Moscariello et al. (2021), the price of HSs, the main product of hemp for food and feed purposes, is around 1.33/1.99 €/kg with a production of 0.6-0.9 ton/ha per year, a price significantly higher than that of soybeans (0.42/0.50 €/kg) (Granaria Commodity Trading Association Milano, 2024), the main protein source used in the diet of livestock animals. However, although cost is a critical point, the interesting nutritional but above all functional profile of hemp-based products compared to soy products, as also demonstrated in our previous work (Lanzoni et al., 2023), suggest their use in the diets of monogastric animals.

For these reasons, to stimulate the use of hemp-based products and their co-products, it is necessary to increase cultivation and production by creating new markets for hemp, thus ensuring the core principles of the Green Deal, One Health and F2F.

## Conclusions

Hemp co-products showed an interesting nutritional profile, especially in terms of protein and lipids, comparable or superior to that of GMs, a matrix widely used in the feed industry. For HLs and MFL, valuable results were obtained in terms of TPC and antioxidant activity (ABTS, FRAP and DPPH), both following green chemical extraction and ex vivo digestion. Extraction with 50% EtOH showed to be the most effective for extracting phenolic compounds and antioxidants from plant-derived matrices. This work represents a preliminary report on the functional profile of hemp co-products for their future application in the feed industry. In vivo trial will be necessary to evaluate their efficacy on the health and growth performance of farm animals, taking into account the high variability of these matrices. The presented data support the potential future growth of hemp processing industries, thus creating markets capable of absorbing large quantities of scraps and consequently valorising it into co-products for the feed industry.

**Disclosure statement**

No potential conflict of interest

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**Ethical approval**

Not applicable

**Data availability statement**

The dataset generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Not applicable

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# CHAPTER 4

## - A proposal for the development of a feed consumption database using a standardised feed classification system

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# A proposal for the development of a feed consumption database using a standardised feed classification system

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**Abstract:** There is currently no comprehensive feed consumption database using a harmonised feed classification system. This means there is a lack of accurate information on the individual amount of feed consumed daily by farmed and companion animals. Such a database would support exposure assessments of feed. This report provides a complete overview of the main feed databases, their structure, and how feed materials are classified. It highlights their limits and potential and reports any differences from FoodEx2. A proposal for future updates of FoodEx2 and the development of a comprehensive feed consumption model database is provided. The proposed model database is based on three information areas represented by three Excel sheets. These represent Animal, Feed, and Consumption, allowing the determination of animal dietary exposure. A proof of concept of the developed model database was carried out by performing two case studies focused on genetically modified feed and feed contaminants. For genetically modified feed, a reduced animal dietary exposure was obtained compared to estimations reported in the scientific opinions and obtained using Excel calculators proposed by EFSA. For the contaminants in feed, differences were limited, resulting in slightly higher or lower exposure values. Weaknesses and possible mitigations are also addressed, and recommendations are made for a comprehensive feed consumption database. These include recommendations establishing an EU classification system in which the main features and items reported in the European Catalogue of Feed Materials are harmonised with FoodEx2. It is also recommended that real-life animal consumption data are collected by the feed industry, animal nutritionists, breeders, and farmers, along with the collaboration of stakeholders.

**Keywords**

Feed classification, Feed consumption database, Animal species, Feed categories, exposure assessment

**Question number**

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**Summary**

The overall purpose of this project (procurement OC/EFSA/GMO/2021/05) is to develop preparatory work for future implementation of a feed classification and feed consumption database. This supports a more accurate estimation of animal dietary exposure by attempting to reach the standards in place for human dietary exposure.

The project pursued four major tasks, as explained below:

**Task 1**

A thorough mapping of existing systems and methodologies for feed classification and feed consumption data collection was conducted. Databases, classification systems, terminology, inclusion rates, and feed consumption in each feed database were analysed. The databases showed a significant lack of homogeneity and high variability in the feed classification system. They provide limited information on the inclusion rate of each ingredient and many fail to report a reference mixture or compound feed. This limited information, combined with the absence of data on feed consumption for each species and category of animals, makes it difficult to define exposure to animal feed and to make accurate EU-wide analyses. It also complicates harmonised country-to-country comparisons, potentially introducing over or underestimations which could translate into inadequate risk characterisation.

**Task 2**

A further step was to develop a proposal for a future update of the current EFSA food and feed classification system (FoodEx2). Recommendations for harmonising terminology and descriptions through the same feed materials and compound feed across different databases are proposed. An analysis of "Hierarchies" and "Facets" reported in FoodEx2 was performed focusing on feed materials. When the selected databases were compared with FoodEx2, a great overlap with the European Catalogue of Feed Materials was found, while the other databases were far off in terms of structure and content from FoodEx2.

**Task 3**

Our proposal for a feed consumption database was developed with a focus on recommendations for data collection and structure. This considered SSD2 controlled terminologies and ensured future interoperability requirements. The model database uses standardised diets and feed classification harmonised with EFSA's current food and feed classification system (FoodEx2). The data on feed materials used in selected animal diets was obtained using the “Global Livestock Environmental Assessment Model – Interactive GLEAM-i” and scientific literature. These diets were used to

populate the model database, which consists of three different Excel sheets named Animal, Feed, and Consumption.

Our model database is designed to improve and harmonise various aspects of feed exposure assessments. However, using diets from the literature in our database highlighted the lack of comprehensive and real-life feed consumption data. It also emphasised the importance of collaborating with stakeholders and the feed industry to collect such data.

**Task 4**

A proof of concept for the database developed in Task 3 was conducted by retrieving animal and feed consumption data from this model database. Two case studies focused on genetically modified (GM) feed (maize) and feed contaminants (ochratoxin A) were performed. Our database first included animal diets for specific animal categories to enable a comparison of animal dietary exposure in the two case studies. The proof of concept took into account four EFSA scientific opinions published in the EFSA Journal. We compared the results of animal dietary exposure reported in the scientific opinions with those obtained using Excel calculators proposed by EFSA. Finally, we compared these results with the values obtained from the extrapolated animal and feed consumption data from the model database. Generally, for the selected case studies, we observed reduced animal dietary exposure from the input data extracted from the model database.

Overall, we found a wide variety of feed materials used in ration and diet formulations have not yet been classified under a single classification system. In fact, we revealed differences in databases published by national and international organisations. The disparities in the feed classification system, alongside different nomenclatures for animals and the lack of feed consumption data, may cause miscommunication among operators and professionals in the feed industry (e.g., farmers, feed manufacturers). It may also represent a limitation in the evaluation of animal exposure assessments.

For the future development of a feed consumption database, further work is needed. We suggest establishing an EU classification system where the main features and items reported in the European Catalogue of Feed Materials are harmonised with FoodEx2. After this harmonisation, collecting consumption data to populate the database from the feed industry, animal nutritionists, breeders, and farmers is recommended, along with collaboration with stakeholders.

During this project, initial stakeholder involvement was facilitated by organising a meeting to share preliminary information about the project and collect feedback. At the meeting, discussions revolved around setting up a uniform classification system for feed ingredients. A further workshop with stakeholders has been planned for the end of the project to present and discuss the final results and to collect final input for the development and implementation of an EU feed consumption database.

# 1 Introduction

## 1.1 Terms of reference as provided by the requestor

This contract was awarded by EFSA to a consortium headed up by the University of Milan:

Contractor: University of Milan

Members of the consortium:

- University of Milan
- ToxHub Srl
- ISA Digital Consulting.

Contract: OC/EFSA/GMO/2021/05

## 1.2 Background

Food producing and non-food producing animals[1] need a balanced diet containing all the necessary nutrients, fluids, minerals, and vitamins. Proper nutrition allows animals to meet their physiological needs, grow, reproduce, defend against infections, and produce food for humans (e.g., milk, eggs, meat). For animals raised for food production, such as poultry, pigs, and aquaculture, it is crucial to optimise production and allocate nutrient supplies efficiently for maximum societal benefit. Decisions about what and how to feed animals are based on reliable information regarding the composition of all feed materials used in animal feeding. This information is fundamental in assigning priorities to the use of available feed supplies.

Animal feed is typically divided into those that come from traditional sources and those that are a mixture of additives and other raw materials. Factors such as type (e.g., classification), role (e.g., ingredient, additives), quality, and safety are essential in defining not only nutrient supply but also the welfare and health of the animals.

The risk assessment of feed involves several steps: hazard identification and characterisation to define a safe intake level for the hazard under assessment; dietary exposure assessment to estimate

1 According to Reg 767/2009, companion animals are classified as non-food producing animals

the exposure to the hazard linking data on feed consumption with the concentration of biological, chemical and physical agents in feed, and risk characterisation to define the overall risk.

In the European Union (EU), a comprehensive legislative framework is in place which addresses the risk assessment of biological, chemical, and physical hazards in feed. This includes undesirable substances, pesticides, and veterinary drug residues, feed additives, and new or endogenous constituents with altered levels due to genetic modification.

In 2019, an EFSA task force reviewed the different approaches used to estimate animal dietary exposure across scientific areas involved in the risk assessment of feed. EFSA highlighted the need to develop a harmonised feed classification system and a comprehensive feed consumption database for both food producing and non-food producing animals. This aims to approach the standard in place for human dietary exposure (EFSA, 2019), which is based on a comprehensive food classification system (FoodEx2 system) (EFSA, 2015) and a food consumption database for the EU human population[2].

This technical report summarises the main relevant findings on the main feed databases, the methodologies for feed classification, and the collection of data on feed consumption. The information was complemented by a proposal for harmonisation with EFSA's current food and feed classification system (FoodEx2). A model database[3] for a feed consumption database was then proposed. To verify the functioning and viability of the proposed model, two distinct case studies were carried out, one focusing on genetically modified (GM) feed and the other on feed contaminants.

### 1.3 Objectives as provided by EFSA

The aim of this procurement was to outsource the preparatory work for further implementation of a harmonised feed classification system and the development of an EU feed consumption database.

The project meets EFSA's recommendations to support a more accurate estimation of animal dietary exposure by approaching the standards in place for human dietary exposure (EFSA, 2019).

The outsourced activities include:

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2 Food consumption data are made available in the EFSA Comprehensive European Food Consumption Database at: https://www.efsa.europa.eu/en/data-report/food-consumption-data

3 Hereinafter, we refer to the proposed feed consumption database model (section 2.3.2) with the term “model database”

1. mapping existing systems and methodologies for feed classification and feed consumption data collection

2. developing a proposal for future update of the current EFSA food and feed classification system (FoodEx2)

3. developing a proposal for a feed consumption database that includes data structures and guidance for data collection. This is supported by a case study applicable to the areas of EFSA risk assessment where improvements in animal dietary exposure are most urgently needed.

The long-term objectives to implement a harmonised feed classification system and to develop an EU feed consumption database represent a complex project. However, it is of recognised interest not only by EFSA but also by other organisations at the international level. A stepwise approach will reduce the overall complexity by providing the necessary preparatory work, and the tasks below have been requested.

Task 1: Map existing systems and methodologies for feed classification and feed consumption data collection.

Task 2: Develop a proposal for future update of the current EFSA food and feed classification system (FoodEx2)

Task 3: Develop a proposal for a feed consumption database, including data structures and recommendations for data collection.

Task 4: Proof of concept for the development of a feed consumption database

Task 5: Final report

# 2 Data and methodologies

## 2.1 Mapping of existing systems and methodologies for feed classification and feed consumption data collection

### 2.1.1 Analysis of the classification system adopted in each feed database

The first step in mapping existing systems was analysing the classification system adopted in each feed database The complete list of databases indicated in the OC/EFSA/GMO/2021/05 tender was:

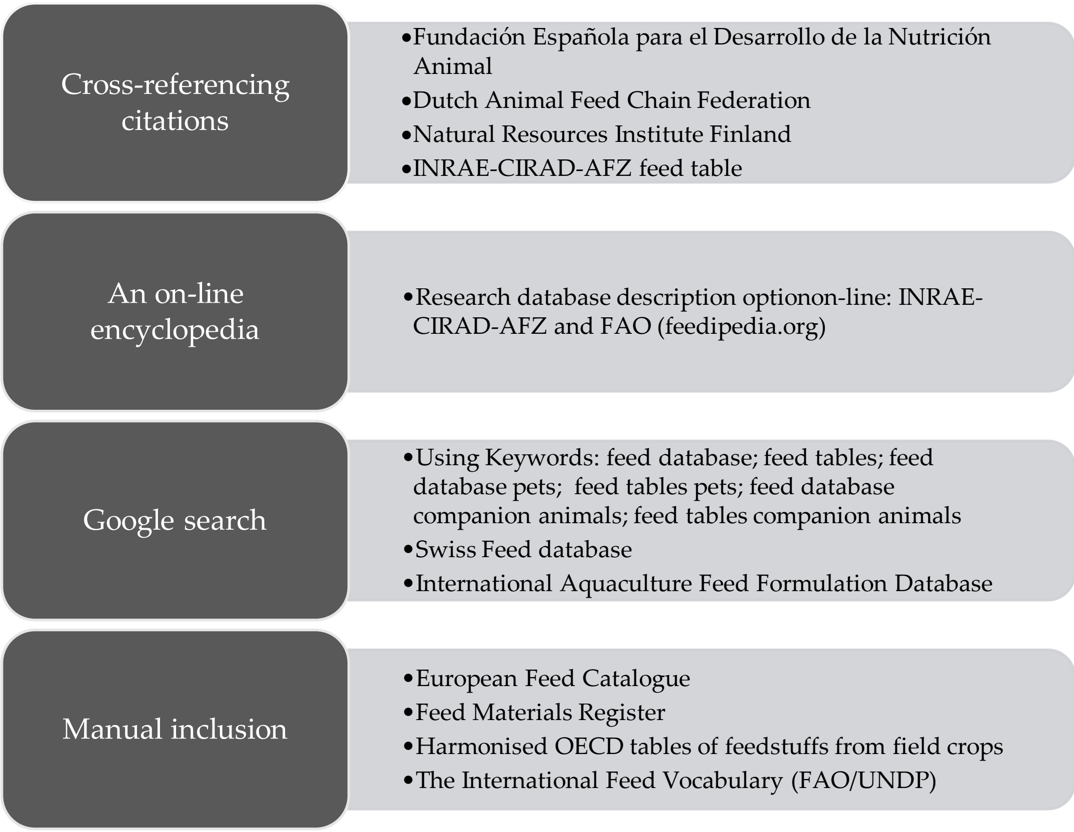
- Fundación Española para el Desarrollo de la Nutrición Animal (FEDNA)
- Dutch Animal Feed Chain Federation (CVB System)
- Natural Resources Institute of Finland (LUONNONVARATIETO)
- INRAE-CIRAD-AFZ (FEED TABLES)
- INRAE-CIRAD-AFZ and FAO (Feedipedia)

The list was integrated by combining different approaches, as summarised in Figure 1. Specifically, a manual search was conducted using specific keywords within the Google search engine. Keywords used included feed database; feed tables; feed database pets; feed tables pets; feed database companion animals; and feed tables companion animals. Only European databases were considered; therefore, all databases based on non-EU data were excluded, with the exception of the International Aquaculture Feed Formulation Database (IAFFD).

From mapping the existing feed databases, emerged the presence of other classification systems that are even more narrative-based and informative than the classic databases considered in the previous section.

For this reason, as reported in Figure 1, the following classification systems were included manually:i) European Feed Catalogue; ii) Feed Materials Register; iii) Harmonised OECD tables of feedstuffs from field crops; iv) The International Feed Vocabulary (FAO/UNDP).

Figure 1: Methodology approach adopted to select databases and other classification systems.




### 2.1.2 Analysis of terminology and description of feed ingredients in selected feed databases

The analysis of terminology and description used in each feed database verified whether:

• there was an internal search engine in all the databases found when the feed databases were mapped.

• the terminology and description used in each feed database, using Corn (grain), Maize, and Zea Mays as keywords. Corn was chosen as the reference material due to its worldwide employment.

• the following information was reported: Name (Acronym); Origin; Language; Website; Downloadable; Narrative description of the information reported for the test feed ingredient (i.e., corn).

### 2.1.3 Collection of information on inclusion rates and feed consumption

Feed consumption and its efficient utilisation is one of the major concerns regarding animal nutrition. Feed consumption varies and is influenced by several factors, including animal species and size, physiological state of animals, number of animals on the farm, energy content of the diet, ambient temperature, hygienic conditions, and rearing environment.

Given the scarcity of information regarding feed consumption in the selected feed databases, the search was expanded to a non-European context with the report presented in December 2017 by the American Institute for Feed Education (IFEEDER) and Research in collaboration with Decision Innovation Solutions (DIS). This publication addresses feed consumption for food-producing animals only. It estimates the amount of feed consumed by different animal species throughout their lifetime in certain states and regions.

Another non-European source of information about feed consumption for food-producing animals is “The Agricultural Market Information System” (AMIS). In 2014, this inter-agency platform reported the pros and cons of various methodologies, discussed the data requirements for estimating feed use in the context of cereal balance sheets, and reviewed some of the options for better estimating cereal feed use.

The availability of information on feed consumption was also verified with associations or groups of stakeholders with expertise in collecting and managing data related to feed consumption in the EU for both food-producing and non-food producing animals1 (e.g. feed producer associations, food producing animal associations, veterinary associations, pet food industry). This approach was implemented through questionnaires and online consultation (workshops).

## 2.2 Developing a proposal for future update of the current EFSA food and feed classification system (FoodEx2)

### 2.2.1 Analysis of FoodEx2: functioning, structure and available information

FoodEx2 is a comprehensive food classification and description system designed to address the need for detailed food data collection across various food safety domains (EFSA, 2015). Since its first release in 2011, the system has been extensively tested for its effectiveness in collecting data on food consumption and chemical occurrences. It has involved several Member State organisations that operate in practical data collection scenarios.

The testing phase identified strengths and weaknesses of the food classification system, leading to suggestions for improvement and the identification of specific areas for enhancement. Consequently, FoodEx2 was revised to better align with the needs of its diverse users. The current version includes nine hierarchies: eight domain-specific and one service hierarchy for terminology management. EFSA has consistently emphasised the importance of capturing detailed information about food groups in exposure assessments. Now, it advocates applying the same detailed process to animal feed as well.

FoodEx2 categorises feed and food items into groups and broader categories within a hierarchical parent-child relationship structure. This allows each term to be expanded to reveal more specific terms within each category. The terms in the hierarchy are denoted by a blue pyramid symbol, while generic or non-specific terms are denoted by a white or yellow sphere symbol. The red sphere represents the core terms, which indicate the minimum level of specificity recommended for data encoding. The green sphere represents the extended terms, which provide the highest level of specificity. Lastly, the orange diamond represents facets and other service terms. The entire system is code-based, with each item uniquely identified by a five-digit alphanumeric code.

The initial step in evaluating the FoodEx2 system involves analysing its functionality, structure, and the information it provides. When the FoodEx2 Matrix is accessed via the EFSA catalogue browser (EFSA & Ioannidou, 2019), users can select among different hierarchies displayed in the upper section of the Catalogue Browser. At the hierarchy level, a general description of the feed item is provided, while the facets detail additional specific aspects. The detailed analysis of these hierarchies and facets will be discussed in the following sections.

### 2.2.2 FoodEx2: hierarchies' analysis and comparison with selected feed databases

In the upper section of the Catalogue Browser, besides the sections for Hierarchy and Facet, a drop-down menu lists all the hierarchies. The "MTX (FoodEx2 Matrix)" serves as the main hierarchy containing all the FoodEx2 codes in the catalogue, which are then further organised into different hierarchies. Of the eight hierarchies, we only considered the Feed hierarchy for detailed evaluation.

After identifying the categories in the Feed hierarchy of FoodEx2 we compared these categories with those in other databases/sources. These sources include FEDNA, feedipedia.org, and the European Catalogue of Feed Materials. Through this evaluation, discrepancies and similarities were documented and discussed, providing a basis for proposing amendments to FoodEx2.

### 2.2.3 FoodEx2: facets analysis

Facets provide additional detail on a selected feed item through a list of 28[4] groups

of descriptors. Facets are collections of single descriptors defined from specific viewpoints applicable to particular food items; examples include source (F01), packaging material (F19), and production method (F21).

4 At the time of the facet analysis in May 2023

Many facets are included, but not all are applicable to all terms. Some terms are only appropriate for a subset of base terms, such as age classes which can only be used for animals. There are general facets usable across all domains, such as part-nature, source, source-commodities, and process. Specialised facets, applicable only to specific domains such as purpose-of-raising, reproductive-level, animal-age-class, and gender, are relevant only in the animal domain. The first critical aspect to consider is "applicability," which also indicates whether a facet is intended for feed. Another significant aspect is the "cardinality" of facets, which refers to the number of descriptors of the same facet that can be applied to a single base term. Cardinality and applicability can be "single" (e.g., F01 Source) or "repeatable" (e.g., F04 Ingredient). Both aspects should be considered when coding with FoodEx2, as defined in EFSA 2015: "The food classification and description system FoodEx2 - revision 2".

Facets have two roles within the system:

• Implicit Facets: These descriptors always apply to a specific food group and are pre-assigned in FoodEx2. Users coding a food item do not need to enter these facets, and it is advised never to report implicit facets in coded datasets.

• Added Facets: These descriptors are added to a code while coding a food item. They describe a characteristic that distinguishes one specific food item from others within the same food group, as defined by a chosen base term.

Facets are reported with a code that includes a header defining the facet (e.g., "Fxx") and a unique term code defining the descriptor. For example, a code for a facet might be: F02.A0F0Y (Feed-related as part-nature).

The FoodEx2 code integrates various pieces of coded information into a single string. The code includes a base term (the food or feed being analysed), followed by a hash "#" and a sequence of facet codes. These codes are added at the coder's discretion, separated by dollar signs "$". The number of added facets is unlimited, and it is best to arrange facets in alphabetical order.

Regarding the two case studies on maize/corn and soy, typical FoodEx2 codes are reported below:

A07XG#F02.A0F0Y$F01.A059G$F27.A07XG$F23.A07TV$F28.A07LA

The first part of the code (A07XG) means "Maize grain (feed)". After the hash, the four implicit facets follow automatically (F02 Part-nature=Feed-related (as part-nature), F01 Source, F27 Source-commodities and F23 Target-consumer). Considering maize/corn as a feed ingredient, some other information can be added (added Facets). For example, selecting Facet F28 (process), Grinding/milling/crushing (A07LA).

With regards to Processed maize-based flakes (feed):

A0EFQ#F02.A0F0Y$F27.A07XG$F23.A07TV$F28.A07GP

The first part of the code (A0EFQ) means "Processed maize-based flakes (feed)". After the hash, the four implicit facets follow automatically (F02 Part-nature=Feed-related (as part-nature), F27 Source-commodities, and F23 Target-consumer). After that, other Facets can be added, for example, F28 Process, "steaming" (A07GP).

With regards to soy:

A0EHX#F02.A0F0Y$F01.A05HX$F27.A0EHX$F23.A07TV.A07GQ$F28.A07HC

The first part of the code (A0EHX) means "Soya (beans) (feed)". After the hash, four implicit facets follow, (F02 Part-nature=Feed-related (as part-nature), F01 Source, F27 Source-commodities, and F23 Target-consumer). Considering soy as a feed ingredient, other information, for example can be added, F28 Process, "Toasting" (A07HC).

With regards to soy (bean) meal (feed):

A0BFJ#F02.A0F0Y$F27.A0EHX$F23.A07TV$F10A0CJE

The first part of the code (A0BFJ) means "Soya (bean) meal (feed)". After the hash, the implicit facets are automatically added (F02 Part-nature=Feed-related (as part-nature), one implicit facet, like F27 Source-commodities, and F23 Target-consumer that is used when not obvious, or when this information makes the difference). After that, the added facet is F10 Qualitative-info, "Proteins-related info" (A0CJE).

### 2.2.4 Comparison of terminology between FoodEx2 and selected feed databases/sources

We compared the terminology and level of detail between FoodEx2 and some selected feed databases/sources regarding two feed ingredients, namely corn/maize and soy.

### 2.2.5 MTX (FoodEx2 Matrix) integrations

The integrations that need to be done to the structure of FoodEx2 have been analysed to make it usable in the animal nutrition field. Then, we analysed how to integrate and/or link the data stored in the FoodEx2 database with the information found in other selected databases. The aim was to make the terminology uniform.

The analysis of the possible integrations was based on the MTX version of the Excel file MTX_13.2.xlsx, and the corresponding graphical interface provided by EFSA (EFSA Catalogue browser).

Specifically, the following were investigated

- Feed Hierarchy structure analysis and facets refinements
- Harmonisation of FoodEx2 with other selected databases

## 2.3 Developing a proposal for a feed consumption database, including data structures and recommendations for data collection

For the development of a proposal for a feed consumption database, we consulted recommendations on methodologies for the collection of feed consumption data representative for food producing and non-food producing1 animals in the EU Member States for use in dietary exposure assessments. A feed consumption model database and data structure was then built. These are available as Excel files under the Supporting Information Section of the online version of this output.

### 2.3.1 Test diets for modelling a feed consumption database

Harmonised classification and quantification of daily feed consumption are fundamental for accurate estimations of animal exposure to potential hazards. However, the absence of a comprehensive and centralised database containing accurate feed consumption data remains a challenge. This lack of validated and robust data on feed intake and consumption, which includes both food-producing and non-food-producing animals, significantly limits accurate risk assessments due to potential over or underestimations of feed consumption.

Consequently, the goal is to lay the groundwork for amending the current feed section of the FoodEx2 system by developing a proposal for a feed consumption database that integrates feed classification, animal categories, and consumption data. To achieve this goal, selecting specific diets for various species across different growth, maintenance, and production phases is essential. In this context, the FAO "Global Livestock Environmental Assessment Model – interactive GLEAM-i" (https://gleami.apps.fao.org) was utilised. GLEAM-i is a unique source of information that offers reference diets applicable to specific countries and production systems, including industrial and backyard setups. However, the FAO "Global Livestock Environmental Assessment Model – interactive GLEAM-i" often uses generic terms for feed ingredients and does not account for variations in age or weight among the animals considered for specific diets. To address these limitations, we supplemented GLEAM-i with diet recommendations from the literature, particularly for species not initially covered by GLEAM-i. By merging these data sources, a selection of representative diets for each feed consumption simulation was developed.

### 2.3.2 Development of a proposal for a feed consumption database

A feed consumption model database was proposed. Pigs, dairy cows, and laying hens' diets were selected as test cases due to their widespread presence across the European Union. Moreover, simplified test diets for Atlantic salmons and adult dogs were included to provide a wider selection of food-producing animals and to include a non-food-producing1 animal category.

As a data structure for the feed consumption database, an Excel file was compiled using the Standard Sample Description 2 (SSD2) (EFSA, 2013) controlled terminologies, particularly the FoodEx2 classification system. The file is organised into three different sheets called Animal, Feed, and Consumption. The Animal and Feed sheets store information on animals and feed, respectively, and are linked by the Consumption which specifies the type and quantity of feed consumed by each animal per day.

The Animal sheet describes properties/features that are relevant for determining their feed consumption. A row regards a specific kind of animal with related features, and is associated with a unique identifier and a textual description. The animal characteristics are listed in columns, and they can be gender, physiological conditions (e.g. pregnancy or lactation), health possible dietary requirements (e.g. maintenance, veterinary diets), and details about the geographic area/country where the animals are raised. Other properties regard the weight, the method used to measure the animal weight, and the overall daily feed consumption of the animal expressed in kg of dry matter (DM). A given animal type, for example the fattening pig, can appear in multiple rows, each characterised by different identifiers and property values. For instance, “Fattening pig20” and “Fattening pig40” can be the identifiers of two distinct rows of pigs characterised by a weight of 20 and 40 kg, respectively, with different amounts of daily feed consumption. Further rows differentiate the daily feed consumptions of pigs when they reach a greater weight, or if they are farmed in different geographic areas e.g. northern or southern Europe.

The Feed sheet describes the properties/features that are relevant for categorising feed composition and nutritional information. Rows refer to a specific feed with related features, and are associated with a unique identifier as well as a textual description, both in the original language and in English. Columns are used to store the corresponding FoodEx2 code of the considered feed and for any comment. A row can be used to describe individual feed materials, which can then be included in a complete diet for a specific animal type (e.g. “Complete feed for fattening pigs”, “Complete feed for dairy cows”), or which can be given to the animals as a single feed material alone, like for example “Corn”, “Soybean meal”, or “Corn silage”.

The Consumption sheet links the other two sheets, and details the type and the quantity of feed consumed daily by each kind of animal. Rows regard the consumption of a specific feed by a specific kind of animal. They are associated with a unique identifier, a reference to the identifier of the considered animal in the Animal sheet, and a reference to the identifier of the considered feed in the Feed sheet. Other columns specify the inclusion rate of such a feed in the daily diet, the quantity in kg of such a feed eaten by the given animal in a day, the brand of the feed, and its corresponding FoodEx2 code. When the feed material is part of a complete diet, information about the total amount in kg of such a recipe is also recorded. For instance, a row can be used to specify that fattening pigs of 20 kg, referenced in the Animal sheet with the identifier "Fattening pig20", eat 0.325 kg of corn per day as part of a complete diet of 1.3 kg per day.

### 2.4 Proof of concept for the development of a feed consumption database

To validate our database, two case studies were conducted: the first on genetically modified (GM) feed, specifically maize, and the second on contaminants, particularly ochratoxin A.

First, three EFSA scientific opinions published in the EFSA Journal were considered. An additional EFSA scientific opinion on GM feed was considered to include Atlantic salmon and dogs, i.e. to represent the fish and companion animal categories (Appendix C).

The selected EFSA scientific opinions, published in the EFSA Journal, are:

• GM feed Example 1: Assessment of genetically modified maize MON 95379 for food and feed uses, under Regulation (EC) No 1829/2003 (EFSA GMO Panel 2022a)

• GM feed Example 2: Assessment of genetically modified maize DP4114 x MON89034 x MON 87411 x DAS-40278-9 and sub-combinations, for food and feed uses, under Regulation (EC) No 1829/2003 (EFSA GMO Panel 2022b)

• Contaminants Example: Risks for animal health related to the presence of ochratoxin A (OTA) in feed (EFSA CONTAM Panel 2023)

GM feed Example 3 (Appendix C): Assessment of genetically modified maize DP202216 for food and feed uses, under Regulation (EC) No 1829/2003 (EFSA GMO Panel 2024)

#### 2.4.1 Elaboration of the model database

To directly compare animal exposure data reported in the EFSA scientific opinions (EFSA GMO Panel 2022a, EFSA GMO Panel 2022b, EFSA CONTAM Panel, 2023) with the results obtained using the model database, the model database was further elaborated. The elaboration included animal data and diets for specific animal categories into physiological states, with relative feed materials and feed products (e.g. maize gluten feed) intakes, in order to compare animal dietary exposure.

Animal data (body weight, total daily intake) are reported in Appendix A.1.1. Tables 27, 29, 31, 33, 35 and the diets with inclusion rates were reported in Appendix A.1.1. Tables 28, 30, 32, 34, and 36.

### 2.4.2 Step-by-step procedure for retrieving data from the model database

The model database (see Section 2.3.2) was generated in Excel and each Excel sheet is organised into columns, which are the variables of the data model (Figures 2, 3 and 4).

| | A | B | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ORANIMALCODE | ANIMALDESC | GENDER | WEIGHT | MWEIGHT | TOTALDAILYINTAKE | SPECIALCON | SPECDIET | COUNTRY | AREA | COMMENTSANIMAL |

Figure 2: Animal data sheet variables of the database

| | A | B | C | D | E |
|---|---|---|---|---|---|
| 1 | ORFEEDCODE | ORFEEDNAME | ENFEEDNAME | FOODEXCODE | COMMENTSFEED |

Figure 3: Feed sheet variables of the database

| | A | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | RECORDIDENTIFIER | ORANIMALCODE | ORRECIPECODE | FOODEXRCODE | AMOUNTRECIPE | ORFEEDCODE | IR | AMOUNT | BRAND | FOODEXCODE |

Figure 4: Consumption sheet variables of the database

For the proof of concept of the model database, we had to retrieve animal, feed material and consumption data relative to six animal categories (broiler, fattening pig, finishing pig, lactating dairy cows, dairy sheep, Atlantic salmon, dog) of interest.

Here is a step-by-step procedure of how we retrieved this data:

1. In the Animal sheet, we searched for our animal of interest (e.g. dairy cow) (column B, “ANIMALDESC”)

2. We selected the animal with the diet of interest from the correct source (e.g. Darabighane et al., 2020) (column K, “COMMENTSANIMAL”).

3. We noted down the unique animal code (column A, “ORANIMALCODE”) and used it in Step 5

4. We retrieved the animal body weight in column D (“WEIGHT”) and total daily intake in column F (“TOTALDAILYINTAKE”) corresponding to the selected animal.

5. In the Consumption sheet, we searched for the unique animal code (e.g. Dairy cow623) noted down in Step 3 (column C, “ORANIMALCODE”)

6. We retrieved all the feed materials inclusion rates (column H, “IR”) of the rows presenting the unique animal code of interest (column C, “ORANIMALCODE”)

7. We retrieved the feed code (column G, “ORFEEDCODE”) corresponding to each IR (column H, “IR”).

8. In the Feed sheet, we used the feed codes (e.g. R09.I01) retrieved in Step 7 in order to trace the corresponding feed names (column C, “ENFEEDNAME”) of the feed materials in the diet.

The feed names (Feed sheet, column C, “ENFEEDNAME”) correspond to the same naming of feed materials as reported in the EU feed catalogue. Their FoodEx2 codes can be found in the Feed sheet, column D, “FOODEXCODE”.

### 2.4.3 Standardisation of feed material and animal categories nomenclature

Data regarding body weight and feed consumption (feed materials total daily intake, feed materials inclusion rate) of each selected animal category were extrapolated from our model database, as outlined in Section 2.4.2.

To estimate animal dietary exposure, EFSA employs various approaches across several Panels/Units. These methodologies assess the risks associated with GM feed, feed contaminants, pesticide residues, and feed additives. In 2019, EFSA provided a comprehensive overview of these current approaches and introduced a general formula for estimating animal dietary exposure to a chemical of interest. This calculation requires data on the chemical concentration in the feed and the daily consumption (intake) of that feed. By combining these variables and considering the animals' body weight, dietary exposure can be calculated as follows:

(concentration of chemical in feedstuff) x (amount of feedstuff consumed) / (body weight)

In GM feed, the concentration data for newly expressed proteins primarily derives from experimental data, and literature data can facilitate a comparative exposure approach.

In studies involving dietary exposure to contaminants in feed, the daily intake of feed material for each animal category is multiplied by its corresponding OTA contamination level, as outlined in the OTA scientific opinion (EFSA CONTAM Panel 2023). The feed materials are then aggregated to calculate the entire daily diet for the specific animal category.

The amount of feedstuff consumed (intake) is typically estimated based on default values, such as animal body weight and nutrient requirements, which are available from various sources, including scientific literature and guidance documents from European and international organisations, e.g. FAO, OECD, EFSA).

# 3 Assessment/Results

## 3.1 Mapping of existing systems and methodologies for feed classification and feed consumption data collection

In order to map the feed classification systems, a total of 12 International and European databases and other classification systems were considered. This included the five databases listed in the EFSA procurement, further augmented by three databases found on the web, and four other classification systems. For each database considered, the classification of feed ingredients was assessed, including their division into categories and sub-categories.

The analysis highlighted significant variability in the complexity of the classification of feed material. Some databases, such as the Dutch CVB system, rely on a limited number of categories or do not have sub-categories, while others adopt a complex and detailed classification (e.g. Fundacion Espanola para el Desarollo de la Nutrition Animal). The analysis highlighted that there is no clear consensus on the classification of feed ingredients among the different databases considered (Appendix B.1. Table 40).

### 3.1.1 Analysis of the classification system adopted in each feed database

<u>Fundación española para el desarrollo de la nutrición animal (FEDNA)</u>

FEDNA is the Spanish Foundation for the Development of Animal Nutrition, created in 1989 by a collaboration between Spanish universities and private industry. The website covers different topics on animal nutrition. In accordance with the classification of feed material, which is provided in the section 'Tablas FEDNA - Compositión Alimentos Valor Nutritivo', the feed ingredients are allocated into three main groups, each corresponding to a specific table, as follows:

- Ingredientes para piensos (feed ingredients – compound feeds)
- Forrajes (fodder – forage)
- Subproductos fibrosos humedos (wet fibrous by products)

<u>INRAE-CIRAD-AFZ (feed tables)</u>

The INRAE-CIRAD-AFZ feed tables are a French feed database resulting from the collaboration between National Research Institute for Agriculture, Food and the Environment (INRAE), Centre de Coopèration Internationale en la Recherche Agronomique pour le development (CIRAD), and Association Francaise de Zootechnie (AFZ). The INRAE-CIRAD-AFZ feed tables contain chemical data, nutritional data, as well as environmental data on feeds for ruminants, pigs, poultry, rabbits, horses, and fish (salmonids). No information is given on the inclusion limits of the raw materials in the diets of the different animal species, and there are no examples of feed consumption or formulations.

Dutch animal feed chain federation (CVB SYSTEM)

The CVB system operates under the CVB foundation, with activities conducted by Wageningen Livestock Research (WLR) and the Flemish Research Institute for Agriculture, Fisheries and Food (ILVO). The CVB collects data on the chemical composition of feedstuffs and feed materials, as well as data on digestibility for different farm animal categories. However, it does not provide information on the inclusion limits of raw materials in the diets of various animal species, nor does it offer examples of feed consumption or formulations.

Natural Resources Institute of Finland - Finnish feed tables (Luonnonvaratieto)

The Finnish feed tables are published by Natural Resources Institute Finland (Luke), under a mandate from the Finnish Ministry of Agriculture and Forestry. The Feed Tables and Nutrient Requirements website offers official Finnish Feed Tables and equations for calculating feed values and nutrient requirements. The animal species include ruminants, pigs, poultry, horses, and fur animals.

Nordic feed evaluation system (NorFor)

In the late 1990s, dairy farmers from the Nordic countries created a common feed evaluation system and replace various existing systems in Denmark, Norway, Sweden, and Iceland. The NorFor Model serves as an evaluation system, featuring the NorFor Feed Table as one of its main components. This model does not provide details about the inclusion limits for raw materials or examples of feed consumption or formulations.

The Swiss feed database (FeedBase)

FeedBase is a Swiss system that provides information on the composition and nutritional value of over 600 raw materials and roughages in Switzerland. Users can freely search the database for averaged feed data on all individual feeds and roughages. Results from the roughage survey are also available for open access (single value query). The recorded feed values in the database represent processed averages based on dry matter, with considerations for data coherence and representativeness. FeedBase also allows users to retrieve data on feed ingredients based on their Nutrients and Nutritive Values, tailored to various animal species, including ruminants, fattening calves, pigs, poultry, and horses.

International aquaculture feed formulation database (IAFFD)

The IAFFD is an open-access database designed to support aquaculture feed formulation. It provides industry stakeholders with essential information on the nutritional requirements of numerous species at different life stages and the characterisation of the nutritive value of various feed ingredients. The ingredients are listed without categorical or alphabetical organisation but each is assigned a numerical code, allowing for selection and comparison.

Feedipedia

Feedipedia is an online encyclopaedia of animal feed, jointly developed principally by INRAE, CIRAD, AFZ, and the Food and Agriculture Organization (FAO). Feedipedia is open access and provides information on the nature, occurrence, chemical composition, nutritional value, and safe use of nearly 1400 livestock feeds worldwide. Generally no information is provided regarding the inclusion limits for the raw materials, except for that based on data retrieved from the scientific literature.

The European Catalogue of Feed Materials

The EU Catalogue of Feed Materials was established by Article 24 of Regulation (EC) No 767/2009 to improve the labelling of feed materials and compound feed. It provides a detailed, though non-exhaustive, classification and description of feed materials. The first version of the catalogue was adopted with Regulation (EU) 242/2010, then repealed and reinstated in a much more complete form by Regulations (EU) 575/2011 and (EU) 68/2013. The version of the catalogue currently in force under Regulation (EU) 2017/1017 has two major sections: a glossary of 69 procedures applicable to feed, which includes descriptions of processes with definitions of the terms currently in use (as Part B), and the list of feed materials containing 650 feed items (as Part C), subdivided into 13 macro categories listed below. For each feed item listed in the catalogue, descriptions of the source and the process it has undergone are also given. The feed materials are presented in the form of tables, where the name of the feed ingredient, the identification number, a brief description, and, when relevant, the compulsory declarations are reported.

The harmonised OECD tables of feedstuffs from field crops

The Harmonised OECD tables of feedstuffs from field crops have been published by the Organisation for Economic Co-operation and Development (OECD), an intergovernmental organisation composed of representatives from industrialised countries in North America, Europe, Asia, the Pacific Region, and the European Commission. These tables classify individual feed commodities into four specific feedstuff categories and aim to determine the quantity of pesticide residues in products of animal origin that result from residues in feedstuffs (including fodder crops) or from direct application to livestock. Information on the percentage of the diet for each feedstuff is provided, based on national agricultural practices, the typical body weight of the finished animal, and daily feed consumption parameters. Additionally, for each animal species and category, information is divided into regions (USA/Canada, EU, Australia, and Japan). For each region, the reference body weight (kg) and daily intake (DM in kg) are reported. No information on the chemical composition of the different feedstuffs is provided, nor are any examples of formulas given.

The International Feed Vocabulary (FAO/UNDP)

The International Feed Vocabulary is not a real database but is helpful for our proposed classification "levels." It was published in 1978 as part of the report on the FAO/UNDP Training Course in Fish Feed Technology, held at the College of Fisheries, University of Washington, Seattle, Washington (USA), where a new international system was proposed by Harris (1963) and Harris et al. (1968) to overcome inconsistencies in naming feeds. This system was modified and is now known as The International Feed Vocabulary. The International Feed Vocabulary is designed to name each feed as concisely as possible. Each feed name is coined using descriptors taken from one or more of six facets. In this way, over 18,000 feeds have been recorded and assigned International Feed Descriptions or Names in English, German, and French. Additionally, descriptions of the chemical and non-chemical characteristics and quality of the main cereal grains are available.

### 3.1.2 Analysis of terminology and description of feed ingredients in selected feed databases

For each of the databases and other classification systems, the terminology and description of feed ingredients were assessed following a systematic approach, using Corn as reference material.

Three keywords, namely Corn, Maize, and Zea mays, were selected and searched for within each database. The information provided in each database was compared. We found that some databases provide a detailed description of the feed ingredients, sometimes also including images and photos. In other databases there is just a summary description.

Fundación española para el desarrollo de la nutrición animal (FEDNA)

There is no 'Search' functionality on the Spanish feed database FEDNA. Consequently, to find a feed material within the feed database, one needs to know to which feed category the material belongs. By narrowing down the search to Corn, only two items are left: Maíz nacional (Corn from Spain) and Maíz rico en aceite (Corn, high in oil). In the case of corn from Spain, a detailed description is provided. This includes the scientific name of the product, the various types of corn available on the market (including the genetic lines that have been selected for their specific features), the characteristics of the feed in terms of energetic value, fibrous fraction, mineral-vitamin content. It also includes the digestibility of proteins and amino acids, fermentability in ruminants and monogastric, storage stability and mycotoxins content. This information is also reported in tables.

INRAE-CIRAD-AFZ (Feed Tables)

In the French feed database, there is a "Search box" in each section of the website (Home, Tables, Charts, Nomenclature, Construction). By typing a specific quest within this box (e.g. the name of a feed ingredient), the research is performed within the entire website and not only within the feed tables. Therefore, many of the results obtained are not consistent with the search performed and need to be filtered. In Feed Profiles, tables with the nutritional values of the ingredients are reported. In this section, it is not possible to type and search for a specific feed ingredient, but a complete list of all the feed ingredients is provided, where the feed materials are listed in alphabetical order. By default, maize is shown as the first ingredient. In this section, the search for the keywords Maize, Corn, and Zea Mays returns 17, 2, and 0 items, respectively.

Dutch Animal Feed Chain Federation (CVB SYSTEM)

The CVB system consists of a .pdf file, so navigation is limited. However, by typing a query into the search box, which covers the entire document and not only within the section dedicated to the feed ingredients. With previous knowledge of the category to which the feed ingredients belong, searches can be made in the appropriate categories. The CVB system has five main categories: Compound feedstuffs, High moisture industrial co-products, Roughages and related products, Mineral feedstuffs, and Miscellaneous. In the example of corn, within the category Compound feedstuffs, the search returns 0 items for Corn and Zea Mays and 18 items for Maize. For High moisture industrial co-products, the search returns 3 items for Corn and Maize and 0 items for Zea Mays. For Roughages and related products, the search returns 1 item for Corn, 4 items for Maize and 0 items for Zea Mays. By narrowing down the search to corn grain, only 1 item is left: Maize 1002.000/0/0.

Natural Resources Institute of Finland - Finnish feed tables (Luonnonvaratieto)

On the Natural Resources Institute of Finland website, there are feed tables, and it is possible to select the animal species (ruminants, pigs, poultry, horses, fur animals). There are also tables for Minerals and Amino acids. If users do not know the category to which the feed material of interest belongs, they can select All feeds in the first box. Selecting Ruminants and typing in the Search box of the first box Feed (mandatory) the keywords Corn, Maize, and Zea Mays, the output is 0, 5, and 0, respectively. While in the second box Feed data (mandatory) there are 19 nutrient items (chemical composition, feed values, digestibility coefficients). After this selection, the output is a table with all the data that was chosen. In the case of ruminants and horses, the 5 items with the keyword Maize are maize grain, maize gluten, maize gluten feed, maize stand, and maize silage.

Nordic Feed Evaluation System (NorFor)

The feed table of the Nordic Feed Evaluation System (NorFor) is an interactive database, where it is possible to actively search for specific terms, e.g. feed ingredients. If users do not know the category to which the feed material of interest belongs, they can type the name of the feed ingredient in the "Feed name" tab, without selecting any other parameter. This means that the search will be performed within the entire database. By searching for the keywords Corn, Maize, and Zea Mays in the "Feed name" tab, a total of 0, 38, and 0 results are obtained, respectively.

The Swiss feed database (FeeDBase)

The Swiss feed database "FeeDBase" is a very interactive database but specific feed ingredients cannot be found directly as there is no search engine. Therefore, to find feed material within the feed database, a good knowledge of how the database works is required. To find a feed material within the feed database, users need to know to which feed category the material belongs. To search for a feed ingredient, users can select Reference Data and choose between Raw materials or Roughage in the section Feed Type. Additionally, in the section Nutrients, users can choose from different nutrients and different animal categories (ruminants, fattening calves, pigs, poultry, and horses). When selecting Raw Materials, a window with different feed categories appears, and at the bottom of this window, a search box is provided, where the name of the feed ingredient can be inserted. Searching for the keywords Corn, Maize, and Zea Mays, the output is 7, 10, and 1, respectively. Selecting Roughage and searching in the box at the bottom of the box for the keywords, the output is 19, 28, and 20, respectively.

International aquaculture feed formulation database (IAFFD)

The IAFFD database is divided into three different modules. Here, for the sake of this document, the focus is on the Feed Ingredient Composition Database (FICD), dedicated to feed ingredients, which provides tables of the chemical composition of feed ingredients. This database is focused on aquaculture, but some data also refer to pig and poultry production. In the FICD, there is a search box named Description. The search by the keyword Corn returns 38 items, while no items are reported searching by Maize and Zea Mays as keywords.

Feedipedia

Feedipedia's search engine can be used from a section on the website, including Feed categories, Scientific names, Tool, and Resources. Additionally, in the "All Feeds" section under Feed Categories, there is a list of 778 feeds. Here, searches can be performed by category, datasheet name, common name, scientific name synonym, and status. All feed datasheets consistently present information about world production, the use of the feed ingredient in different livestock animals, and the possible environmental impact. For example, setting “any” as a category and searching for Corn or Maize yields 20 and 12 items, respectively; searching for Zea mays yields 14 items. It is also possible to select a specific feed category, which requires previous knowledge of the category to which the feed ingredient belongs. Searching the keywords Corn, Maize, and Zea mays in the category Cereal and grass forages returns 9, 5, and 5 items respectively; while in the category Cereal Grains and by-products, the same search returns 12 items for Corn, 10 for Maize, and 12 for Zea mays. In the Forage plants category, the search returns 8, 3, and 3 items for Corn, Maize, and Zea mays, respectively; while in the Legume forages category, it results in only 1 item for all the keywords. In the Oil plants and by-products categories, the search returns only 1 item for all the keywords. In the “Other plants by-products” category, it returns no items for Corn and 1 item for Maize and Zea mays. Similarly, searching the keywords in the “Other products” category yields 1 item for all the keywords. Finally, searching in the Plant product and by-products category returns 11 items for Corn, 8 for Maize, and 10 for Zea mays.

The European Catalogue of Feed Materials

The European Catalogue of Feed Materials is a .pdf file with limited navigation. However, entering a query in the search box scans the entire document. Having prior knowledge of the category to which the feed ingredient belongs, one can search in the appropriate category. Ingredients are classified into 13 main categories. When searching the keywords Corn, Maize, and Zea Mays focusing exclusively on the results in the "Name" column of the table, the outcomes are 1, 18, and 0 results, respectively. Specifically, for Corn, only one item is found under the category “1. Cereal grains and products derived thereof.” For Maize, 17 results are returned under the same category, and 1 under the category “6. Forages and roughage, and products derived thereof.”

The Harmonised OECD Tables of feedstuffs from field crops

The Harmonised OECD tables of feedstuffs from field crops is a .pdf file but the search engine works the entire document. With prior knowledge of the category to which the feed ingredient belongs, in the tables, ingredients are sorted into four main categories: Forages/Fodders, Roots & Tubers, Cereal Grains/Crop Seeds, and By-products. In the example of corn, within the Forages/Fodders category, the search returns 5 items for Corn, and 0 for Maize and Zea Mays; no items are found in the Roots & Tubers category for any terms; 2 items are found for Corn and none for Maize and Zea Mays in the Cereal Grains/Crop Seeds category; and 6 items for Corn and none for Maize and Zea Mays in the by-products category.

The International Feed Vocabulary (FAO/UNDP)

The International Feed Vocabulary serves as a basic guide to overcoming inconsistencies in naming feeds but is not accessible as a system; though the structure is described. One significant limitation highlighted by The International Feed Vocabulary is the use of short names for feed materials, which are commonly utilised in Feed Composition Tables for specific countries or regions when it is impractical to use the longer, more precise International Feed Description. However, this short nomenclature is not recommended for describing a feed in a database. To address naming issues in some countries, feeds have been given official names. Generally, these names are not utilised as international feed descriptions because they are either incomplete or do not start with the origin or parent material.

### 3.1.3 Collection of information on inclusion rates and feed consumption

While studying the databases and other considered classification systems, it was assessed whether information on inclusion rates of feed materials in rations, diets, and formulas for compound feed fed to food-producing and non-food-producing animals was available. For each source, the presence or absence of inclusion rates of feed ingredients in the diets for all animal species was assessed, and where available, information on the inclusion limits was noted. It was found out that information on this topic was scarce and that most of the databases and classification systems do not provide information on mixtures or examples of compound feed, with the exception of NorFor and IAFFD. No information on feed consumption was found.

Due to the lack of information on feed consumption and the methodologies currently used to measure it, it was decided to extend the search outside Europe. A relevant report by the Institute for Feed Education and Research (IFEEDER) in collaboration with Decision Innovation Solutions (DIS) was included, which addresses the Animal Food Consumption (https://ifeeder.org/feeddata/). This publication, which was drafted in the USA, discusses feed consumption across different food-producing and non-food-producing animals1, aiming to estimate the amount of feed consumed by different animal species throughout their lifetimes, in selected states and regions. These estimations are based on the total quantities of feed ingredients that typically pass through feed mills. This entails determining and assigning a general diet to each livestock species and using the quantity of animal-source food produced to estimate total feed consumption.

The Agricultural Market Information System (AMIS), an inter-Agency platform, describes three methods for estimating feed use in the context of cereal balance sheets: the survey approach, the balance sheet approach, and the demand approach for estimating animal feed requirements.

Even though these examples from outside Europe can provide predictive information on feed consumption data and market supply and demand, they are derived from production estimations and not directly from actual animal consumption.

Precise and comprehensive information on inclusion rates and consumption of feed materials in rations and diets of food-producing and non-food-producing animals1 is very limited. Given the importance of such information, especially in the context of risk assessment related to animal dietary exposure, addressing this aspect is crucial.

Although an estimation approach like that described by IFEEDER could be used for general predictions, it might be more insightful and beneficial to gather information through direct contact with industries involved in the feed supply chain to obtain actual consumption data. However, considering the extensive nature of the feed industry and the broad range of feed ingredients and animal species involved, this approach would be challenging and time-consuming. Therefore, if this approach is to be implemented, it would require a dedicated project.

## 3.2 Developing a proposal for future update of the current EFSA food and feed classification system (FoodEx2)

### 3.2.1 FoodEx2 Feed hierarchy

The Feed hierarchy consists of 14 main feed categories/groups, listed below:

1. Cereal grains and products derived thereof (feed) [A0BBA],

2. Oil seeds, oil fruits, and products derived thereof (feed) [A0BEB],

3. Legume seeds and products derived thereof (feed) [A0BG2],

4. Tubers, roots and products derived thereof (feed) [A0BH7],

5. Other seeds and fruits, and products derived thereof (feed) [A0BJL],

6. Forages and roughage, and products derived thereof (feed) [A0BKT],

7. Other plants, algae and products derived thereof (feed) [A0BLK],

8. Milk products and products derived thereof (feed) [A0BM9],

9. Land animal products and products derived thereof (feed) [A0BMY],

10. Fish, other aquatic animals and products derived thereof (feed) [A0BNJ],

11. Minerals and products derived thereof (feed) [A0BP4],

12. Fermentation (by-)products from microorganism the cells of which have been inactivated or killed (feed) [A0BRA],

13. Miscellaneous (feed) [A0BRR],

14. Compound feed (feed) [A0BT0]

### 3.2.2 FoodEx2 feed categories comparison with selected feed databases

Table 1 compares the feed categories proposed in the selected databases/sources and those in FoodEx2. It highlights the discrepancies and similarities, starting with the categories.

The FoodEx2 Feed hierarchy was developed based on a comprehensive list of feed materials and related processing factors established in Regulation (EU) No 68/2013 of 16 January 2013 (EFSA, 2019). It thus completely aligns with the first 13 categories of feed from FoodEx2 and those from the European Catalogue of Feed Materials, except for the 14th category (i.e., Compound feed), which is absent in the European Catalogue of Feed Materials.

When comparing FoodEx2 with FEDNA, using Maize/Corn as an example, it is evident that FoodEx2 provides more detailed information, corresponding to a greater number of entries/items. For instance, FEDNA lists an item as "Corn heat treated"; however, according to its website description which states that "Thermal processing includes procedures such as cooking-lamination, micronization, expansion, extrusion, etc. […]", it is challenging to align this term with the corresponding terms in FoodEx2, where multiple entries refer to corn treated with heat, such as Processed maize-based flakes [A0EFQ] or Maize puffed [A0BC5]. In some instances, FEDNA offers more details than FoodEx2.

FEDNA further categorises items based on the percentage of crude protein (CP), resulting in different items such as "Corn gluten feed 19% CP" and "Corn gluten feed 20.5% CP". Similarly, Distillers' grain and solubles. In FoodEx2, they are represented by one item [A0BE3], while FEDNA subdivides them according to the percentage of ether extract (EE) and starch, resulting in three different items: "Corn DGGs 7.5% EE-6.8% starch", "Corn DGGs 12.5% EE- 3.5% starch", and "Corn DGGs 12.5% EE-6.8% starch".

A similar situation is observed with soy products. In FEDNA, there are four different items for soybean meal, differentiated by CP content: "Soybean meal 44%", "Soybean meal 45.5%", "Soybean meal 47%", and "Soybean meal 48.5%". Conversely, in FoodEx2, there is only one item listed as "Soya (bean) meal feed [A0EHT]".

In comparing Feedipedia.org with the FoodEx2 system, the pattern mirrors the comparison with FEDNA. FoodEx2 typically offers more detailed entries than Feedipedia.org. However, there are some items featured by Feedipedia.org that are absent or unmatchable in the FoodEx2 system, such as Ear Maize, Maize green forage, and Maize stover. These items should be integrated into FoodEx2 for completeness.

Table 1: Comparison between FoodEx2 main categories and European Catalogue of feed materials, FEDNA and Feedipedia.org main categories.

| **European Catalogue of Feed Materials** | **FEDNA** | **Feedipedia.org** | **FoodEx2** |
|---|---|---|---|
| Cereal grains and products derived thereof<br>Oil seeds, oil fruits, and products derived thereof<br>Legume seeds and products derived thereof<br>Tubers, roots, and products derived thereof<br>Other seeds and fruits, and products derived thereof<br>Forage and roughage, and products derived thereof<br>Other plants, algae and products derived thereof<br>Milk products and products derived thereof<br>Land animal products and products derived thereof<br>Fish, other aquatic animals and products derived thereof<br>Minerals and products derived thereof<br>Products and by-products obtained by fermentation using micro-organism, inactivated resulting in absence of live micro-organisms<br>Miscellaneous. | 1. Granos de cereales (cereal grains)<br>2. Cereales procesados por calor (heat treated cereals)<br>3. Subproductos de cereals (cereal by-products)<br>4. Frutos y tubérculos. Melazas y vinazas (Fruits & tubers. Molasses & vinasses)<br>5. Concentrados proteína vegetal (vegetal protein concentrates)<br>6. Alimentos fibrosos (fibrous feeds)<br>7. Concentrados de proteína animal (animal protein concentrates)<br>8. Productos lácteos (dairy procucts)<br>9. Grasas y aceites (fats and oils)<br>10. Minerales (minerals)<br>11. Microingredientes (microingredients) | 1.Forage plants<br>2.Plant products/ by-products<br>3. Feed of animal origin<br>4. Other feeds | Cereal grains and products derived thereof (feed)<br>Oil seeds, oil fruits, and products derived thereof (feed)<br>Legume seeds and products derived thereof (feed)<br>Tubers, roots and products derived thereof (feed)<br>Other seeds and fruits, and products derived thereof (feed)<br>Forages and roughage, and products derived thereof (feed)<br>Other plants, algae and products derived thereof (feed)<br>Milk products and products derived thereof (feed)<br>Land animal products and products derived thereof (feed)<br>Fish, other aquatic animals and products derived thereof (feed)<br>Minerals and products derived thereof (feed)<br>Fermentation (by-)products from microorganism the cells of which have been inactivated or killed (feed)<br>Miscellaneous (feed)<br>Compound feed (feed) |

### 3.2.3 FoodEx2 facets analysis

Considering the 28 facets available in the FoodEx2 catalogue, F01 source, F02 part-nature, F04 ingredient, F07 fat-content, F09 fortification-agent, F10 qualitative info, F19 packaging material, F21 production method, F22 preparation-production-place, F23 target consumer, F26 generic term, F27 source commodities, F28 process, F29 purpose of raising, F30 reproductive-level, F31 animal-age-class, F32 gender, and F33 legislative-class were deemed suitable for feed description. Additionally, six facets were potentially suitable for feed description: F03 physical state, F06 surrounding-media, F08 sweetening-agent, F18 packaging-format, F20 part-consumed-analysis, and F25 risky-ingredient. Four facets were not suitable for feed description: F11 alcohol-content, F12 dough-mass, F17 extent-of-cooking, and F24 intended-use.

Among these facets, some are particularly important for amendments in the feed area, having been mentioned in other documents (EFSA 2021 and 2022), such as in the case of F23. Other relevant facets that require amendments for feed include F25, F29, and F30. Notably, F06 refers to fortification, which has a completely different nutritional implication in the case of animals.

### 3.2.4 Proposal for amendments to FoodEx2 catalogue

The first amendment to be implemented is to change the name of the category "Fermentation (by-) products from microorganisms, the cells of which have been inactivated or killed (feed)" (i.e., the 12th category) to align with the European Catalogue of Feed Materials as shown in Table 2.

Table 2: Update of the name of the 12th category of the feed Hierarchy

| termCode | termExtendedName | termScopeNote |
|---|---|---|
| A0BRA | Products and by-products obtained by fermentation using micro-organism, inactivated resulting in absence of live micro-organisms (feed) | Feed ingredient group consisting of Fermentation (by-) products from microorganisms the cells of which have been inactivated or killed |

The values of the termExtendedName and termScopeNote need updating.

A significant change is required to address the limitations on the use of the Compound feed category (i.e., the 14th category) of the Feed Hierarchy, which is absent in the European Catalogue of Feed Materials. Currently, when a coder wants to add ingredients to compound feed, only food-related items appear for selection/use. For this reason, we recommend adding a new "Feed Ingredient Facet" to allow the use of feed materials as part of Compound feed.

Further integrations of the FoodEx2 catalogue, stemming from the case studies considered, have been made on corn/maize and soy items. Additionally, the catalogue could be enriched with new information such as links to external databases and images. The following table highlights the inserted and updated items (Table 3).

The following information could be added/updated in the FoodEx2 catalogue:

- FEDNA id of the corresponding item in the FEDNA database, if there is a match,
- Feedipedia.org link to the corresponding item in the Feedipedia.org database, if there is a match,
- termExtendedName to align the item name with the one used in the European Catalogue of Feed Materials,
- termScopeNote to align the item description with the one used in the European Catalogue of Feed Materials,

commonNames to store all the alternative names for a specific item.

Table 3: Example of integrations and updates regarding the corn/maize and the soy items

| termCode | termExtended Name | termScopeNote | Common Names | EUFeed Reg | FEDNA | Feedipedia. org |
|---|---|---|---|---|---|---|
| **A07XG** | Maize (feed) | Grains of Zea mays L. ssp. mays | Corn grain$Maize grain$Corn$ Maíz [Spanish]$M ais [Italian, German]$Ma ïs [French] | 1.2.1 | https://www.f undacionfedn a.org/node/37 0 | https://www.f eedipedia.org/ node/556 |
| **A0EFQ** | Maize flakes (feed) | Product obtained by steaming or infrared micronising and rolling dehusked maize. It may contain a small proportion of maize husks | | 1.2.2 | | |
| **A0BC0** | Maize protein [Maize Gluten] (feed) | Product from the manufacture of maize starch. It consists principally of protein (prolamins) obtained during separation of starch | Corn gluten meal | 1.2.8 | http://www.fu ndacionfedna. org/ingredient es_para_piens os/gluten-de- maíz-60 | https://www.f eedipedia.org/ node/715 |
| **A0BC1** | Maize protein feed [Maize | Product obtained during the manufacture of maize | | | | |

| | | | |
|---|---|---|---|
| | Maize Gluten feed] (feed) | starch. It is composed of bran and maize solubles. The product may also include broken maize and co-products from oil extraction of maize germs. Other products derived from starch and from the refining or fermentation of starch products may be added. May contain up to 2 % sodium and 2 % chloride | |
| **A0BC7** | Sweet maize silage (feed) | Co-product of the sweet corn processing industry, composed of centre cobs, husks, base of the kernels, chopped and drained or pressed. Generated by chopping sweet corn cobs, husks and leaves, with presence of sweet corn kernels | 1.2.16 |
| **A0BFR** | Co-product from soybean preparation (feed) | Products obtained when processing soybeans to obtain soybean food preparations | 2.18.10 |

### 3.3 Developing a proposal for a feed consumption database, including data structures and recommendations for data collection

#### 3.3.1 Test diets for modelling a feed consumption database suitable for FoodEx2 development

<u>Pigs</u>

Using the "Global Livestock Environmental Assessment Model – Interactive GLEAM-i," it was possible to gather information about "typical" industrial pig diets across the first seven EU countries in pig production. For the potential test diets for the simulations proposed in this report, four major feed materials were identified from the list utilised in the seven leading pig-producing countries: grains from maize, barley, wheat, and soy by-products. Subsequently, data from GLEAM were combined with diets detailed in selected papers (Pastorelli et al. 2022 and Millet et al. 2012), which considered various pig rearing phases. This integration was essential as GLEAM diets are average industrial diets without specific references to the target age of animals. Consequently, two reference diets for pigs—both weaned and growing—have been developed and are shown in Table 4 and Table 5.

Table 4: Feed materials (%) of weaning diet (DM). Pigs of 9-20 kg of body weight.

| Feed materials | Feed materials code and terminology referred to European Catalogue of Feed Materials (Reg. EU 2022/1104) | 9-20 kg (%) |
|---|---|---|
| **Corn grain** | 1.2.1 Maize | 29 |
| **Barley grain** | 1.1.1. Barley | 25 |
| **Whey powder** | 8.17.1 Whey/whey powder | 12.5 |
| **Hulled barley** | 1.1.6 Barley hulls | 12.5 |
| **Soybean meal 48%** | 2.18.4 Soya (bean) meal, dehulled | 11.5 |
| **Wheat bran** | 1.11.7 Wheat bran | 6.0 |
| **Coconut oil** | 2.20.1 Vegetable oil and fat | 1.0 |
| **Dextrose monohydrate** | 13.2.2. Dextrose | 1 |
| **Vitamin-Mineral premix+AA** | N.A. | 2.35 |
| **Feed consumption (kg/die)** | | 1.1 |

Table 5: Feed materials (%) of growing diets (DM). Pigs of 20-110 kg of body weight.

| Feed materials | Feed materials code and terminology referred to European Catalogue of Feed Materials (Reg. EU 2022/1104) | 20-40 kg (%) | 40-70 kg (%) | 70-110 kg (%) |
|---|---|---|---|---|
| **Corn grain** | 1.2.1 Maize | 25.00 | 25.00 | 20.4 |
| **Barley grain** | 1.1.1. Barley | 22.11 | 25.00 | 25.00 |
| **Wheat grain** | 1.11.1 Wheat | 20.00 | 21.37 | 25.00 |
| **Soybean meal (48% CP)** | 2.18.4 Soya (bean) meal, dehulled | 19.01 | 12.51 | 11.58 |
| **Molasses** | 4.1.4. (Sugar) beet molasses | 4.00 | 5.00 | 6.00 |
| **Alfalfa meal** | 6.10.5 Lucerne meal (alfalfa meal) | 3.90 | 3.56 | 3.41 |
| **Full fat soybeans** | 2.18.6 Soya beans, extruded | 1.35 | 4.34 | 5.96 |
| **Soybean oil** | 2.18.10 Co-product from soybean preparation | 1.41 | 0.17 | - |
| **Vitamin and mineral premix+AA** | N.A. | 3.22 | 3.07 | 2.64 |

| | | | |
|---|---|---|---|
| **Feed consumption (kg/die)** | 1.30 | 1.98 | 2.40 |

Dairy cows

Using the "Global Livestock Environmental Assessment Model – Interactive GLEAM-i"," information was obtained about "typical" industrial diets for lactating dairy cows in the top seven EU dairy production countries. To establish potential reference diets for the simulations, six primary feed materials commonly used in cows' diets were identified from the list available in these countries: fresh grass, hay or silage from cultivated grass, silage from the whole maize plant, grains, a fresh mixture of grass and legumes, and maize.

Given that GLEAM proposes these diets as average industrial standards without specific age references and uses some generic terms, these data were supplemented with information from a study (Phipps et al., 2003) that considered lactating cows. In this study, all cows (BW 641kg; 61 days in milk (DIM); Milk Yield 46.7 kg/day) received a total mixed ration with a forage to concentrate ratio of 55:45 DM. The ingredients of these total mixed diets are detailed in Table 6.

Table 6: Feed materials (%) of lactating cows' diet (DM).

| Feed materials | Feed materials code and terminology referred to European Catalogue of Feed Materials (Reg. EU 2022/1104) | (%) |
|---|---|---|
| **Corn silage** | 6.11.1 Maize silage | 41.2 |
| **Grass silage** | 6.6.4 Green silage | 13.8 |
| **Ground corn grain** | 1.2.17 crushed degerminated (degermed) maize | 18.5 |
| **Soybean meal (48% CP)** | 2.18.4 Soya (bean) meal, dehulled | 13.0 |
| **Rapeseed meal** | 2.14.3 Rape seed meal | 12.5 |
| **Minerals** | N.A. | 1.0 |
| **Feed consumption (kg/die)** | | 24 |

The diet specified does not include fresh grass due to its generic definition and variability in DM content and types of grasses (e.g., gramineous vs. leguminous) across different countries. Thus, a silage-based diet was chosen for its consistency.

Laying hens

Similarly, using the "Global Livestock Environmental Assessment Model – Interactive GLEAM-i" we obtained information about diets for laying hens in the top seven EU producers. From the list of potential feed materials in these countries, four major feed materials typically used in laying hens' diets were identified: maize grain, wheat grain, soybeans, and rapeseed.

This baseline information proposed by GLEAM reflects average industrial diets without reference to specific life stages of animals. To develop potential reference diets for our simulations, a diet incorporating specific ingredients such as tallow and oyster shell was created, drawing on selected scientific literature. The approach was to also include feed materials like tallow and oyster shell, to cover specific ingredients. Table 7 reports a diet for laying hens (Saky et al., 2019), considering a body weight of 1.5 kg and a daily feed consumption of 0.080 kg/bird/per day (NRC 1994).

Table 7: Feed materials (%) of laying hens' diet (DM).

| **Feed materials** | **Feed materials code and terminology referred to European Catalogue of Feed Materials (Reg. EU 2022/1104)** | **(%)** |
|---|---|---|
| **Corn** | 1.2.1 Maize | 60.75 |
| **Soybean meal (44%)** | 2.18.3 Soya (bean) meal | 21.38 |
| **Corn gluten meal** | 1.2.9 Maize protein feed (Maize gluten feed) | 2 |
| **Oats** | 1.4.1 Oats | 1 |
| **Corn oil** | 1.2.13 Crude maize germ oil | 0.63 |
| **Tallow** | 9.13.1 Greaves | 3 |
| **Oyster Shell** | 11.1.2 calcareous marine shells | 1.5 |
| **Vitamin and mineral premix** | N.A. | 9.74 |
| **Feed consumption (kg/die)** | | 0.080 |

<u>Atlantic salmons</u>

Fish nutrition has high protein requirements, specific requirements for n-3 fatty acids, and is low in carbohydrates and dietary fibre. The formulation of fish feeds is thus based on a relatively small number of raw materials. Therefore, we have provided information for feed materials that are currently used in aquaculture. To propose potential reference diets for our simulations, a diet for Atlantic salmon was based on a scientific study (Weththasinghe et al. 2021). Table 8 reports a diet for Atlantic salmon with a body weight of 2 kg and a feed consumption of 0.040 kg per day.

Table 8: Feed materials (%) of Atlantic salmon diet (DM).

| **Feed materials** | **Feed materials code and terminology referred to European Catalogue of Feed Materials (Reg. EU 2022/1104)** | **(%)** |
|---|---|---|

| | | |
|---|---|---|
| **Soy protein concentrate** | 2.18.7 Soya (bean) protein concentrate | 34.5 |
| **Fishmeal** | 10.4.2 fish meal | 22.5 |
| **Fish oil** | 10.4.6 Fish oil | 16 |
| **Wheat meal** | 1.11.1 Wheat | 14 |
| **Wheat bran** | 1.11.7 Wheat bran | 5.5 |
| **Corn gluten** | 1.2.8 Maize protein (Maize gluten) | 5.5 |
| **Vitamin and mineral premix** | N.A. | 2 |
| **Feed consumption (kg/die)** | | 0.040 |

Adult dogs

A balanced diet is critical to a dog's maintenance, growth, and health. The amount of feed for adult dogs should be based on their size and energy output. Activity levels can vary dramatically among dogs and play an important role in determining caloric intake. Their tooth structure and intestinal tract have become adapted to an omnivorous diet, so under normal circumstances, dogs can meet their nutritional needs by consuming a combination of plant and animal foods. Table 9 presents a premium diet for an adult dog (Biagi et al. 2021). For this report, we used an adult dog with a body weight ranging from 10 kg to 25 kg and a feed consumption ranging from 0.2 kg per day to 0.4 kg per day, as outlined in the feeding guide available at https://alacarte4k9.com.au/feeding-guide/.

Table 9: Feed materials (%) of adult dog (10 kg) diet (DM).

| Feed materials | Feed materials code and terminology referred to European Catalogue of Feed Materials (Reg. EU 2022/1104) | (%) |
|---|---|---|
| **Dried Chicken** | 9.4.1 Processed animal protein | 27 |
| **Rice** | 1.6.16 Rice | 26 |
| **Potato** | 4.8.1 Potatoes | 24 |
| **Fresh chicken meat** | 9.14.1 Products of animal origin | 14 |
| **Chicken fat** | 9.2.1 Animal Fat | 4.3 |
| **Animal protein (from heart and liver)** | 9.1.1 Animal by-products | 1.5 |
| **Flaxseed/linseeds** | 2.8.1 Linseed | 1.4 |
| **Brewer's yeast** | 12.1.5 Yeast, inactivated (brewer's yeast, inactivated, if appropriate) | 0.5 |
| **Fish oil** | 10.4.6 Fish oil | 0.4 |

| | | |
|---|---|---|
| **Chicory pulp dried** | 4.4.5 Dried chicory pulp | 0.2 |
| **Sunflower oil** | 2.20.1 Vegetable oil and fat | 0.1 |
| **Mix of aromatic herbs and spices** | 7.7.1 Leaves, dried | 0.5 |
| **Dried Yucca Schidigera** | 7.12.1 Mojave yucca | 0.1 |
| **Feed consumption (kg/die)** | | 0.2 |

### 3.3.2 Development of a proposal for a feed consumption database

This section details the structure of the proposed feed consumption database, and some examples of database population are provided. As mentioned in Section 2.3.2, the database has three sheets: Animal, Feed, and Consumption.

<u>Animal sheet</u>

In the animal sheet, each row describes a specific animal/livestock category. The following properties are in columns:

- ORANIMALCODE (mandatory): unique animal identifier.
- ANIMALDESC (mandatory): description/name of the animal.
- GENDER (mandatory): gender of the animal.
- WEIGHT (mandatory): weight of the animal, expressed in kg, at the beginning of the given diet.
- MWEIGHT (mandatory): method used to measure the weight of the animal.
- TOTALDAILYINTAKE (mandatory): total daily feed consumption, expressed in kg of DM.
- SPECIALCON (mandatory): condition of the animal.
- SPECDIET (mandatory): diet of the animal.
- COUNTRY: country in which the animal was raised.
- AREA: geographical area in which the animal was raised (northern/southern Europe).
- COMMENTSANIMAL: text field for additional information about the animals or the related diet.

An example of the Animal sheet is given in Table 10, where some rows referring to pigs are shown.

Table 10: Example of Animal sheet referred to pigs.

| ORANIMAL CODE | ANIMAL DESC | GENDER | WEIGHT | MWEIGHT | TOTAL DAILY INTAKE | SPECIAL CON | SPEC DIET | COUNTRY | AREA | COMMENTS ANIMAL |
|---|---|---|---|---|---|---|---|---|---|---|
| **Piglet9** | Piglet | G3 | 9 | X1 | 0.50 | C1 | D1 | IT | Southern Europe | Pastorelli et al., 2022 |
| **Fattening pig20** | Fattening pig | G3 | 20 | X1 | 1.30 | C1 | D1 | BE | Northern Europe | Millet et al., 2012 |
| **Piglet10** | Piglet | G3 | 10 | X3 | 0.50 | C5 | D5 | | | EFSA 2019 (50 g DM / kg BW) |

This example shows the values associated with piglets of 9 kg, and the general information regarding their diet. The weight of 9 kg is intended as the weight that the piglets have at the beginning of the proposed diet; as soon as they reach a weight of 20 kg, the next diet is provided to them, that is the one for the fattening pigs of 20 kg, corresponding to the second row in the example. The ORANIMALCODE (“Piglet9”) is generated by linking the ANIMALDESC value (“Piglet”) and the WEIGHT value (“9”). The GENDER value is set as unclassified (“G3”), as the proposed diet can be given to both male and female piglets. The weight of the piglets is measured (the MWEIGHT value is set to “X1”).

In this example, the diet given to piglets of 9 kg is 0.5 kg of DM per day, and this value is stored in the TOTALDAILYINTAKE field. This diet is defined as a normal diet (the SPECDIET value is set to “D1”), and it is meant for piglets in normal conditions (the SPECIALCON value is set to “C1”). The COUNTRY and the AREA values specify that the breeding takes place in Italy (“IT”) and “southern Europe”. Finally, the COMMENTSANIMAL field specifies the reference to this diet for the piglets of 9 kg, namely “Pastorelli et al., 2022”.

In the second row, the general information regarding the diet given to the fattening pigs which start from a weight of 20 kg is provided. In particular, the ORANIMALCODE (“Fattening pig20”) is generated by linking the ANIMALDESC value (“Fattening pig”) and the WEIGHT value (“20”). The GENDER value is set as unclassified (“G3”), as the proposed diet can be given to both male and female fattening pigs. The weight of the fattening pig is measured (the MWEIGHT value is set to “X1”). The diet considered for fattening pigs of 20 kg is 1.30 kg of DM per day, and this value is stored in the TOTALDAILYINTAKE field. Again, the diet is defined as a normal diet (the SPECDIET value is set to “D1”), and is meant for fattening pigs in normal conditions (the SPECIALCON value is set to “C1”). The COUNTRY and the AREA values specify that the breeding takes place in Belgium (“BE”) and “northern Europe”. The diet is taken from the reference “Millet et al. 2012”, as specified in the COMMENTSANIMAL field value.

In the third row, the ORANIMALCODE (“Piglet10”) is generated by linking the ANIMALDESC value (“Piglet”) and the WEIGHT value (“10”). In this case, the diet given to Piglet10 corresponds to an amount of 0.5 kg of DM per day, and this value is stored in the TOTALDAILYINTAKE field. The COMMENTSANIMAL field specifies the reference (EFSA, 2019) in which, for the piglet category, daily feed intake is calculated applying the formula 50 g DM/kg BW.

Feed sheet

In the feed sheet, each row represents a specific feed for animals, which can be part of a complete diet, or which can be given to the animals as a single feed material alone. The sheet is modelled according to the following properties on columns:

- ORFEEDCODE (mandatory): unique feed identifier
- ORFEEDNAME (mandatory): feed description in the original language.
- ENFEEDNAME (mandatory): feed description in the English language.
- FOODEXCODE (mandatory): EFSA feed identifier
- COMMENTSFEED: text field for additional information about the feed.

An example of the Feed sheet is given in Table 11.

Table 11: Example of Feed sheet.

| ORFEEDCODE | ORFEEDNAME | ENFEEDNAME | FOODEXCODE | COMMENTS FEED |
|---|---|---|---|---|
| **R01.I01** | Farina di mais | Corn meal | A07XG | |

This example shows the description of the feed ingredient “Corn meal”, in English (ENFEEDNAME value), namely “Farina di mais” in Italian (ORFEEDNAME value). The FOODEXCODE field is filled with the value of the EFSA FoodEx2 feed identifier (“A07XG”).

Consumption sheet

In the consumption sheet, each row specifies an animal with the corresponding type and quantity of feed per day. Both the animal and the feed have references to corresponding records in the Animal and Feed sheets, respectively. The sheet is modelled according to the following properties on columns:

- RECORDIDENTIFIER (mandatory): unique record identifier.
- ORANIMALCODE (mandatory): unique animal identifier. The values in this column refer to the records of the Animal sheet.
- FOODEXRCODE (mandatory only if the row refers to a feed material in a complete diet): EFSA feed identifier belonging to the “Complete feed” category.
- AMOUNTRECIPE (mandatory only if the row refers to a feed material in a complete diet): amount of the complete feed eaten by the animal in a day, expressed in kg. This amount must be repeated for each feed material belonging to the complete feed diet.
- ORFEEDCODE (mandatory): unique identifier of a specific feed material. The values of this column refer to the records of the Feed sheet.
- IR (mandatory): inclusion rate of the single feed material in the daily diet (percentage).
- AMOUNT (mandatory): amount of the single feed material eaten by the animal in a day, expressed in kg.
- BRAND: name of the brand of a specific feed.
- FOODEXCODE (mandatory): EFSA feed identifier.

An example of the Consumption sheet referring to pigs is given in Table 12.

Table 12: Example of Consumption sheet referred to pigs.

| RECORD IDENTIFIER | OR ANIMAL CODE | FOODEX RCODE | AMOUNT RECIPE | ORFEED CODE | IR | AMOUNT | BRAND |
|---|---|---|---|---|---|---|---|
| **Piglet9_1** | Piglet9 | A0BT6 | 0.50 | R01.I01 | 29.00 | 0.1450 | |
| **Piglet9_2** | Piglet9 | A0BT6 | 0.50 | R01.I02 | 25.00 | 0.1250 | |

This example shows the daily feed consumption of two feed materials in the diet of piglets weighing 9 kg. The RECORDIDENTIFIER (“Piglet9_1”) is generated by linking the ORANIMALCODE value (“Piglet9”) with a progressive number. The FOODEXRCODE value (“A0BT6”) is the EFSA identifier for “Piglet Starter I (pre-starter) / Complete feed (feed)”. The AMOUNTRECIPE value (“0.50”) reports, in kg, the total amount of the complete feed provided. The ORFEEDCODE value of the first row in the example (“R01.I01”) is a reference to the record of the Feed sheet describing the corn meal, which is a single feed material that is part of the complete feed diet provided to the piglets weighing

9 kg. The IR value ("29,00") indicates the inclusion rate, that is, the percentage, of corn meal eaten in a day by the piglets. The AMOUNT value ("0.1450") indicates, in kg, the amount of this specific feed ingredient (corn meal) eaten in a day by the piglets. The BRAND value of such feed material is not specified since it is not available. Finally, the FOODEXCODE value reports the EFSA FoodEx2 identifier for corn meal ("A07XG").

In the second row of the example, the ORFEEDCODE value ("R01.I02") is a reference to the record of the Feed sheet describing the barley meal, which is another feed material that is part of the complete feed diet provided to the piglets weighing 9 kg. The IR value ("25,00") indicates the inclusion rate of barley meal eaten in a day by the piglets. The AMOUNT value ("0,1250") indicates, in kg, the amount of this specific feed material (barley meal) eaten in a day by the piglets. The BRAND value of such feed material is not specified too since it is not available. Finally, the FOODEXCODE value reports the EFSA FoodEx2 identifier for barley meal ("A05CR"). Thus, to provide the description of a complete feed diet for piglets of 9 kg, the sum of the values in the IR column must be of 100.00 (100%), and the sum of the values in the AMOUNT column must correspond to the value specified in the AMOUNTRECIPE column ("0.50").

The same approach was used for dairy cows, laying hens, Atlantic salmons, and adult dogs. Specific examples of the Consumption sheet referring to these species are reported in Table 13.

Table 13: Example of Consumption sheet referred to dairy cows, laying hens, Atlantic salmons and adult dogs.

| RECORD IDENTIFIER | OR ANIMAL CODE | FOODEX RCODE | AMOUNT RECIPE | ORFEED CODE | IR | AMOUNT | BRAND |
|---|---|---|---|---|---|---|---|
| **Dairy cow641_1** | Dairy cow641 | A0BT5 | 24.00 | R03.I01 | 41.20 | 9.8880 | |
| **Laying hen1,5_1** | Laying hen1,5 | A0BTK | 0.80 | R04.I01 | 60.75 | 0.4860 | |
| **Dog10_1** | Dog10 | A0BTV | 0.20 | R05.I01 | 27.00 | 0.0540 | |
| **Salmon2_1** | Salmon2 | A16AX | 0.04 | R06.I01 | 34.50 | 0.0138 | |

### 3.4 Proof of concept for the development of a feed consumption database

#### 3.4.1 Animal selection

Animal categories selected for the two case studies were chosen to ensure representation of large ruminants, small ruminants, monogastric animals, and poultry. For the GMO case, dairy cows, dairy sheep, finishing pigs, and broilers were chosen for their respective categories. Similarly, for the contaminant case, dairy cows, dairy sheep, fattening pigs, and broilers were selected. All animal categories were chosen as they are most comparable to those present in each scientific opinion. Additionally, a GM case study including Atlantic salmon and dogs, representing fish and companion animal categories, is included in Appendix C.

#### 3.4.2 First case study: GM feed

<u>GM feed Example 1</u>

The scientific opinion for GM feed Example 1 (EFSA GMO Panel 2022a) assesses the risk of GM maize MON 95379, to confer insect protection against certain lepidopteran species through the expression of Cry1Da_7 and Cry1B.868 proteins.

According to the scientific opinion (EFSA GMO Panel 2022a), dietary exposure to Cry1Da_7 and Cry1B.868 proteins was estimated through the consumption of maize grains, gluten feed, gluten meal, and silage across three animal categories: broilers, finishing pigs, and lactating dairy cows. The intake of maize grains, gluten feed, and gluten meal was evaluated for broilers and finishing pigs, as well as maize gluten feed, gluten meal, and silage for lactating dairy cows.

Dietary exposure estimates to Cry1Da_7 and Cry1B.868 proteins relied on default values for animal body weight, daily feed intake, and inclusion rates of maize feedstuffs in diets, as recommended by the OECD (2009). A scenario assuming 100% substitution of traditional maize products with maize MON 95379 products was considered.

Mean protein levels of Cry1Da_7 and Cry1B.868, measured in grains and forages, served as occurrence data to estimate the average levels (dry weight) of these proteins in the maize by-products gluten feed and gluten meal, which were expected to be 2.6 and 7.1 times higher, respectively, than in grains. This adjustment accounted for the relative protein content in these feed materials compared to maize grain (OECD, 2002), assuming no protein degradation during processing.

In developing the proof of concept for the proposed database, animal dietary exposure to Cry1Da_7 and Cry1B.868 proteins, measured in mg/kg BW per day based on the intake of maize grains, gluten feed, gluten meal, and silage, was detailed as outlined in the selected scientific opinion (EFSA GMO Panel 2022a) (see Appendix B.2.1. Table 41).

The EFSA statement from 2023 (EFSA GMO Panel, 2023) regarding “animal dietary exposure in the risk assessment of feed derived from genetically modified plants”, " suggested using an Excel calculator to consistently report predicted dietary exposure estimates for newly expressed proteins. Implementing this recommendation, the animal dietary exposure to Cry1Da_7 and Cry1B.868 was recalculated using the proposed Excel calculator for the designated animal species. This calculator facilitated daily dietary exposure calculations, as reported in Appendix B.2.1, Table 42.

Subsequently, feed intake data for the selected feed materials (grain, gluten feed, gluten meal) were extracted from the elaborated model database, which incorporated additional dietary formulations from the literature relevant to the GM feed and contaminants case studies. An assessment of animal dietary exposure to Cry1Da_7 and Cry1B.868 proteins was conducted by substituting the standard animal and consumption data with the data derived from the expanded model database (Appendix B.2.1. Table 43).

Dairy sheep were included as representatives of the small ruminant category to enhance the comparison between GM feed Examples 1 and 2. During the use of the expanded model database for animal dietary assessment, discrepancies in animal and feed material nomenclature were noted compared to those in the Examples. To address this, three tables (Appendix A.1.2, Tables 37, 38, and 39) were prepared to align animal names with the database and feed material names with the EU Catalogue of Feed Materials.

Finally, daily dietary exposure (DDE) results from the EFSA scientific opinion (EFSA GMO Panel 2022a), findings from the Excel calculator (EFSA GMO Panel 2023), and outcomes from the model database are presented in Appendix B.2.1, Table 44.

The table highlights a noticeable reduction in DDE for finishing pigs consuming maize gluten feed, as detailed with the data from the model database. This variance is attributed to differing inclusion rates of maize gluten feed in the diets; whereas in GM feed Example 1 the inclusion rate was 20%, it was only 5% in the model database diet. Furthermore, there was a notable difference in animal body weight, shifting from 100 kg in GM feed Example 1 (Appendix B.2.1. Table 41) to 121.6 kg (Appendix B.2.1. Table 43) in the model database, although total daily intake amounts were similar at approximately 2.875 kgDM/animal in the model database and 3 kgDM/animal in GM feed Example 1.

The only slight increase in DDE obtained with the input data from the model database, compared to the DDE reported in the scientific opinion (EFSA GMO Panel 2022a), was in dairy cows consuming maize gluten feed. This is due to the combination of a lower animal body weight (623 kg compared to 650 kg) and a slightly higher total daily intake (25.82 kgDM/animal compared to 25 kgDM/animal) in the model database compared to GM), despite a slightly lower maize protein feed inclusion rate (18.81% compared to 20% in GM feed Example 1) in the model database.

GM feed Example 2

The scientific opinion (EFSA GMO Panel 2022b) selected for the GM feed Example 2 regards the risk assessment of the GM maize DP4114 x MON 89034 x MON 87411 x DAS-40278-9 (four-event stack maize). This GM maize was developed to help protect the crop against lepidopterans (through the expression of Cry1F, Cry1A.105 and Cry2Ab2 proteins), coleopterans (Cry34Ab1/Cry35Ab1 and Cry3Bb1 proteins), western corn rootworms (DvSnf7 dsRNA). It also deals with tolerance to glufosinate-ammonium herbicide (PAT proteins), glyphosate herbicide (CP4 EPSPS protein), as well as aryloxyphenoxypropionate (AOPP) herbicide degradation and tolerance to 2,4-D herbicides (through expression of AAD-1 protein).

Dietary exposure to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1A.105, Cry2Ab2, Cry3Bb1, CP4 EPSPS and AAD-1 proteins in the four-event stack maize was estimated by the consumption of maize grains and forage. Nine animal categories were considered: beef cattle, dairy cattle, rams/ewes, lambs, breeding pigs, finishing pigs, broilers, layers, and turkeys.

The consumption of maize grain was reported for all nine species. The consumption of forage and forage inclusion were reported only for beef cattle, dairy cattle, lambs, breeding pigs and layers. The estimation of dietary exposure to newly expressed proteins was based on default values for animal body weight, daily feed intake, and inclusion rates (percentage) of maize grain and forage in diets/rations (OECD, 2013).

A conservative scenario with 100% replacement of conventional maize products by four-event stack maize products was considered. Mean protein levels of Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1A.105, Cry2Ab2, Cry3Bb1, CP4 EPSPS and AAD-1 proteins analytically determined in grain and forage were used as occurrence data. The animal dietary exposure (mg/kg BW per day) based on the consumption of maize grain and forage was reported in EFSA GMO Panel 2022b.

As with the GM feed Example 1, in the GM feed Example 2 we also reported the animal dietary exposure as outlined in EFSA GMO Panel 2022b (Appendix B.2.1. Table 45).

Next, we calculated the animal dietary exposure to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1A.105, Cry2Ab2, Cry3Bb1, CP4 EPSPS and AAD-1 proteins using the Excel calculator proposed in the EFSA (2023) statement (EFSA GMO Panel 2023). The Excel calculator was used for the DDE calculation (Appendix B.2.1. Table 46).

Finally, the animal dietary exposure to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1A.105, Cry2Ab2, Cry3Bb1, CP4 EPSPS and AAD-1 proteins was assessed by replacing the default animal and consumption data with the extracted values from the model database (Appendix B.2.1. Table 47).

Appendix B.2.1. Table 48 shows the DDE results from i) EFSA GMO Panel 2022b, ii) the Excel calculator (EFSA GMO Panel 2023), and iii) the model database.

Table 48 shows a notable DDE decrease in the results obtained with the input data from the model database for dairy cows consuming maize silage. This can be explained by the different inclusion rates of the maize silage in the dairy cow diets. However, in the GM feed Example 2, the maize silage inclusion rate was 60% (Appendix B.2.1. Table 45), and in the model database the inclusion rate in the diets was 21.23% (Appendix B.2.1. Table 47).

The inclusion rate difference is higher than the slight difference in animal body weight, from 650 kg of the GM feed Example 2 to 623 kg and the small total daily intake increase from 25 kgDM/animal of the GM feed Example 2 to 25.82 kgDM/animal from the model database. All the other animals consuming maize also showed a notable decrease in DDEs. In the case of dairy sheep, the inclusion rate of maize (41% compared to 30%) in the model database was higher than in GM feed Example 2 and the animal body weight (57 kg compared to 75 kg) was lower. Despite these changes, the total daily intake (1.22 kgDM/animal compared to 2.5 kgDM/animal) was significantly lower in the model database compared to GM feed Example 2, leading to a decrease in the DDE value.

### 3.4.3 Discussion and comparative considerations of GM feed Examples 1 and 2

To evaluate the animal daily dietary exposure, the DDE was calculated, considering the following input values:

- Total daily intake
- Animal body weight
- Inclusion rate of feed materials

Any variation in these parameters leads to a proportional difference in daily dietary intake, as the daily dietary intake is calculated as follows:

[(Total Daily Intake X Inclusion rate %)/ Body Weight] X 1000.

A difference in the daily dietary intake would directly influence the DDE, as it is calculated as follows:

Daily dietary intake X NEP level.

While the total daily intake, feed material inclusion rate, daily dietary intake and NEP level are directly proportional to the DDE values, animal body weight is inversely proportional to the DDE values.

NEP levels exhibited a direct correlation with the DDE values, however they are obtained analytically or calculated in derived or processed feed by conversion factors. They are specific to the respective genetically modified plant and therefore do not contribute to the differences in the DDE value reported in the GM feed examples and the DDE value obtained using input data from the model database.

Another factor that could lead to a further change in DDE values is the selection of feed materials to estimate exposure. This selection could include a single feed material (e.g. maize), but also its products (e.g. maize gluten feed, gluten meal) and by-products (e.g. milled by-products).

This is crucial because it impacts the total inclusion rate of individual maize feed materials, maize products and by-products in the total DDE estimation. For example, the diets in the model database include maize, maize gluten feed, maize gluten and maize forage. Maize milled by-products are not considered in any of the proposed diets, thus no DDE is derived from them, whereas they are included in the current GMO approach (EFSA GMO Panel 2023). This leads to a further difference in the total DDE. Additionally, with a consumption database that includes different diets, the incorporation of further maize feed materials and maize by-products cannot be precluded, which thus affects the estimation.

Another key factor is the methodology for animal dietary exposure (ADE).

In the EFSA 2023 statement (EFSA GMO Panel, 2023), the GMO Panel reported a notable absence of specific recommendations of methodologies to estimate and present ADE associated with GM feed, thus leading to inconsistent approaches.

As evident from Examples 1 and 2, different approaches have also been adopted in these examples. In GM Feed Example 1, only three animal categories (broilers, finishing pigs, lactating dairy cows) were considered, while GM Feed Example 2 covers a broader range of nine animal categories (beef cattle, dairy cattle, rams/ewes, lambs, breeding pigs, finishing pigs, broilers, layers, turkeys). Furthermore, there is a disparity in the evaluation of maize itself. In the GM Feed Example 1, the animal dietary exposure to maize, gluten feed, gluten meal and silage was assessed, whereas, in GM Feed Example 2, the focus was on the exposure to maize grain and forage.

To further harmonise the application dossiers, the GMO Panel provided advice in their 2023 statement (EFSA GMO Panel, 2023) on the selection of appropriate feed consumption and feed concentration data, and on the reporting of exposure estimates. The panel proposed a list of animal species, recommending limiting the estimations of expected animal dietary exposure only to some animal species and using selected feed materials. These recommendations would facilitate the selection of animal species and feed materials, harmonising the reporting of the information in the application dossiers.

Given that there is no comprehensive feed consumption database, the EFSA GMO panel recommended using default values for feed consumption, including animal body weight, daily feed intake and inclusion rates of feed materials in diets, derived from the most pertinent sources in the literature. The implementation of such a database, as proposed in this project, could help prevent the reliance on default values. In fact our database could facilitate precise EU-wide analyses, thus minimising the introduction of over or underestimations that could result in an inadequate risk characterisation.

The overestimations observed for both Example 1 and Example 2 are due to a very conservative scenario where an animal diet consisted of 90-100% maize feed materials for the GM feed Example 1 and 30-90% maize feed materials in GM feed Example 2, for the selected animal categories. These diets would be nutritionally unbalanced and impractical for real-world applications because they do account for the inclusion rates of other feed materials in the diet.

The importance of the model database and of developing a European Union feed consumption database lies in its potential to address these limitations. It would provide more comprehensive and realistic feed consumption data and contribute to the development of more accurate and harmonised risk assessments.

#### 3.4.4 Second case study: feed contaminant

Ochratoxin A, produced by fungi such as Aspergillus and Penicillium, belongs to the class of mycotoxins, which are natural products generated as secondary metabolites during fungal development. Mycotoxins are commonly found in food, feed, and agricultural environments due to the growth of toxin-producing fungi in soil, hay, decaying vegetation, and grains (Dhakal et al. 2023).

The Commission Recommendation 2006/576/EC, amended by Commission Recommendation 2016/1319/EC, provides guidance values for ochratoxin A (OTA) in feed, expressed as mg/kg based on a dry matter of 88%. For cereals and cereal products, the OTA's guidance value levels are set at 0.25 mg/kg of feed.

The EFSA expert panel estimated the animal dietary exposure to OTA according to data from the EFSA Data Warehouse, accessed on 28 November 2022. The exposure considered intervals of exposure, including the mean lower bound (LB), upper bound (UB), and 95th percentile LB and UB, taking into account compound and/or complementary feed, and the following feed materials: barley grain, maize grain, maize protein, oat grains, oat feed, rice, broken, wheat grains, wheat feed, rape seed, rape seed (expeller + meal), soybean (expeller + meal), soybean hulls, soybean, protein concentrate, sunflower (expeller + meal), carobs, horse beans, pea flour, grass meal, hay and alfalfa, lucerne meal (alfalfa meal), and maize silage.

The scientific opinion (EFSA CONTAM Panel 2023) considered dairy cows, cattle for fattening, veal calves, dairy goats, fattening goat kids, dairy sheep, fattening lambs, horses, weaned piglets, fattening pigs, lactating sows, fattening chickens, laying hens, turkeys, ducks, fattening rabbits, salmon, dogs, dogs fed a vegetarian diet, and cats.

The default values for average feed intakes and body weights used to calculate animal dietary exposure to OTA were based on published guidelines on nutrition and feeding (NRC, 2006; Leeson and Summers, 2008; EFSA FEEDAP Panel, 2017; see Annex C, Table C.1 of the OTA scientific opinion), and have been extensively described in previous scientific opinions (EFSA CONTAM Panel 2011, 2012) and subsequently updated in 2023.

Various scenarios, using model diets or compound feed (complete feed or complementary feed plus forage), were employed for dietary exposure assessment. For the model diet, the scientific opinion (EFSA CONTAM Panel 2023) lists the feed intake of each animal category considering 100% dry matter. The animal dietary exposure to OTA (mg/kg dry matter, μg/kg BW/day and μg/day) was determined, based on a model diet and on compound feed, considering mean LB and UB, median LB and UB, 75th percentile LB and UB and 95th percentile LB and UB as exposure scenarios. In the scientific opinion (EFSA CONTAM Panel 2023), only the 95th percentile mean LB, UB and the high LB, UB exposures were reported. Additionally, for feed ingredients for which data for the 95th percentile contamination was not available, the 75th percentile LB and UB data were considered.

The proof of concept for the proposed feed consumption database for OTA was performed by replacing the animal and consumption data provided in the EFSA scientific opinion (EFSA CONTAM Panel 2023) with the corresponding extracted values from the model database. The Excel calculator originally used to calculate the OTA exposure in the opinion (EFSA CONTAM Panel 2023) was used, inserting feed data from the model diets retrieved from the model database.

Animal exposure to OTA was thus obtained, expressed as μg/kg feed, μg/day feed, 100% dry matter, and μg/kg BW/day.

The following tables show the results obtained by applying the contamination data reported in the EFSA scientific opinion (EFSA CONTAM Panel 2023) and in the Excel calculator fed with the model diets retrieved from the model database. Model diets as retrieved from the model database, and default values for live weight and feed intake of animal categories are reported in Appendix A.

Dairy Cows

Table 14: Dairy cow exposure to OTA (mg/kg, 100% Dry Matter) based on model database diet, as calculated in the Excel calculator, considering 1 kg/feed, compared to exposure to OTA residues (mg/kg, 100% DM; 70% concentrate and 30% forages) as reported in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023).

| | **OTA model database diet (µg/kg feed)** | **EFSA OTA scientific opinion diet (mg/kg Dry Matter; 70% concentrate and 30% forages)** | **Variation** |
|---|---|---|---|
| Mean lower bound | 1.71 | 1.80 | ↓ |
| Mean upper bound | 3.60 | 3.80 | ↓ |
| High lower bound | 3.50 | 3.20 | ↑ |
| High upper bound | 7.83 | 9.20 | ↓ |

Table 15: Dairy cow exposure to OTA (mg/day, 100% Dry Matter) based on model database diet, as calculated in the Excel calculator, taking into account the amount of feed material consumption per day, compared to exposure to OTA (mg/kg, 100% Dry Matter; 70% concentrate and 30% forages) as reported in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023).

| | **OTA model database diet (µg/day)** | **EFSA OTA scientific opinion diet (µg/day)** | **Variation** |
|---|---|---|---|
| Mean lower bound | 44.22 | 35.00 | ↑ |
| Mean upper bound | 92.90 | 77.00 | ↑ |
| High lower bound | 90.46 | 64.00 | ↑ |
| High upper bound | 202.27 | 183.00 | ↑ |

Table 16: Dairy cow exposure to OTA (mg/kg BW/day, 100% dry matter) based on model database weight as calculated in the Excel calculator, compared to exposure to OTA residues (mg/kg BW/day, 100% Dry Matter) as reported in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023).

| | **OTA model database diet (µg/kg BW/day)** | **EFSA OTA scientific opinion diet (µg/kg BW/day)** | **Variation** |
|---|---|---|---|
| Mean lower bound | 0.07 | 0.05 | ↑ |
| Mean upper bound | 0.15 | 0.12 | ↑ |

| | | | |
|---|---|---|---|
| High lower bound | 0.14 | 0.10 | ↑ |
| High upper bound | 0.32 | 0.28 | ↑ |

For the cow diets, some adjustments were made: for the feed material maize gluten feed (20% CP), OTA contamination data from maize protein were considered, and for the feed material maize grain, ground, data from maize grain were considered. No contamination data was available for the feed material mineral and vitamin mix, beet pulp and fat powder.

Dairy Sheep

Table 17: Dairy sheep exposure to OTA (mg/kg 100% Dry Matter, 35% concentrate and 65% forage) based on model database diet, as calculated in the Excel calculator, considering 1 kg/feed compared to exposure to OTA (mg/kg 100% Dry Matter; 35% concentrate and 65% forage) as reported in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023).

| | **OTA model database diet (μg/kg feed, μg/kg feed, 35% concentrate and 65% forage)** | **EFSA OTA scientific opinion diet (mg/kg Dry Matter; 35% concentrate and 65% forage)** | **Variation** |
|---|---|---|---|
| Mean lower bound | 1.94 | 1.40 | ↑ |
| Mean upper bound | 4.59 | 3.80 | ↑ |
| High lower bound | 2.22 | 2.10 | ↑ |
| High upper bound | 11.27 | 10.80 | ↑ |

Table 18: Dairy sheep exposure to OTA (mg/day 100% Dry Matter, 35% concentrate and 65% forage) based on model database diet, as calculated in the Excel calculator, considering the amount of feed material consumption per day.

| | **OTA model database diet (μg/day, 35% concentrate and 65% forage, 88% dry matter)** |
|---|---|
| Mean lower bound | 2.36 |
| Mean upper bound | 5.60 |
| High lower bound | 2.70 |
| High upper bound | 13.74 |

Table 19: Dairy sheep exposure to OTA (mg/kg BW/day, 100% dry matter, 35% concentrate and 65% forage) based on model database weight as calculated in the Excel calculator.

| | OTA model database diet (μg/kg BW/day, 35% concentrate and 65% forage) |
|---|---|
| Mean lower bound | 0.04 |
| Mean upper bound | 0.09 |
| High lower bound | 0.04 |
| High upper bound | 0.24 |

For the dairy sheep diet, the following adjustments were made: for the feed material Maize gluten meal (60% CP) and Maize gluten feed (20% CP), OTA contamination data were retrieved from maize protein. Additionally, no data were reported for the alfalfa hay high (95%) UB and LB contamination level, thus only mean LB and UB contamination data were taken into consideration, using the same approach as the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023). No contamination data was available for the feed materials wheat straw and mineral and vitamin mix.

A comparison of the results in Tables 18 and 19 with the data in the EFSA scientific opinion (EFSA CONTAM Panel 2023) was not possible

Fattening Pigs

Table 20: Fattening pig exposure to OTA (mg/kg 100% Dry Matter) based on model database diet, as calculated in the Excel calculator, taking into account 1 kg/feed, compared to exposure to OTA (mg/kg 100% Dry Matter) as reported in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023).

| | OTA model database diet (μg/kg feed) | EFSA OTA scientific opinion diet (mg/kg feed) | Variation |
|---|---|---|---|
| Mean lower bound | 1.74 | 1.80 | ↓ |
| Mean upper bound | 3.08 | 3.00 | ↑ |
| High lower bound | 5.57 | 4.10 | ↑ |
| High upper bound | 6.21 | 6.10 | ↑ |

Table 21: Fattening pig exposure to OTA (mg/day 100% Dry Matter) based on model database diet, as calculated in the Excel calculator, taking into account the amount of feed material in each animal

diet per day, compared to exposure to OTA (mg/kg BW/day 100% Dry Matter) as reported in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023).

| | OTA model database diet (µg/day) | EFSA OTA scientific opinion diet (µg/day) | Variation |
|---|---|---|---|
| Mean lower bound | 3.80 | 3.90 | ↓ |
| Mean upper bound | 6.73 | 6.70 | = |
| High lower bound | 12.13 | 9.00 | ↑ |
| High upper bound | 13.54 | 13.50 | = |

Table 22: Fattening pig exposure to OTA (mg/kg BW/day, 100% dry matter) based on model database weight as calculated in the Excel calculator, compared to exposure to OTA (mg/kg bw/day 100% Dry Matter) as reported in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023).

| | OTA model database diet (µg/kg BW/day) | EFSA OTA scientific opinion diet (µg/kg BW/day) | Variation |
|---|---|---|---|
| Mean lower bound | 0.09 | 0.06 | ↑ |
| Mean upper bound | 0.15 | 0.11 | ↑ |
| High lower bound | 0.28 | 0.15 | ↑ |
| High upper bound | 0.31 | 0.22 | ↑ |

For the fattening pig diet, the following adjustments were made: for the feed material maize, OTA data contamination for maize grain was considered, while for the maize gluten feed material (20% CP), OTA contamination data were retrieved from maize protein. No contamination data was available for the molasses cane and mineral and vitamin mix feed materials.

Broilers

Table 23: Broiler exposure to OTA (mg/kg, 100% Dry Matter) based on model database diet, as calculated in the Excel calculator, taking into account 1 kg/feed, compared to exposure to OTA (mg/kg BW/day 100% Dry Matter) as reported in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023).

| | OTA model database diet (µg/kg feed) | EFSA OTA scientific opinion diet (mg/kg feed) | Variation |
|---|---|---|---|

| | | | |
|---|---|---|---|
| Mean lower bound | 1.03 | 1.20 | ↓ |
| Mean upper bound | 1.91 | 2.60 | ↓ |
| High lower bound | 2.99 | 4.10 | ↓ |
| High upper bound | 4.06 | 5.70 | ↓ |

Table 24: Broiler exposure to OTA (mg/day 100% Dry Matter) based on model database diet, as calculated in the Excel calculator, taking into account the amount of feed material in each animal diet per day, compared to exposure to OTA (mg/kg BW/day 100% Dry Matter) as reported in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023).

| | **OTA model database diet (μg/day)** | **EFSA OTA scientific opinion diet (μg/day)** | **Variation** |
|---|---|---|---|
| Mean lower bound | 0.14 | 0.20 | ↓ |
| Mean upper bound | 0.25 | 0.40 | ↓ |
| High lower bound | 0.40 | 0.65 | ↓ |
| High upper bound | 0.54 | 0.90 | ↓ |

Table 25: Broiler exposure to OTA (mg/kg bw/day, 100% Dry Matter) based on model database weight as calculated in the Excel calculator, compared to exposure to OTA (mg/kg BW/day 100% Dry Matter) as reported in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023).

| | **OTA model database diet (μg/kg BW/day)** | **EFSA OTA scientific opinion diet (μg/kg BW/day)** | **Variation** |
|---|---|---|---|
| Mean lower bound | 0.08 | 0.10 | ↓ |
| Mean upper bound | 0.15 | 0.20 | ↓ |
| High lower bound | 0.23 | 0.33 | ↓ |
| High upper bound | 0.32 | 0.45 | ↓ |

For the broiler diet, the following adjustments were made: for the feed material maize, ground OTA data contamination for maize grain was considered, for the maize gluten feed (20% CP), OTA contamination data were retrieved from maize protein, while for sunflower oil contamination data were retrieved from sunflowers (expeller + meal). No contamination data was available for the feed materials sorghum, ground and mineral and vitamin mix.

### 3.4.5 Discussion and considerations of contaminant case.

To evaluate the animal daily dietary exposure to OTA, the following parameters were considered:

- Contamination levels of feed material;
- Diet composition
- Total daily feed intake;
- Animal body weight.

OTA contamination levels of feed materials were expressed as lower and upper bounds levels (LB and UB, respectively), thus the exposure results to OTA, expressed in µg/kg dry matter, µg/kg bw/day and µg/day, were reported considering the mean LB, UB and the high LB, UB exposures. Calculations were performed using the Excel calculator provided by EFSA, as in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023).

As can be seen in the tables reported in the Results section, different exposure values were obtained for the animal categories, due to differences in the animal weights, feed intake and diet composition compared to those reported in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023). For instance, fattening pigs showed an overall higher exposure to OTA than the diet considered in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023) for all exposure scenarios except for the mean LB, which was due to the different diet compositions.

For dairy cows, the OTA exposure calculated per kilogram of body weight/day was greater for the model database diet, due to body weight differences considered for this animal species. Nevertheless, such differences were limited, resulting in slightly higher or lower exposure values than those reported in the EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023). As for dairy cows, the diet retrieved from the model database already contained maize silage as a feed material. It was thus not possible to determine the OTA contamination exposure following the approach reported in the EFSA OTA scientific opinion (EFSA CONTAM Panel 2023), in which the contamination for dairy cows was calculated considering 70% concentrated dry matter and 30% forage dry matter, maize silage. In our case this method would lead to an overestimation of OTA exposure due to the redundant inclusion of maize silage. The comparisons reported in Tables 14, 15 and 16 thus only consider the model diet proposed in the model database, which already contains forage, without adapting the values for 70% concentrate and 30% forage.

Animal body weight is a crucial factor in exposure estimation, as different physiological stages and breeds can result in a wide range of weights that impact the calculations. Another significant factor influencing the daily exposure values is the selection of feed materials in the diet composition. This selection can include individual feed materials (e.g., molasses cane), processed products (e.g., soybean meal), and by-products (e.g., milled byproducts). The choice of feed materials significantly affects the total inclusion rate of individual components and the related contamination levels in the overall exposure estimation.

Looking at CONTAM Panel evaluations, the recent evaluation on OTA exposure reported in the considered EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023) was performed according to methodologies already used and described in previous scientific opinions, with some modifications related to feed intake and diet composition assessed by the CONTAM Panel in May 2023 in line with current with common practices and published guidelines. The amendments aimed to harmonise the CONTAM Panel and the FEEDAP Panel when dealing with compounds in feed. The estimated feed intakes were based on published guidelines on nutrition and feeding (NRC, 2006; Leeson and Summers, 2008; EFSA FEEDAP Panel, 2017).

Without a comprehensive feed consumption database, the EFSA CONTAM panel used default values for feed consumption, including animal body weight, daily feed intake and inclusion rates of feed materials in diets, derived from the most pertinent and applicable sources within the published literature. These recommendations are based on a scientific analysis of findings from the literature, given the current lack of a comprehensive feed consumption database. Similar to GMO evaluations, the implementation of such a database, as proposed in this project, has the potential to eliminate reliance on default values. Such a database could facilitate precise EU-wide analyses, minimising the risk of overestimations or underestimations that could lead to inadequate risk characterisation.

Potential errors in reporting the contaminant occurrence data in the classification of the feed category in EFSA CONTAM Panel OTA scientific opinion (EFSA CONTAM Panel 2023) were identified, for example the use by data providers of feed categories at high (often not enough specified) FoodEx/FoodEx2 levels. The correct classification and harmonisation of feed nomenclature proposed in the present project could help prevent these kinds of biases.

A comprehensive database encompassing more variability in weights, feed intakes and feed materials, would not only enhance its applicability across a wider range of cases and uses, but would also contribute to a more accurate and harmonised exposure evaluation and risk assessment. End users would be able to tailor the database outcomes to their specific situations, rather than depending on default values.

## 4. Weaknesses and recommendations for future developments

• Feed materials:

The results reported in the previous sections revealed several differences among databases and classification systems. Of these different systems, the European Catalogue of Feed Materials is the best-known and has the strongest legal basis.

Although it only provides information of a qualitative nature (description of the product), stakeholders see it as the main reference in terms of feed classification and the majority use it within a legal framework, thus for placing products on the market and for labelling purposes.

Our first proposed intervention for the development of a feed consumption database is to define a unique EU classification system in which the main features and items reported in the European Catalogue of Feed Materials are harmonised with FoodEx2. This involves a systematic review of the contents of both the European Catalogue of Feed Materials and FoodEx2. This systematic review would represent not only a milestone for defining a complete list of similarities and differences but also for harmonising feed materials reported in FoodEx2 and in the European Catalogue of Feed Materials. Therefore, a future step towards an exhaustive feed classification would be to align the FoodEx2 feed hierarchy with any updated version of the EU Catalogue of Feed Material, which currently seems to be the most complete list available.

• Animals:

The animal categories included in our database are focused on a limited number of selected animal categories, as requested by the procurement for this preparatory work.

The proof of concept was thus conducted considering a limited number of animals, representative of large ruminants, small ruminants, monogastric animals, poultry animals, fish and companion animals.

For a more comprehensive feed consumption database, a wider range of food producing animals and non-food-producing animals is needed, e.g. those published in the overview of the EFSA current approaches to animal dietary exposure, reporting animal and production categories per Panel/Unit (Table 2, EFSA 2019). All these categories with different ages (e.g. cattle, young cattle, calves, heifers), physiological and productive phases (e.g. dairy calves, veal calves) should be included in the feed consumption database. However they should also have a harmonised nomenclature, not only to prevent different naming across different areas of risk assessment (e.g. swine vs pig, salmonids vs salmon) but also to create a harmonised database.

• Feed consumption data:

This project highlighted the lack of a reference feed consumption database that is recognised by the entire scientific community. Such a database could be used to harmonise animal exposure estimations and risk assessments. To address this issue, we have developed a proposal for a reference feed consumption database. To date, the model database only includes data generated from the FAO "Global Livestock Environmental Assessment Model – interactive GLEAM-i" along with selected scientific literature, given the limited access to exhaustive real-life animal consumption data. For future developments, the model database should be populated with thousands of records regarding real-life feed consumption data.

A good starting point would be the "Feed Modelling" project supported by DG-AGRI. This was mentioned in a workshop held with stakeholders within the framework of the present EFSA procurement. The aim was to acquire knowledge on the consumption of cereals and other crops by food producing animals. To improve the European Commission's ability to assess the demand for EU raw materials used in animal feed, the Directorate General for Agriculture and Rural Development of the European Commission (DG AGRI) decided to develop a quantitative model via the study 'Modelling feed consumption in the EU'. This model, called FeedMod, is a computer-based model that estimates the tonnage of raw materials used to produce industrial and on-farm compound feed in the EU 28. In 2009, the first version, jointly created by Tallage and Céréopa, was presented to the European Commission. A further study[5] conducted in 2014 led to an update of the model, which considered new raw materials and introduced a new method for an on-farm feed consumption forecast. This approach represents an excellent starting point especially if the feed industry and farmers/breeders are involved.

A key milestone will thus be to collect information from industry, nutritionists, breeders, farmers, etc. regarding the representative/typical diets that can be used for simulations and as reference formulas. Methodologies for data collection, such as surveys (which could mirror surveys used for human consumption) can provide accurate insights into diet compositions and comprehensive feed material inclusion rates covering real-world diets and applications. This will help identify the key ingredients and define possible scenarios for future animal dietary exposures, which is one of the pillars of risk assessment (EFSA 2019). Obtaining information on how much an animal species (and within a species for each animal category) eats is thus indispensable. This would entail collecting a large amount of data on different ingredients, combined in different diets and different inclusion rates, as well as a variety of animal species (e.g. bovines, poultry, pigs, fish, pets) with their

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5 Available online: https://agriculture.ec.europa.eu/system/files/2019-12/ext-study-feed-mod-fulltext_2014_en_0.pdf

age/weight stages (e.g. calves under 1-year, young cattle, adult cattle, piglets, sows, laying hens, chicks, dogs, puppies).

The collection of survey data from farmers could be further facilitated by the development of a web application with a smart, intuitive interface. A harmonised list of feed ingredients and animals could be pre-entered, so that those filling in the survey could choose the specific feed material and animal of interest for a considered diet to input into the database. This list should be open-ended, meaning users could propose new feed materials as needed so as to keep pace with the evolving market. A database manager could evaluate each submission to verify whether it truly represents a new feed material thus ensuring there is no overlap of feed materials under different names. Additionally, the web application should include a feed recipe database enabling users to submit, update and adjust feed recipes along with specifying the quantities or weights of each ingredient. The main weaknesses and recommendations are summarised in Table 26.

Table 26: Main weaknesses and recommendations

| Category | Weaknesses | Recommendations |
|---|---|---|
| Feed Materials | Differences among databases and classification systems | Define a complete list of feed materials by aligning the FoodEx2 feed hierarchy with any updated version of the EU Catalogue of Feed Material |
| | Limited feed materials in the proposed model database | Include the defined and harmonised feed materials in the future feed consumption database with an open-ended option |
| | Limited feed recipe in the proposed model database | Include a feed recipe database |
| Animals | Lack of harmonised nomenclature across different risk assessment areas | Harmonise nomenclature of food-producing and non-food-producing animals, starting from those listed in the EFSA current approaches for animal dietary exposure (EFSA, 2019) |
| | Limited animal categories in the proposed model database | Include the harmonised list of food-producing and non-food-producing animals in the future feed consumption database |
| Feed Consumption Data | Absence of a reference feed consumption database recognized by the scientific community | Develop a web application for survey data collection, with pre-entered harmonised lists of feed ingredients and animals |
| | Limited real-life animal consumption data available | Collect data consumption information from the industry, stakeholders, nutritionists, breeders and farmers |
| | Consumption data from the literature in the proposed model database | Include the real-life data consumption in the future feed consumption database |

## 5. Conclusion

Task 1

The classification systems and databases analysed in this project have highlighted a considerable lack of homogeneity in terms of the subdivision into groups or categories and subcategories, as well as in the description and nomenclature of each feed.

The classification systems and databases have different approaches to classifying feed ingredients. Some provide a general division between forage and concentrate feeds (e.g. FEDNA and FeeDBase), while others provide no such distinction. Some databases do not even include forages, such as INRAE-CIRAD-AFZ Tables. Some databases present an additional level of classification that includes the use of numerical and/or alphanumerical codes, while others do not.

This heterogeneity and variability could be problematic and present limitations in the attempt to harmonise the different feed classification systems. Of the databases and classification systems analysed, the European Catalogue of Feed Materials is considered the most reputable and widely used within the legal framework for placing products on the market and for labelling purposes.

Moreover, precise and comprehensive information is severely lacking on the inclusion rates and consumption of feed materials in the rations and diets of food producing and non-food producing1 animals. Due to the importance of such information, especially within the context of risk assessments related to animal dietary exposure, this data gap should be addressed.

Task 2

Our analysis of FoodEx2 revealed that the hierarchical structure closely aligns with the classification of feed materials in the European Catalogue of Feed Materials. For an exhaustive and harmonised feed classification system, further alignment of the FoodEx2 feed hierarchy with the EU Catalogue of Feed Materials is recommended.

We propose the following additions/updates for the FoodEx2 catalogue:

• FEDNA id of the corresponding item in the FEDNA database, if there is a exists,

• Feedipedia.org link to the corresponding item in the Feedipedia.org database, if there is a match,

• termExtendedName to align the item name with the one used in the European Catalogue of Feed Materials,

• termScopeNote to align the item description with the one used in the European Catalogue of Feed Materials,

• commonNames to store all the alternative names for a specific item

Task 3

A model feed consumption database was created consisting of three different Excel sheets: Animal, Feed and Consumption. The model database was populated by feed consumption data obtained using FAO "Global Livestock Environmental Assessment Model – interactive GLEAM-i" along with the selected scientific literature. This highlighted the lack of comprehensive and real-life feed consumption data and the importance of collaborating with stakeholders and the feed industry to collect such data.

Task 4

To evaluate the feasibility and limitations of our model database, two case studies were conducted, one involving NEPs expressed in a GM crop, and the second involving a contaminant (OTA). The results of animal dietary exposure reported in the selected EFSA scientific opinions were compared with those obtained using Excel calculators and those obtained from animal dietary exposure assessments using data extracted from the model database. Overall, lower animal dietary exposure levels were obtained using data extracted from the model database in the selected case studies, which were more marked in the GMO examples, except for Atlantic salmon (Appendix C). In the OTA example, lower animal dietary exposure was also observed, although some animal categories showed slightly higher dietary exposure.

Overall conclusion

To date no common database is available that can predict feed intake for food producing animals and non-food-producing animals in the European Union. Different approaches therefore rely on different sources of default values for animal body weights, total amount of daily consumed feed and inclusion rates of feedstuffs (e.g. OECD 2009, 2013; EFSA CONTAM Panel, 2011; EFSA FEEDAP Panel, 2017; US National Research Council; INRA; CIRAD; AFZ and FAO). Thus, collecting real-life data on feed consumption from stakeholders, industry, nutritionists, breeders, and farmers is recommended. However, considering the extensive nature of the feed industry and the wide range of feed ingredients and animal species involved, this approach would be difficult and time-consuming, and therefore a dedicated project is needed. The collected data should be used to develop a comprehensive feed consumption database for a selected and harmonised list of food producing and non-food producing animals. This would enable more realistic exposure assessments and more accurate and harmonised risk assessments.

## 5. Abbreviations

| | |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| AA | Amino acids |
| AAD-1 | aryloxyalkanoate dioxygenase |
| ADE | animal dietary exposure |
| AFZ | Association Francaise de Zootechnie |
| AMIS | Agricultural Market Information System |
| AOPP | aryloxyphenoxypropionate |
| BW | body weight |
| CIRAD | Centre de Coopèration Internationale en la Recherche Agronomique pour le Development |
| CONTAM | EFSA Panel on Contaminants in the Food Chain |
| CP | Crude protein |
| CVB | Centraal Veevoerder Bureau |
| DDE | daily dietary exposure |
| DG-AGRI | Directorate-General for Agriculture and Rural Development |
| DIM | days in milk |
| DIS | Decision Innovation Solutions |
| DM | dry matter |
| DW | dry weight |
| EE | ether extract |
| EFSA | European Food Safety Authority |
| EPSPS | 5-enolpyruvylshikimate-3-phosphat synthase |
| EU | European Union |
| F | forage |
| FAO | Food and Agriculture Origanization of the United Nations |
| FEDNA | Fundaciòn Española para el Desarrollo de la Nutrición Animal |
| FeeDBase | Swiss Feed Database |
| FEEDAP | EFSA Panel on on additives and products or substances used in animal feed |
| FICD | Feed Ingredients Composition Database |
| FoodEx2 | EFSA food and feed classification system |
| G | grain |
| GLEAM-i | Global Livestock Environmental Assessment Model – interactive |
| GM | genetically modified |
| GMO | genetically modified organism |
| GMO Panel | EFSA Panel on Genetically Modified Organisms |
| IAFFD | International Aquaculture Feed Formulation Database |
| ICAR | International Committee for Animal Recording |
| IFEEDER | Institute for Feed Education and Research |
| ILVO | Flemish Research Institute for Agriculture, Fisheries and Food |
| INRA | Institut National de la Recherche Agronomique |
| IR | inclusion rate |
| LB | lower bound |
| LOQ | limit of quantification |
| MTX | matrix |
| NEP | newly expressed proteins |

| | |
|---|---|
| NorFor | Nordic Feed Evaluation System |
| NRC | National Research Council |
| OECD | Organisation for Economic Co-operation and Development |
| OTA | ochratoxin A |
| PAFF | Practical aquaculture feed formulation database |
| PAT | phosphinothricin acetyltransferase enzyme |
| PoC | Proof of concept |
| SSD2 | Standard Sample Description 2 |
| TDI | tolerable daily intake |
| RPCs | Raw primary commodities |
| SCF | Scientific Committee on Food |
| UB | upper bound |
| UNDP | United Nations Development Programme |
| WHO | World Health Organization |
| WLR | Wageningen Livestock Research |

# Appendix A

## Data and Methodology Tables

# Appendix A – Data and Methodology Tables

## A.1 Proof of concept for the development of a feed consumption database

### A.1.1. Analysis of the model database

Table 27: Animal data on dairy cow (Darabighane et al., 2020).

| ANIMAL | Animal body weight (kg) | Total daily intake ($kg^{DM}$/animal) |
|---|---|---|
| Dairy cow | 623 | 25.82 |

Table 28: Dairy cow diet, with corresponding feed materials inclusion rates (Darabighanee et al., 2020)

| FEED MATERIALS | | Inclusion rate (IR%) |
|---|---|---|
| Literature terminology | Code and terminology referred to European Catalogue of Feed Materials (Reg. EU 2022/1104) | |
| Corn silage | 6.11.1 Maize silage | 21.23 |
| Corn gluten feed (20% CP) | 1.2.9 Maize gluten feed | 18.81 |
| Beet pulp | 4.1.10 Dried (sugar) beet pulp | 2.64 |
| Corn grain, ground | 1.2.1 Maize | 18.09 |
| Barley grain, ground | 1.1.1 Barley | 14.57 |
| Soybean meal (44%) | 2.18.3 Soya (bean) meal | 14.5 |
| Canola meal | 2.14.3 Rape seed meal | 5.00 |
| Fat powder | 13.6.4 Salts of fatty acids | 2.30 |
| Mineral & vitamin mix | N.A. | 2.86 |
| Total | | 100 |

Table 29: Animal data on fattening pigs (Sevillano et al., 2018).

| ANIMAL | Animal body weight (kg) | Total daily intake ($kg^{DM}$/animal) |
|---|---|---|
| Fattening pig | 43.5 | 2.187 |

Table 30: Fattening pig diet, with corresponding feed materials inclusion rates (Sevillano et al., 2018).

| FEED MATERIALS | | Inclusion rate (IR%) |
|---|---|---|
| **Literature terminology** | **Code and terminology referred to European Catalogue of Feed Materials (Reg. EU 2022/1104)** | |
| Corn | 1.2.1 Maize | 69.84 |
| Soybean meal (48%) | 2.18.4 Soya (bean) meal, dehulled | 18.05 |
| Molasses cane | 7.6.1 (Sugar) cane molasses | 5.00 |
| Corn Gluten Feed (20% CP) | 1.2.9 Maize gluten feed | 2.50 |
| Mineral & vitamin mix | N.A. | 4.61 |
| TOTAL | | 100 |

Table 31: Animal data on finishing pigs (Sevillano et al., 2018)

| ANIMAL | Animal body weight (kg) | Total daily intake ($kg^{DM}$/animal) |
|---|---|---|
| Finishing pig | 121.6 | 2.875 |

Table 32: Finishing pig diet, with corresponding feed materials inclusion rates (Sevillano et al., 2018)

| FEED MATERIALS | | Inclusion rate (IR%) |
|---|---|---|
| **Literature terminology** | **Code and terminology referred to European Catalogue of Feed Materials (Reg. EU 2022/1104)** | |
| Corn | 1.2.1 Maize | 75.51 |
| Soybean meal (48%) | 2.18.4 Soya (bean) meal, dehulled | 9.83 |
| Molasses cane | 7.6.1 (Sugar) cane molasses | 5.00 |
| Corn Gluten Feed (20% CP) | 1.2.9 Maize gluten feed | 5.00 |
| Mineral & vitamin mix | N.A. | 4.66 |
| TOTAL | | 100 |

Table 33: Animal data on broilers (Abdel-Wareth et al., 2019).

| ANIMAL | Animal body weight (kg) | Total daily intake ($kg^{DM}$/animal) |
|---|---|---|
| Broiler | 1.707 | 0.1343 |

Table 34: Broiler diet, with corresponding feed materials inclusion rates (Abdel-Wareth et al., 2019).

| FEED MATERIALS | | Inclusion rate (IR%) |
|---|---|---|
| **Literature terminology** | **Code and terminology referred to European Catalogue of Feed Materials (Reg. EU 2022/1104)** | |
| Corn, ground | 1.2.1 Maize | 30.00 |
| Sorghum, ground | 1.8.1 Sorghum | 30.00 |
| Soybean meal (44%) | 2.18.3 Soya (bean) meal | 25.00 |
| Corn Gluten Meal (60% CP) | 1.2.8 Maize Gluten | 6.00 |
| Sunflower Oil | 2.20.1 Vegetable oil and fat | 5.52 |
| Mineral & vitamin mix | N.A. | 3.48 |
| TOTAL | | 100 |

Table 35: Animal data on dairy sheep (Milis et al., 2004).

| ANIMAL | Animal body weight (kg) | Total daily intake ($kg^{DM}$/animal) |
|---|---|---|
| Dairy sheep | 57 | 1.22 |

Table 36: Dairy sheep diet, with corresponding feed materials inclusion rates (Milis et al., 2004).

| FEED MATERIALS | | Inclusion rate (IR%) |
|---|---|---|
| **Literature terminology** | **Code and terminology referred to European Catalogue of Feed Materials (Reg. EU 2022/1104)** | |
| Corn grain | 1.2.1 Maize | 41.00 |
| Alfalfa hay | 6.10.2 Lucerne, field dried; alfalfa field dried | 30.00 |
| Wheat Straw | 6.3.1 Cereals Straw | 5.00 |

| | | |
|---|---|---|
| Corn Gluten Meal (60% CP) | 1.2.8 Maize gluten | 10.00 |
| Corn Gluten Feed (20% CP) | 1.2.9 Maize gluten feed | 10.00 |
| Mineral & vitamin mix | N.A. | 4.00 |
| TOTAL | | 100 |

### A.1.2. Standardisation of feed material and nomenclature of animal categories

Table 37: Standardisation of feed materials for GMO case

| GMO case | | |
|---|---|---|
| **Feed materials as reported in GM feed Example 1** | **Feed materials as reported in model database** | **Feed materials chosen for standardisation** |
| Maize Grain | Maize | Maize |
| Maize Gluten feed | Maize gluten feed | Maize gluten feed |
| Maize Gluten meal | Maize gluten | Maize gluten |
| Maize Forage/Silage | Maize silage | Maize silage |
| Feed materials as reported in GM feed Example 2 | Feed materials as reported in model database | Feed materials chosen for standardisation |
| Maize grain | Maize | Maize |
| Maize Forage | Maize silage | Maize silage |

Table 38: Standardisation of feed materials for feed contaminant case

| GMO case | | | |
|---|---|---|---|
| **Feed materials as reported in EFSA OTA scientific opinion – Annex A** | **Feed materials as reported in EFSA OTA scientific opinion – Table 7** | **Feed materials as reported in model database** | **Feed materials chosen for standardisation** |
| Wheat | Wheat grains | Wheat (Grains of Triticum aestivum L., Triticum durum Desf. and other wheat cultivars) | Wheat |
| Wheat feed | Wheat feed | Wheat feed | Wheat Feed |
| Barley | Barley grain | Barley | Barley grain |
| Maize | Maize grain | Maize | Maize |
| | Maize protein | Maize Protein | Maize Protein |

| | | | |
|---|---|---|---|
| | | (Maize Gluten)<br><br>Maize protein feed (Maize gluten feed) | |
| | Maize silage | Maize silage | Maize silage |
| Soybean meal | Soybean (expeller + meal) | Soya (bean) meal<br><br>Soya (bean) expeller<br><br>Soya (bean) meal, dehulled | Soybean (expeller + meal) |
| | Soybean hulls | Soya (bean) hulls | Soybean hulls |
| | Soybean, protein concentrate | Soya (bean) protein concentrate | Soybean, protein concentrate |
| **Rapeseed meal** | Rape seed | Rape seed | Rape seed |
| | Rape seed (expeller + meal) | Rape seed expeller<br><br>Canola meal | Rape seed (expeller + meal) |
| **Vegetable oils and fats** | -- | Vegetable oil and fat | Vegetable oil and fat |
| **Sugar beet pulp** | -- | Dried (sugar) beet pulp | Beet pulp |
| **Molasses** | -- | (Sugar) beet molasses | (Sugar) beet molasses |
| **Sunflower meal** | Sunflower (expeller + meal) | Sunflower Oil | Sunflower Oil |
| **Horse beans** | Horse beans | Horse Beans | Horse Beans |
| **Mineral salts** | -- | Minerals and products derived thereof | Minerals and products derived thereof |
| **Premix** | Premix | Premix | Premix |
| **Forage** | Grass meal, hay and alfalfa | Forage meal, grass meal, green meal<br><br>Hay | Forage |
| | Lucerne meal (Alfalfa meal) | Lucerne meal (alfalfa meal) | Lucerne meal (alfalfa meal) |

Table 39: Standardisation of animal categories

| GMO case | | |
|---|---|---|
| **Animal categories as reported in GM feed**<br><br>**Example 1** | **Animal categories as reported in model database** | **Animal categories chosen for standardisation** |
| Lactating dairy cow | Dairy cow | Dairy cow |
| Finishing pig | Finishing pig | Finishing pig |
| Broiler | Broiler | Broiler |
| **Animal categories as reported in GM feed Example 2** | Animal categories as reported in model database | Animal categories chosen for standardisation |
| Dairy cattle | Dairy cow | Dairy cow |
| Ram/ewe | Dairy sheep | Dairy sheep |
| Finishing pigs | Finishing pig | Finishing pig |
| Broiler | Broiler | Broiler |
| **Animal categories as reported in GM feed**<br><br>**Example 2** | **Animal categories as reported in model database** | **Animal categories chosen for standardisation** |
| Dairy cattle | Dairy cow | Dairy cow |
| Ram/ewe | Dairy sheep | Dairy sheep |
| Finishing pigs | Finishing pig | Finishing pig |
| Broiler | Broiler | Broiler |
| **Feed contaminant case** | | |
| **Animal categories as reported in EFSA OTA scientific opinion** | **Animal categories as reported in model database** | **Animal categories chosen for standardisation** |
| Dairy cows | Dairy Cow | Dairy cow |
| Dairy sheep | Dairy sheep | Dairy sheep |
| Pigs for fattening | Fattening pig | Fattening pig |
| Chickens for fattening | Broiler | Broiler |

# Appendix B

## Assessment/Result Tables

# Appendix B – Assessment/Result Tables

## B.1. Methodologies for mapping and describing the classification system adopted in the selected feed database

Table 40: List of the classification categories in each selected feed database

| Database | Groups/Categories |
|---|---|
| FEDNA | 1.Granos de cereals (cereal grains) |
| | 2. Cereales procesados por calor (heat treated cereals) |
| | 3. Subproductos de cereals (cereal by-products) |
| | 4. Frutos y tubérculos. Melazas y vinazas (Fruits & tubers. Molasses & vinasses) |
| | 5. Concentrados proteína vegetal (vegetal protein concentrates) |
| | 6. Alimentos fibrosos (fibrous feeds) |
| | 7. Concentrados de proteína animal (animal protein concentrates) |
| | 8. Productos lácteos (dairy procucts) |
| | 9. Grasas y aceites (fats and oils) |
| | 10. Minerales (minerals) |
| | 11. Microingredientes (microingredients) |
| INRAE-CIRAD-AFZ | Cereal grains |
| | Cereal by-products |
| | Legumes and oilseeds |
| | Oil by-products |
| | Roots and by-products |
| | Fruits and by-products |
| | Other plant products |
| | Animal products |
| | Dairy products |
| | Oils and fats |
| | Mineral products |
| | Amino acids |
| CVB | Compound feedstuffs[a] |
| | High moisture industrial co-products |
| | Roughages and related products |
| | Mineral feedstuffs |
| | Miscellaneous |
| Luonnonvaratieto | 01. Grains and seeds |
| | 02. Cakes and meals |
| | 03. Plant by-products |
| | 04. Feeds of animal origin |
| | 05. Roots, tubers, fruits and cabbages, |
| | 06. Fresh forages and pasture |
| | 07. Grass silages |
| | 08. Other silages |
| | 09. Hay and artificially dried grasses |
| | 10. Straw |
| | 11. Minerals |

| | |
|---|---|
| | 90. Other feeds |
| **NorFor** | 1. Grains<br>2. Oil seeds<br>3. Legume seeds<br>4. Tubers and roots<br>5. Other seeds and fruits<br>6. Forages and roughage<br>7. Other plants<br>8. Milk products<br>9. Animal products<br>10. Marine products<br>11. Minerals<br>12. Miscellaneous<br>13. Feed additives<br>14. Standard feed mixtures<br>18. Commercial products<br>97. Special feedstuff company mixture |
| **FeedBase** | Raw materials<br>Roughage |
| **IAFFD** | Feed Ingredient composition database (FICD)- no categories |
| **Feedipedia** | Forage plants<br>Plant products/ by-products<br>Feed of animal origin<br>Other feeds |
| **The European Catalogue of Feed Material[b]** | 1. Cereal grains and products derived thereof<br>2. Oil seeds, oil fruits, and products derived thereof<br>3. Legume seeds and products derived thereof<br>4. Tubers, roots, and products derived thereof<br>5. Other seeds and fruits, and products derived thereof<br>6. Forage and roughage, and products derived thereof<br>7. Other plants, algae and products derived thereof<br>8. Milk products and products derived thereof<br>9. Land animal products and products derived thereof<br>10. Fish, other aquatic animals and products derived thereof<br>11. Minerals and products derived thereof<br>12. Products and by-products obtained by fermentation using micro-organism, inactivated resulting in absence of live micro-organisms<br>13. Miscellaneous |
| **The Harmonised OECD tables of feedstuffs from field crop** | 1. Forages/Fodders<br>2. Roots & Tubers<br>3. Cereal Grains/Crops Seeds<br>4. By-products |
| **The international Feed Vocabulary (FAO/UNDP)** | 1. Dry forages and roughages<br>2. Pasture, range plants, and forages fed green<br>3. Silages<br>4. Energy feeds |

| | |
|---|---|
| | 5. Protein supplements<br>6. Mineral supplements<br>7. Vitamin supplements (including ensiled yeast)<br>8. Additives |

(a): In CVB the term compound feed is used instead of raw materials (single ingredients)
(b): The Feed Register has been not included since it uses the same categories of the European Catalogue of Feed material

## B.2. Proof of concept for the development of a feed consumption database

### B.2.1. First case study: GM feed

**GM feed Example 1**

Table 41: Animal dietary exposure to Cry1Da_7 and Cry1B.868 proteins (mg/kg BW per day) based on the consumption of maize grains, gluten feed, gluten meal and silage in selected animals, as reported in the GM feed Example 1 (EFSA GMO Panel 2022a)

| Animal categories BW (kg)/total diet intake (kg dw) | Feed material | IR% | Cry1Da_7 | Cry1B.868 |
|---|---|---|---|---|
| **Broiler** **1.7/0.12** | Grain | 70 | 12.35 | 1,285 |
| | Gluten feed | 10 | 4.6 | 477.1 |
| | Gluten meal | 10 | 12.5 | 1,305 |
| | Total | 90 | 29 | 3,065 |
| **Finishing pig** **100/3** | Grain | 70 | 5.25 | 546 |
| | Gluten feed | 20 | 3.9 | 405.6 |
| | Gluten meal | 10 | 5.3 | 555 |
| | Total | 100 | 14 | 1,505 |
| **Lactating dairy cow** **650/25** | Gluten feed | 20[(a)] | 5 | 520 |
| | Gluten meal | 20 | 13.6 | 1,423 |
| | Forage/Silage | 60 | 600 | 2,538 |
| | Total | 100 | 619 | 4,478 |

Table 42: Animal daily dietary exposure to Cry1Da_7 and Cry1B.868 proteins (mg/kg BW per day) performed with the Excel calculator (EFSA GMO Panel 2023)

| Protein | Feed material | Animal species | Inclusion rate | NEP level | Daily dietary exposure to NEP |
|---|---|---|---|---|---|
| | | Category | IR% | $\mu g^{NEP}/g^{DM}$ | $\mu g^{NEP}/kg^{BW}$ |

| | | | | | |
|---|---|---|---|---|---|
| Cry1B.868 | Grain | Dairy cow 650/25 | 30 | 0.25 | 2.88 |
| | | Finishing pig 100/3 | 70 | 0.25 | 5.25 |
| | | Broiler 2/0.158 | 70 | 0.25 | 13.83 |
| | | Dairy Sheep 80/2.8 | 25 | 0.25 | 2.19 |
| | Gluten feed | Dairy cow 650/25 | 30 | 0.65 | 7.47 |
| | | Finishing pig 100/3 | 20 | 0.65 | 3.89 |
| | | Broiler 2/0.158 | 10 | 0.65 | 5.12 |
| | | Dairy Sheep 80/2.8 | 10 | 0.65 | 2.27 |
| | Gluten meal | Dairy cow 650/25 | 20 | 1.79 | 13.77 |
| | | Finishing pig 100/3 | 10 | 1.79 | 5.37 |
| | | Broiler 2/0.158 | 10 | 1.79 | 14.14 |
| | | Dairy Sheep 80/2.8 | 10 | 1.79 | 6.27 |
| | Forage/silage | Dairy cow 650/25 | 60 | 26.00 | 600.00 |
| | | Finishing pig 100/3 | NA | NA | NA |
| | | Broiler 2/0.158 | NA | NA | NA |
| | | Dairy Sheep 80/2.8 | 80 | 26.00 | 728.00 |
| | Milled by-products | Dairy cow 650/25 | 30 | 0.25* | 2.88 |
| | | Finishing pig 100/3 | 75 | 0.25* | 5.63 |
| | | Broiler 2/0.158 | 60 | 0.25* | 11.85 |
| | | Dairy Sheep 80/2.8 | NA | NA | NA |
| | TOTAL | Dairy cow 650/25 | 170 | NA | 627.01 |
| | | Finishing pig 100/3 | 175 | NA | 20.13 |
| | | Broiler 2/0.158 | 150 | NA | 44.93 |

| | | | | | |
|---|---|---|---|---|---|
| | | Dairy Sheep 80/2.8 | 125 | NA | 738.72 |
| Cry1B.868 | Grain | Dairy cow 650/25 | 30 | 26.00 | 300.00 |
| | | Finishing pig 100/3 | 70 | 26.00 | 546.00 |
| | | Broiler 2/0.158 | 70 | 26.00 | 1437.80 |
| | | Dairy Sheep 80/2.8 | 25 | 26.00 | 227.50 |
| | Gluten feed | Dairy cow 650/25 | 30 | 67.34 | 777.00 |
| | | Finishing pig 100/3 | 20 | 67.34 | 404.04 |
| | | Broiler 2/0.158 | 10 | 67.34 | 531.99 |
| | | Dairy Sheep 80/2.8 | 10 | 67.34 | 235.69 |
| | Gluten meal | Dairy cow 650/25 | 20 | 186.00 | 1432.00 |
| | | Finishing pig 100/3 | 10 | 186.00 | 558.48 |
| | | Broiler 2/0.158 | 10 | 186.00 | 1470.66 |
| | | Dairy Sheep 80/2.8 | 10 | 186.00 | 651.56 |
| | Forage/silage | Dairy cow 650/25 | 60 | 110.00 | 2538.46 |
| | | Finishing pig 100/3 | NA | NA | NA |
| | | Broiler 2/0.158 | NA | NA | NA |
| | | Dairy Sheep 80/2.8 | 80 | 110.00 | 3080.00 |
| | Milled by-products | Dairy cow 650/25 | 30 | 26.00* | 300.00 |
| | | Finishing pig 100/3 | 75 | 26.00* | 585.00 |
| | | Broiler 2/0.158 | 60 | 26.00* | 1232.40 |
| | | Dairy Sheep 80/2.8 | NA | NA | NA |
| | TOTAL | Dairy cow 650/25 | 170.00 | NA | 5347.46 |
| | | Finishing pig 100/3 | 175.00 | NA | 2093.52 |

| | | | | |
|---|---|---|---|---|
| | Broiler 2/0.158 | 150.00 | NA | 4672.85 |
| | Dairy Sheep 80/2.8 | 125.00 | NA | 4194.75 |

NA indicates that the inclusion rate or NEP levels were not provided, and therefore no exposure calculations were done. The total inclusion rate for dairy cow, finishing pig, broiler, dairy sheep is >100%, resulting in an overestimation in the DDE to each protein.

*The GMO panel did not recommend specific data for a conversion factor so the value of 1 was selected.

Table 43: Animal daily dietary exposure to Cry1Da_7 and Cry1B.868 proteins (mg/kg BW per day) performed with the model database input data.

| Protein | Feed material | Animal categories BW (kg)/total diet intake (kg dw) | Inclusion rate | NEP level | Daily dietary exposure to NEP |
|---|---|---|---|---|---|
| | | **Category** | **IR%** | **µgNEP/gDM** | **µgNEP/kgBW** |
| **Cry1Da_7** | **Maize** | Dairy cow 623/25.82 | 18.09 | 0.25 | 1.87 |
| | | Finishing pig 121.6/2.875 | 75.51 | 0.25 | 4.46 |
| | | Broiler 1.707/0.1343 | 30 | 0.25 | 5.90 |
| | | Dairy sheep 57/1.22 | 41 | 0.25 | 2.19 |
| | **Maize gluten feed** | Dairy cow 623/25.82 | 18.81 | 0.65 | 5.05 |
| | | Finishing pig 121.6/2.875 | 5 | 0.65 | 0.77 |
| | | Dairy sheep 57/1.22 | 10 | 0.65 | 1.39 |
| | **Maize gluten** | Broiler 1.707/0.1343 | 6 | 1.79 | 8.45 |
| | | Dairy sheep 57/1.22 | 10 | 1.79 | 3.83 |
| | **Maize silage** | Dairy cow 623/25.82 | 21.23 | 26.00 | 228.77 |
| | **TOTAL** | Dairy cow 623/25.82 | 58.13 | NA | 235.69 |
| | | Finishing pig 121.6/2.875 | 80.51 | NA | 5.23 |
| | | Broiler 1.707/0.1343 | 36 | NA | 14.35 |

| | | | | | |
|---|---|---|---|---|---|
| | | Dairy sheep 57/1.22 | 61 | NA | 7.41 |
| Cry1B.868 | Maize | Dairy cow 623/25.82 | 18.09 | 26.00 | 194.93 |
| | | Finishing pig 121.6/2.875 | 75.51 | 26.00 | 464.18 |
| | | Broiler 1.707/0.1343 | 30 | 26.00 | 613.67 |
| | | Dairy sheep 57/1.22 | 41 | 26.00 | 228.16 |
| | Maize gluten feed | Dairy cow 623/25.82 | 18.81 | 67.34 | 524.96 |
| | | Finishing pig 121.6/2.875 | 5 | 67.34 | 79.61 |
| | | Dairy sheep 57/1.22 | 10 | 67.34 | 144.13 |
| | Maize gluten | Broiler 1.707/0.1343 | 6 | 186.00 | 878.78 |
| | | Dairy sheep 57/1.22 | 10 | 186.00 | 398.45 |
| | Maize silage | Dairy cow 623/25.82 | 21.23 | 110.00 | 967.86 |
| | TOTAL | Dairy cow 623/25.82 | 58.13 | NA | 1687.75 |
| | | Finishing pig 121.6/2.875 | 80.51 | NA | 543.78 |
| | | Broiler 1.707/0.1343 | 36 | NA | 1492.45 |
| | | Dairy sheep 57/1.22 | 61 | NA | 770.74 |

NA indicates that an inclusion rate or NEP levels were not provided and therefore no exposure calculations were done.

Table 44: Summary of DDE results to Cry1Da_7 and Cry1B.868 proteins (mg/kg BW per day) reported in the EFSA scientific opinion (EFSA GMO Panel 2022a), results obtained using the Excel calculator (EFSA GMO Panel 2023) and results obtained using the model database.

| Protein | Feed material | Animal species | DDE scientific Opinion | DDE Excel calculator | DDE model database |
|---|---|---|---|---|---|
| | | Category | $\mu g^{NEP}/kg^{BW}$ | $\mu g^{NEP}/kg^{BW}$ | $\mu g^{NEP}/kg^{BW}$ |
| Cry1Da_7 | Maize | Dairy cow | NA | 2.88 | 1.87 |
| | | Finishing pig | 5.25 | 5.25 | 4.46 |

| | | | | | |
|---|---|---|---|---|---|
| | | Broiler | 12.35 | 13.83 | 5.90 |
| | | Dairy Sheep | NC | 2.19 | 2.19 |
| | **Maize gluten feed** | Dairy cow | 5.00* | 7.47 | 5.05 |
| | | Finishing pig | 3.90* | 3.89 | 0.77 |
| | | Broiler | 4.60* | 5.12 | NA |
| | | Dairy Sheep | NC | 2.27 | 1.39 |
| | **Maize gluten** | Dairy cow | 13.60* | 13.77 | NA |
| | | Finishing pig | 5.30* | 5.37 | NA |
| | | Broiler | 12.50* | 14.14 | 8.45 |
| | | Dairy Sheep | NC | 6.27 | 3.83 |
| | **Maize silage** | Dairy cow | 600.00* | 600.00 | 228.77 |
| | | Finishing pig | NA | NA | NA |
| | | Broiler | NA | NA | NA |
| | | Dairy Sheep | NC | 728.00 | NA |
| | **Maize milled by-products** | Dairy cow | NA | 2.88 | NA |
| | | Finishing pig | NA | 5.63 | NA |
| | | Broiler | NA | 11.85 | NA |
| | | Dairy Sheep | NC | NA | NA |
| | **TOTAL** | Dairy cow | 619.00* | 627.01 | 235.69 |
| | | Finishing pig | 14.00* | 20.13 | 5.23 |
| | | Broiler | 29.00* | 44.93 | 14.35 |
| | | Dairy Sheep | NC | 738.72 | 7.41 |
| **Cry1B.868** | **Maize** | Dairy cow | NA | 300.00 | 194.93 |
| | | Finishing pig | 546.00* | 546.00 | 464.18 |
| | | Broiler | 1285.00* | 1437.80 | 613.67 |
| | | Dairy Sheep | NC | 227.50 | 228.16 |
| | **Maize gluten feed** | Dairy cow | 520.00* | 777.00 | 524.96 |
| | | Finishing pig | 405.60* | 404.04 | 79.61 |
| | | Broiler | 477.10* | 531.99 | NA |
| | | Dairy Sheep | NC | 235.69 | 144.13 |

| | | | | |
|---|---|---|---|---|
| **Maize gluten** | Dairy cow | 1423.00* | 1432.00 | NA |
| | Finishing pig | 555.00* | 558.48 | NA |
| | Broiler | 1305.00* | 1470.66 | 878.78 |
| | Dairy Sheep | NC | 651.56 | 398.45 |
| **Maize silage** | Dairy cow | 2538.00* | 2538.46 | 967.86 |
| | Finishing pig | NA | NA | NA |
| | Broiler | NA | NA | NA |
| | Dairy Sheep | NC | 3080.00 | NA |
| **Maize milled by-products** | Dairy cow | NA | 300.00 | NA |
| | Finishing pig | NA | 585.00 | NA |
| | Broiler | NA | 1232.40 | NA |
| | Dairy Sheep | NC | NA | NA |
| **TOTAL** | Dairy cow | 4478.00* | 5347.46 | 1687.75 |
| | Finishing pig | 1505.00* | 2093.52 | 543.78 |
| | Broiler | 3065.00* | 4672.85 | 1492.45 |
| | Dairy Sheep | NC | 4194.75 | 770.74 |

NA indicates that an inclusion rate or NEP levels were not provided and therefore no exposures were calculated.
NC indicates that the animal category was not considered in the scientific opinion (EFSA GMO Panel 2022a)
* Indicates that zero digits were added to the original value (Table 41) to obtain three digits after the decimal point.

**GM feed Example 2**

Table 45: Animal dietary exposure to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1A.105, Cry2Ab2, Cry3Bb1, CP4 EPSPS and AAD-1 proteins (mg/kg BW per day) in selected animals, based on the consumption of maize grain and forage, as reported in the GM feed Example 2 (EFSA GMO Panel 2022b).

| **Cry1F** | **BW (kg)** | **TDI feed (kg DM/animal)** | **IR (%) grains** | **IR (%) forage** | **Grain (G)** | **Forage (F)** | **G + F** |
|---|---|---|---|---|---|---|---|
| Beef cattle[(a)] | 500 | 12 | 80 | 80 | 0.033 | 0.10 | 0.14 |
| Dairy cattle | 650 | 25 | 30 | 60 | 0.02 | 0.12 | 0.14 |
| Ram/ewe | 75 | 2.5 | 30 | NA | 0.017 | NA | NA |
| Lamb | 40 | 1.7 | 30 | 30 | 0.022 | 0.069 | 0.091 |
| Breeding pigs | 260 | 6 | 70 | 20 | 0.027 | 0.025 | 0.052 |
| Finishing pigs | 100 | 3 | 70 | NA | 0.036 | NA | NA |
| Broiler | 1.7 | 0.12 | 70 | NA | 0.084 | NA | NA |
| Layer | 1.9 | 0.13 | 70 | 10 | 0.081 | 0.037 | 0.12 |
| Turkey | 7 | 0.50 | 50 | NA | 0.061 | NA | NA |
| **Cry34Ab1** | **BW (kg)** | **TDI feed (kg DM/animal)** | **IR (%) grains** | **IR (%) forage** | **Grain (G)** | **Forage (F)** | **G + F** |
| Beef cattle[(a)] | 500 | 12 | 80 | 80 | 0.36 | 1.50 | 1.9 |
| Dairy cattle | 650 | 25 | 30 | 60 | 0.22 | 1.80 | 2.0 |
| Ram/ewe | 75 | 2.5 | 30 | NA | 0.19 | NA | NA |
| Lamb | 40 | 1.7 | 30 | 30 | 0.24 | 0.99 | 1.2 |
| Breeding pigs | 260 | 6 | 70 | 20 | 0.31 | 0.36 | 0.67 |
| Finishing pigs | 100 | 3 | 70 | NA | 0.40 | NA | NA |
| Broiler | 1.7 | 0.12 | 70 | NA | 0.94 | NA | NA |
| Layer | 1.9 | 0.13 | 70 | 10 | 0.91 | 0.53 | 1.4 |
| Turkey | 7 | 0.50 | 50 | NA | 0.68 | NA | NA |
| **Cry35Ab1** | **BW (kg)** | **TDI feed (kg DM/animal)** | **IR (%) grains** | **IR (%) forage** | **Grain (G)** | **Forage (F)** | **G + F** |
| Beef cattle[(a)] | 500 | 12 | 80 | 80 | 0.015 | 0.40 | 0.42 |
| Dairy cattle | 650 | 25 | 30 | 60 | 0.009 | 0.48 | 0.49 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Ram/ewe | 75 | 2.5 | 30 | NA | 0.0078 | NA | NA |
| Lamb | 40 | 1.7 | 30 | 30 | 0.010 | 0.27 | 0.28 |
| Breeding pigs | 260 | 6 | 70 | 20 | 0.013 | 0.10 | 0.11 |
| Finishing pigs | 100 | 3 | 70 | NA | 0.016 | NA | NA |
| Broiler | 1.7 | 0.12 | 70 | NA | 0.039 | NA | NA |
| Layer | 1.9 | 0.13 | 70 | 10 | 0.037 | 0.14 | 0.18 |
| Turkey | 7 | 0.50 | 50 | NA | 0.028 | NA | NA |
| **PAT** | **BW (kg)** | **TDI feed (kg DM/animal)** | **IR (%) grains** | **IR (%) forage** | **Grain (G)** | **Forage (F)** | **G + F** |
| Beef cattle[(a)] | 500 | 12 | 80 | 80 | 0.00058 | 0.035 | 0.035 |
| Dairy cattle | 650 | 25 | 30 | 60 | 0.00035 | 0.042 | 0.042 |
| Ram/ewe | 75 | 2.5 | 30 | NA | 0.00030 | NA | NA |
| Lamb | 40 | 1.7 | 30 | 30 | 0.00038 | 0.023 | 0.023 |
| Breeding pigs | 260 | 6 | 70 | 20 | 0.00048 | 0.0083 | 0.0088 |
| Finishing pigs | 100 | 3 | 70 | NA | 0.00063 | NA | NA |
| Broiler | 1.7 | 0.12 | 70 | NA | 0.0015 | NA | NA |
| Layer | 1.9 | 0.13 | 70 | 10 | 0.0014 | 0.012 | 0.014 |
| Turkey | 7 | 0.50 | 50 | NA | 0.0011 | NA | NA |
| **Cry1A.105** | **BW (kg)** | **TDI feed (kg DM/animal)** | **IR (%) grains** | **IR (%) forage** | **Grain (G)** | **Forage (F)** | **G + F** |
| Beef cattle[(a)] | 500 | 12 | 80 | 80 | 0.035 | 0.18 | 0.22 |
| Dairy cattle | 650 | 25 | 30 | 60 | 0.021 | 0.22 | 0.24 |
| Ram/ewe | 75 | 2.5 | 30 | NA | 0.018 | NA | NA |
| Lamb | 40 | 1.7 | 30 | 30 | 0.023 | 0.12 | 0.14 |
| Breeding pigs | 260 | 6 | 70 | 20 | 0.029 | 0.043 | 0.072 |
| Finishing pigs | 100 | 3 | 70 | NA | 0.038 | NA | NA |
| Broiler | 1.7 | 0.12 | 70 | NA | 0.089 | NA | NA |
| Layer | 1.9 | 0.13 | 70 | 10 | 0.086 | 0.064 | 0.15 |
| Turkey | 7 | 0.50 | 50 | NA | 0.064 | NA | NA |

| Cry2Ab2 | BW (kg) | TDI feed (kg DM/animal) | IR (%) grains | IR (%) forage | Grain (G) | Forage (F) | G + F |
|---|---|---|---|---|---|---|---|
| Beef cattle[(a)] | 500 | 12 | 80 | 80 | 0.06 | 1.02 | 1.1 |
| Dairy cattle | 650 | 25 | 30 | 60 | 0.035 | 1.2 | 1.3 |
| Ram/ewe | 75 | 2.5 | 30 | NA | 0.030 | NA | NA |
| Lamb | 40 | 1.7 | 30 | 30 | 0.038 | 0.68 | 0.71 |
| Breeding pigs | 260 | 6 | 70 | 20 | 0.05 | 0.24 | 0.29 |
| Finishing pigs | 100 | 3 | 70 | NA | 0.06 | NA | NA |
| Broiler | 1.7 | 0.12 | 70 | NA | 0.15 | NA | NA |
| Layer | 1.9 | 0.13 | 70 | 10 | 0.14 | 0.36 | 0.51 |
| Turkey | 7 | 0.50 | 50 | NA | 0.11 | NA | NA |
| **Cry3Bb1** | **BW (kg)** | **TDI feed (kg DM/animal)** | **IR (%) grains** | **IR (%) forage** | **Grain (G)** | **Forage (F)** | **G + F** |
| Beef cattle[(a)] | 500 | 12 | 80 | 80 | 0.086 | 1.06 | 1.1 |
| Dairy cattle | 650 | 25 | 30 | 60 | 0.052 | 1.27 | 1.3 |
| Ram/ewe | 75 | 2.5 | 30 | NA | 0.045 | NA | NA |
| Lamb | 40 | 1.7 | 30 | 30 | 0.057 | 0.70 | 0.76 |
| Breeding pigs | 260 | 6 | 70 | 20 | 0.073 | 0.25 | 0.33 |
| Finishing pigs | 100 | 3 | 70 | NA | 0.095 | NA | NA |
| Broiler | 1.7 | 0.12 | 70 | NA | 0.22 | NA | NA |
| Layer | 1.9 | 0.13 | 70 | 10 | 0.22 | 0.38 | 0.59 |
| Turkey | 7 | 0.50 | 50 | NA | 0.16 | NA | NA |
| **CP4 EPSPS** | **BW (kg)** | **TDI feed (kg DM/animal)** | **IR (%) grains** | **IR (%) forage** | **Grain (G)** | **Forage (F)** | **G + F** |
| Beef cattle[(a)] | 500 | 12 | 80 | 80 | 0.056 | 0.31 | 0.36 |
| Dairy cattle | 650 | 25 | 30 | 60 | 0.033 | 0.37 | 0.40 |
| Ram/ewe | 75 | 2.5 | 30 | NA | 0.029 | NA | NA |
| Lamb | 40 | 1.7 | 30 | 30 | 0.037 | 0.20 | 0.24 |
| Breeding pigs | 260 | 6 | 70 | 20 | 0.047 | 0.07 | 0.12 |
| Finishing pigs | 100 | 3 | 70 | NA | 0.061 | NA | NA |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Broiler | 1.7 | 0.12 | 70 | NA | 0.14 | NA | NA |
| Layer | 1.9 | 0.13 | 70 | 10 | 0.14 | 0.11 | 0.25 |
| Turkey | 7 | 0.50 | 50 | NA | 0.10 | NA | NA |
| **AAD-1** | **BW (kg)** | **TDI feed (kg DM/animal)** | **IR (%) grains** | **IR (%) forage** | **Grain (G)** | **Forage (F)** | **G + F** |
| Beef cattle[(a)] | 500 | 12 | 80 | 80 | 0.096 | 0.21 | 0.31 |
| Dairy cattle | 650 | 25 | 30 | 60 | 0.058 | 0.25 | 0.31 |
| Ram/ewe | 75 | 2.5 | 30 | NA | 0.05 | NA | NA |
| Lamb | 40 | 1.7 | 30 | 30 | 0.064 | 0.14 | 0.20 |
| Breeding pigs | 260 | 6 | 70 | 20 | 0.081 | 0.05 | 0.13 |
| Finishing pigs | 100 | 3 | 70 | NA | 0.11 | NA | NA |
| Broiler | 1.7 | 0.12 | 70 | NA | 0.25 | NA | NA |
| Layer | 1.9 | 0.13 | 70 | 10 | 0.24 | 0.075 | 0.31 |
| Turkey | 7 | 0.50 | 50 | NA | 0.18 | NA | NA |

(a): The inclusion rate for beef cattle would be 160% of the diet, resulting in the DDE to each protein an overestimation. NA indicates that a forage inclusion rate was not provided in the reference and therefore no exposure calculations were done.

Table 46: Animal daily dietary exposure to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1A.105, Cry2Ab2, Cry3Bb1, CP4 EPSPS and AAD-1 proteins (mg/kg BW per day) performed with the Excel calculator (EFSA GMO Panel 2023)

| **Protein** | **Feed Material** | **Animal species** | **Inclusion rate** | **NEP level** | **Daily dietary exposure to NEP** |
|---|---|---|---|---|---|
| | **Maize** | **Category** | **IR%** | **µg^NEP/g^DM** | **mg^NEP/kg^BW** |
| **Cry1F** | **Grain** | Dairy cow 650/25 | 30 | 1.70 | 0.020 |
| | | Finishing pig 100/3 | 70 | 1.70 | 0.036 |
| | | Broiler 2/0.158 | 70 | 1.70 | 0.094 |
| | | Dairy Sheep 80/2.8 | 25 | 1.70 | 0.015 |
| | **Gluten feed** | Dairy cow 650/25 | 30 | 4.40 | 0.051 |
| | | Finishing pig 100/3 | 20 | 4.40 | 0.026 |

| | | | | | |
|---|---|---|---|---|---|
| | | Broiler 2/0.158 | 10 | 4.40 | 0.035 |
| | | Dairy Sheep 80/2.8 | 10 | 4.40 | 0.015 |
| | **Gluten meal** | Dairy cow 650/25 | 20 | 12.17 | 0.094 |
| | | Finishing pig 100/3 | 10 | 12.17 | 0.037 |
| | | Broiler 2/0.158 | 10 | 12.17 | 0.096 |
| | | Dairy Sheep 80/2.8 | 10 | 12.17 | 0.043 |
| | **Forage/silage** | Dairy cow 650/25 | 60 | 5.40 | 0.125 |
| | | Finishing pig 100/3 | NA | NA | NA |
| | | Broiler 2/0.158 | NA | NA | NA |
| | | Dairy Sheep 80/2.8 | 80 | 5.40 | 0.151 |
| | **Milled by-products** | Dairy cow 650/25 | 30 | 1.70* | 0.020 |
| | | Finishing pig 100/3 | 75 | 1.70* | 0.038 |
| | | Broiler 2/0.158 | 60 | 1.70* | 0.081 |
| | | Dairy Sheep 80/2.8 | NA | NA | NA |
| | **TOTAL** | Dairy cow 650/25 | 170 | NA | 0.308 |
| | | Finishing pig 100/3 | 175 | NA | 0.137 |
| | | Broiler 2/0.158 | 150 | NA | 0.306 |
| | | Dairy Sheep 80/2.8 | 125 | NA | 0.224 |
| **Cry34Ab1** | **Grain** | Dairy cow 650/25 | 30 | 19.00 | 0.219 |
| | | Finishing pig 100/3 | 70 | 19.00 | 0.399 |
| | | Broiler 2/0.158 | 70 | 19.00 | 1.051 |
| | | Dairy Sheep 80/2.8 | 25 | 19.00 | 0.166 |
| | **Gluten feed** | Dairy cow 650/25 | 30 | 49.21 | 0.568 |

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| | | Finishing pig 100/3 | 20 | 49.21 | 0.295 |
| | | Broiler 2/0.158 | 10 | 49.21 | 0.389 |
| | | Dairy Sheep 80/2.8 | 10 | 49.21 | 0.172 |
| | **Gluten meal** | Dairy cow 650/25 | 20 | 136.04 | 1.046 |
| | | Finishing pig 100/3 | 10 | 136.04 | 0.408 |
| | | Broiler 2/0.158 | 10 | 136.04 | 1.075 |
| | | Dairy Sheep 80/2.8 | 10 | 136.04 | 0.476 |
| | **Forage/silage** | Dairy cow 650/25 | 60 | 78.00 | 1.800 |
| | | Finishing pig 100/3 | NA | NA | NA |
| | | Broiler 2/0.158 | NA | NA | NA |
| | | Dairy Sheep 80/2.8 | 80 | 78.00 | 2.184 |
| | **Milled by-products** | Dairy cow 650/25 | 30 | 19.00* | 0.219 |
| | | Finishing pig 100/3 | 75 | 19.00* | 0.428 |
| | | Broiler 2/0.158 | 60 | 19.00* | 0.901 |
| | | Dairy Sheep 80/2.8 | NA | NA | NA |
| | **TOTAL** | Dairy cow 650/25 | 170 | NA | 3.853 |
| | | Finishing pig 100/3 | 175 | NA | 1.530 |
| | | Broiler 2/0.158 | 150 | NA | 3.415 |
| | | Dairy Sheep 80/2.8 | 125 | NA | 2.999 |
| **Cry35Ab1** | **Grain** | Dairy cow 650/25 | 30 | 0.78 | 0.009 |
| | | Finishing pig 100/3 | 70 | 0.78 | 0.016 |
| | | Broiler 2/0.158 | 70 | 0.78 | 0.043 |
| | | Dairy Sheep 80/2.8 | 25 | 0.78 | 0.007 |

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| | **Gluten feed** | Dairy cow 650/25 | 30 | 2.02 | 0.023 |
| | | Finishing pig 100/3 | 20 | 2.02 | 0.012 |
| | | Broiler 2/0.158 | 10 | 2.02 | 0.016 |
| | | Dairy Sheep 80/2.8 | 10 | 2.02 | 0.007 |
| | **Gluten meal** | Dairy cow 650/25 | 20 | 5.58 | 0.043 |
| | | Finishing pig 100/3 | 10 | 5.58 | 0.017 |
| | | Broiler 2/0.158 | 10 | 5.58 | 0.044 |
| | | Dairy Sheep 80/2.8 | 10 | 5.58 | 0.020 |
| | **Forage/silage** | Dairy cow 650/25 | 60 | 21.00 | 0.485 |
| | | Finishing pig 100/3 | NA | NA | NA |
| | | Broiler 2/0.158 | NA | NA | NA |
| | | Dairy Sheep 80/2.8 | 80 | 21.00 | 0.588 |
| | **Milled by-products** | Dairy cow 650/25 | 30 | 0.78* | 0.009 |
| | | Finishing pig 100/3 | 75 | 0.78* | 0.018 |
| | | Broiler 2/0.158 | 60 | 0.78* | 0.037 |
| | | Dairy Sheep 80/2.8 | NA | NA | NA |
| | **TOTAL** | Dairy cow 650/25 | 170 | NA | 0.569 |
| | | Finishing pig 100/3 | 175 | NA | 0.063 |
| | | Broiler 2/0.158 | 150 | NA | 0.140 |
| | | Dairy Sheep 80/2.8 | 125 | NA | 0.621 |
| **PAT** | **Grain** | Dairy cow 650/25 | 30 | 0.03 | 0.00035 |
| | | Finishing pig 100/3 | 70 | 0.03 | 0.00063 |
| | | Broiler 2/0.158 | 70 | 0.03 | 0.00166 |

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| | | Dairy Sheep 80/2.8 | 25 | 0.03 | 0.00026 |
| | **Gluten feed** | Dairy cow 650/25 | 30 | 0.08 | 0.00090 |
| | | Finishing pig 100/3 | 20 | 0.08 | 0.00047 |
| | | Broiler 2/0.158 | 10 | 0.08 | 0.00061 |
| | | Dairy Sheep 80/2.8 | 10 | 0.08 | 0.00027 |
| | **Gluten meal** | Dairy cow 650/25 | 20 | 0.21 | 0.00165 |
| | | Finishing pig 100/3 | 10 | 0.21 | 0.00064 |
| | | Broiler 2/0.158 | 10 | 0.21 | 0.00170 |
| | | Dairy Sheep 80/2.8 | 10 | 0.21 | 0.00075 |
| | **Forage/silage** | Dairy cow 650/25 | 60 | 1.80 | 0.04154 |
| | | Finishing pig 100/3 | NA | NA | NA |
| | | Broiler 2/0.158 | NA | NA | NA |
| | | Dairy Sheep 80/2.8 | 80 | 1.80 | 0.05040 |
| | **Milled by-products** | Dairy cow 650/25 | 30 | 0.03* | 0.00035 |
| | | Finishing pig 100/3 | 75 | 0.03* | 0.00068 |
| | | Broiler 2/0.158 | 60 | 0.03* | 0.00142 |
| | | Dairy Sheep 80/2.8 | NA | NA | NA |
| | **TOTAL** | Dairy cow 650/25 | 170 | NA | 0.04478 |
| | | Finishing pig 100/3 | 175 | NA | 0.00242 |
| | | Broiler 2/0.158 | 150 | NA | 0.00539 |
| | | Dairy Sheep 80/2.8 | 125 | NA | 0.05169 |
| **Cry1A.105** | **Grain** | Dairy cow 650/25 | 30 | 1.80 | 0.021 |
| | | Finishing pig 100/3 | 70 | 1.80 | 0.038 |

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| | | Broiler 2/0.158 | 70 | 1.80 | 0.100 |
| | | Dairy Sheep 80/2.8 | 25 | 1.80 | 0.016 |
| | **Gluten feed** | Dairy cow 650/25 | 30 | 4.66 | 0.054 |
| | | Finishing pig 100/3 | 20 | 4.66 | 0.028 |
| | | Broiler 2/0.158 | 10 | 4.66 | 0.037 |
| | | Dairy Sheep 80/2.8 | 10 | 4.66 | 0.016 |
| | **Gluten meal** | Dairy cow 650/25 | 20 | 12.89 | 0.099 |
| | | Finishing pig 100/3 | 10 | 12.89 | 0.039 |
| | | Broiler 2/0.158 | 10 | 12.89 | 0.102 |
| | | Dairy Sheep 80/2.8 | 10 | 12.89 | 0.045 |
| | **Forage/silage** | Dairy cow 650/25 | 60 | 9.40 | 0.217 |
| | | Finishing pig 100/3 | NA | NA | NA |
| | | Broiler 2/0.158 | NA | NA | NA |
| | | Dairy Sheep 80/2.8 | 80 | 9.40 | 0.263 |
| | **Milled by-products** | Dairy cow 650/25 | 30 | 1.80* | 0.021 |
| | | Finishing pig 100/3 | 75 | 1.80* | 0.041 |
| | | Broiler 2/0.158 | 60 | 1.80* | 0.085 |
| | | Dairy Sheep 80/2.8 | NA | NA | NA |
| | **TOTAL** | Dairy cow 650/25 | 170 | NA | 0.411 |
| | | Finishing pig 100/3 | 175 | NA | 0.145 |
| | | Broiler 2/0.158 | 150 | NA | 0.324 |
| | | Dairy Sheep 80/2.8 | 125 | NA | 0.340 |
| **Cry2Ab2** | **Grain** | Dairy cow 650/25 | 30 | 3.00 | 0.035 |

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|---|---|---|---|---|
| | Finishing pig 100/3 | 70 | 3.00 | 0.063 |
| | Broiler 2/0.158 | 70 | 3.00 | 0.166 |
| | Dairy Sheep 80/2.8 | 25 | 3.00 | 0.026 |
| **Gluten feed** | Dairy cow 650/25 | 30 | 7.77 | 0.090 |
| | Finishing pig 100/3 | 20 | 7.77 | 0.047 |
| | Broiler 2/0.158 | 10 | 7.77 | 0.061 |
| | Dairy Sheep 80/2.8 | 10 | 7.77 | 0.027 |
| **Gluten meal** | Dairy cow 650/25 | 20 | 21.48 | 0.165 |
| | Finishing pig 100/3 | 10 | 21.48 | 0.064 |
| | Broiler 2/0.158 | 10 | 21.48 | 0.170 |
| | Dairy Sheep 80/2.8 | 10 | 21.48 | 0.075 |
| **Forage/silage** | Dairy cow 650/25 | 60 | 53.00 | 1.223 |
| | Finishing pig 100/3 | NA | NA | NA |
| | Broiler 2/0.158 | NA | NA | NA |
| | Dairy Sheep 80/2.8 | 80 | 53.00 | 1.484 |
| **Milled by-products** | Dairy cow 650/25 | 30 | 3.00* | 0.035 |
| | Finishing pig 100/3 | 75 | 3.00* | 0.068 |
| | Broiler 2/0.158 | 60 | 3.00* | 0.142 |
| | Dairy Sheep 80/2.8 | NA | NA | NA |
| **TOTAL** | Dairy cow 650/25 | 170 | NA | 1.547 |
| | Finishing pig 100/3 | 175 | NA | 0.242 |
| | Broiler 2/0.158 | 150 | NA | 0.539 |
| | Dairy Sheep 80/2.8 | 125 | NA | 1.613 |

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|---|---|---|---|---|---|
| Cry3Bb1 | Grain | Dairy cow 650/25 | 30 | 4.50 | 0.052 |
| | | Finishing pig 100/3 | 70 | 4.50 | 0.095 |
| | | Broiler 2/0.158 | 70 | 4.50 | 0.249 |
| | | Dairy Sheep 80/2.8 | 25 | 4.50 | 0.039 |
| | Gluten feed | Dairy cow 650/25 | 30 | 11.66 | 0.134 |
| | | Finishing pig 100/3 | 20 | 11.66 | 0.070 |
| | | Broiler 2/0.158 | 10 | 11.66 | 0.092 |
| | | Dairy Sheep 80/2.8 | 10 | 11.66 | 0.041 |
| | Gluten meal | Dairy cow 650/25 | 20 | 32.22 | 0.248 |
| | | Finishing pig 100/3 | 10 | 32.22 | 0.097 |
| | | Broiler 2/0.158 | 10 | 32.22 | 0.255 |
| | | Dairy Sheep 80/2.8 | 10 | 32.22 | 0.113 |
| | Forage/silage | Dairy cow 650/25 | 60 | 55.00 | 1.269 |
| | | Finishing pig 100/3 | NA | NA | NA |
| | | Broiler 2/0.158 | NA | NA | NA |
| | | Dairy Sheep 80/2.8 | 80 | 55.00 | 1.540 |
| | Milled by-products | Dairy cow 650/25 | 30 | 4.50* | 0.052 |
| | | Finishing pig 100/3 | 75 | 4.50* | 0.101 |
| | | Broiler 2/0.158 | 60 | 4.50* | 0.213 |
| | | Dairy Sheep 80/2.8 | NA | NA | NA |
| | TOTAL | Dairy cow 650/25 | 170 | NA | 1.755 |
| | | Finishing pig 100/3 | 175 | NA | 0.362 |
| | | Broiler 2/0.158 | 150 | NA | 0.809 |

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|---|---|---|---|---|---|
| | | Dairy Sheep 80/2.8 | 125 | NA | 1.733 |
| CP4 EPSPS | **Grain** | Dairy cow 650/25 | 30 | 2.90 | 0.033 |
| | | Finishing pig 100/3 | 70 | 2.90 | 0.061 |
| | | Broiler 2/0.158 | 70 | 2.90 | 0.160 |
| | | Dairy Sheep 80/2.8 | 25 | 2.90 | 0.025 |
| | **Gluten feed** | Dairy cow 650/25 | 30 | 7.51 | 0.087 |
| | | Finishing pig 100/3 | 20 | 7.51 | 0.045 |
| | | Broiler 2/0.158 | 10 | 7.51 | 0.059 |
| | | Dairy Sheep 80/2.8 | 10 | 7.51 | 0.026 |
| | **Gluten meal** | Dairy cow 650/25 | 20 | 20.76 | 0.160 |
| | | Finishing pig 100/3 | 10 | 20.76 | 0.062 |
| | | Broiler 2/0.158 | 10 | 20.76 | 0.164 |
| | | Dairy Sheep 80/2.8 | 10 | 20.76 | 0.073 |
| | **Forage/silage** | Dairy cow 650/25 | 60 | 16.00 | 0.369 |
| | | Finishing pig 100/3 | NA | NA | NA |
| | | Broiler 2/0.158 | NA | NA | NA |
| | | Dairy Sheep 80/2.8 | 80 | 16.00 | 0.448 |
| | **Milled by-products** | Dairy cow 650/25 | 30 | 2.90* | 0.033 |
| | | Finishing pig 100/3 | 75 | 2.90* | 0.065 |
| | | Broiler 2/0.158 | 60 | 2.90* | 0.137 |
| | | Dairy Sheep 80/2.8 | NA | NA | NA |
| | **TOTAL** | Dairy cow 650/25 | 170 | NA | 0.683 |
| | | Finishing pig 100/3 | 175 | NA | 0.234 |

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|---|---|---|---|---|---|
| | | Broiler 2/0.158 | 150 | NA | 0.521 |
| | | Dairy Sheep 80/2.8 | 125 | NA | 0.572 |
| **AAD-1** | **Grain** | Dairy cow 650/25 | 30 | 5.00 | 0.058 |
| | | Finishing pig 100/3 | 70 | 5.00 | 0.105 |
| | | Broiler 2/0.158 | 70 | 5.00 | 0.277 |
| | | Dairy Sheep 80/2.8 | 25 | 5.00 | 0.044 |
| | **Gluten feed** | Dairy cow 650/25 | 30 | 12.95 | 0.149 |
| | | Finishing pig 100/3 | 20 | 12.95 | 0.078 |
| | | Broiler 2/0.158 | 10 | 12.95 | 0.102 |
| | | Dairy Sheep 80/2.8 | 10 | 12.95 | 0.045 |
| | **Gluten meal** | Dairy cow 650/25 | 20 | 35.80 | 0.275 |
| | | Finishing pig 100/3 | 10 | 35.80 | 0.107 |
| | | Broiler 2/0.158 | 10 | 35.80 | 0.283 |
| | | Dairy Sheep 80/2.8 | 10 | 35.80 | 0.125 |
| | **Forage/silage** | Dairy cow 650/25 | 60 | 11.00 | 0.254 |
| | | Finishing pig 100/3 | NA | NA | NA |
| | | Broiler 2/0.158 | NA | NA | NA |
| | | Dairy Sheep 80/2.8 | 80 | 11.00 | 0.308 |
| | **Milled by-products** | Dairy cow 650/25 | 30 | 5.00* | 0.058 |
| | | Finishing pig 100/3 | 75 | 5.00* | 0.113 |
| | | Broiler 2/0.158 | 60 | 5.00* | 0.237 |
| | | Dairy Sheep 80/2.8 | NA | NA | NA |
| | **TOTAL** | Dairy cow 650/25 | 170 | NA | 0.794 |

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|---|---|---|---|---|---|
| | | Finishing pig 100/3 | 175 | NA | 0.403 |
| | | Broiler 2/0.158 | 150 | NA | 0.899 |
| | | Dairy Sheep 80/2.8 | 125 | NA | 0.522 |

NA indicates that an inclusion rate or NEP levels were not provided and therefore no exposures were calculated.
The total inclusion rate for dairy cow, finishing pig, broiler and dairy sheep is >100%, resulting in an overestimation in the DDE to each protein.
*The GMO panel did not recommend specific data for a conversion factor so the value of 1 was selected.

Table 47: Animal daily dietary exposure to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1A.105, Cry2Ab2, Cry3Bb1, CP4 EPSPS and AAD-1 proteins (mg/kg BW per day) performed with the model database input data

| **Protein** | **Feed Material** | **Animal species** | **Inclusion rate** | **NEP level** | **Daily dietary exposure to NEP** |
|---|---|---|---|---|---|
| | | **Category** | **IR%** | **$\mu g^{NEP}/g^{DM}$** | **$mg^{NEP}/kg^{BW}$** |
| **Cry1F** | **Maize** | Dairy cow 623/25.82 | 18.09 | 1.70 | 0.013 |
| | | Finishing pig 121.6/2.875 | 75.51 | 1.70 | 0.030 |
| | | Broiler 1.707/0.1343 | 30 | 1.70 | 0.040 |
| | | Dairy sheep 57/1.22 | 41 | 1.70 | 0.015 |
| | **Maize gluten feed** | Dairy cow 623/25.82 | 18.81 | 4.40 | 0.034 |
| | | Finishing pig 121.6/2.875 | 5 | 4.40 | 0.005 |
| | | Dairy sheep 57/1.22 | 10 | 4.40 | 0.009 |
| | **Maize gluten** | Broiler 1.707/0.1343 | 6 | 12.17 | 0.057 |
| | | Dairy sheep 57/1.22 | 10 | 12.17 | 0.026 |
| | **Maize silage** | Dairy cow 623/25.82 | 21.23 | 5.40 | 0.048 |
| | **TOTAL** | Dairy cow 623/25.82 | 58.13 | NA | 0.095 |
| | | Finishing pig 121.6/2.875 | 80.51 | NA | 0.036 |
| | | Broiler 1.707/0.1343 | 36 | NA | 0.098 |
| | | Dairy sheep 57/1.22 | 61 | NA | 0.050 |

| | | | | | |
|---|---|---|---|---|---|
| Cry34Ab1 | Maize | Dairy cow 623/25.82 | 18.09 | 19.00 | 0.142 |
| | | Finishing pig 121.6/2.875 | 75.51 | 19.00 | 0.339 |
| | | Broiler 1.707/0.1343 | 30 | 19.00 | 0.448 |
| | | Dairy sheep 57/1.22 | 41 | 19.00 | 0.167 |
| | Maize gluten feed | Dairy cow 623/25.82 | 18.81 | 49.21 | 0.384 |
| | | Finishing pig 121.6/2.875 | 5 | 49.21 | 0.058 |
| | | Dairy sheep 57/1.22 | 10 | 49.21 | 0.105 |
| | Maize gluten | Broiler 1.707/0.1343 | 6 | 136.04 | 0.642 |
| | | Dairy sheep 57/1.22 | 10 | 136.04 | 0.291 |
| | Maize silage | Dairy cow 623/25.82 | 21.23 | 78.00 | 0.686 |
| | TOTAL | Dairy cow 623/25.82 | 58.13 | NA | 1.212 |
| | | Finishing pig 121.6/2.875 | 80.51 | NA | 0.397 |
| | | Broiler 1.707/0.1343 | 36 | NA | 1.091 |
| | | Dairy sheep 57/1.22 | 61 | NA | 0.563 |
| Cry35Ab1 | Maize | Dairy cow 623/25.82 | 18.09 | 0.78 | 0.006 |
| | | Finishing pig 121.6/2.875 | 75.51 | 0.78 | 0.014 |
| | | Broiler 1.707/0.1343 | 30 | 0.78 | 0.018 |
| | | Dairy sheep 57/1.22 | 41 | 0.78 | 0.007 |
| | Maize gluten feed | Dairy cow 623/25.82 | 18.81 | 2.02 | 0.016 |
| | | Finishing pig 121.6/2.875 | 5 | 2.02 | 0.002 |
| | | Dairy sheep 57/1.22 | 10 | 2.02 | 0.004 |
| | Maize gluten | Broiler 1.707/0.1343 | 6 | 5.58 | 0.026 |
| | | Dairy sheep 57/1.22 | 10 | 5.58 | 0.012 |

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| | **Maize silage** | Dairy cow 623/25.82 | 21.23 | 21.00 | 0.185 |
| | **TOTAL** | Dairy cow 623/25.82 | 58.13 | NA | 0.206 |
| | | Finishing pig 121.6/2.875 | 80.51 | NA | 0.016 |
| | | Broiler 1.707/0.1343 | 36 | NA | 0.045 |
| | | Dairy sheep 57/1.22 | 61 | NA | 0.023 |
| **PAT** | **Maize** | Dairy cow 623/25.82 | 18.09 | 0.03 | 0.00022 |
| | | Finishing pig 121.6/2.875 | 75.51 | 0.03 | 0.00054 |
| | | Broiler 1.707/0.1343 | 30 | 0.03 | 0.00071 |
| | | Dairy sheep 57/1.22 | 41 | 0.03 | 0.00026 |
| | **Maize gluten feed** | Dairy cow 623/25.82 | 18.81 | 0.08 | 0.00061 |
| | | Finishing pig 121.6/2.875 | 5 | 0.08 | 0.00009 |
| | | Dairy sheep 57/1.22 | 10 | 0.08 | 0.00017 |
| | **Maize gluten** | Broiler 1.707/0.1343 | 6 | 0.21 | 0.00101 |
| | | Dairy sheep 57/1.22 | 10 | 0.21 | 0.00046 |
| | **Maize silage** | Dairy cow 623/25.82 | 21.23 | 1.80 | 0.01584 |
| | **TOTAL** | Dairy cow 623/25.82 | 58.13 | NA | 0.01667 |
| | | Finishing pig 121.6/2.875 | 80.51 | NA | 0.00063 |
| | | Broiler 1.707/0.1343 | 36 | NA | 0.00172 |
| | | Dairy sheep 57/1.22 | 61 | NA | 0.00089 |
| **Cry1A.105** | **Maize** | Dairy cow 623/25.82 | 18.09 | 1.80 | 0.013 |
| | | Finishing pig 121.6/2.875 | 75.51 | 1.80 | 0.032 |
| | | Broiler 1.707/0.1343 | 30 | 1.80 | 0.042 |
| | | Dairy sheep 57/1.22 | 41 | 1.80 | 0.016 |

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|---|---|---|---|---|---|
| | Maize gluten feed | Dairy cow 623/25.82 | 18.81 | 4.66 | 0.036 |
| | | Finishing pig 121.6/2.875 | 5 | 4.66 | 0.006 |
| | | Dairy sheep 57/1.22 | 10 | 4.66 | 0.010 |
| | Maize gluten | Broiler 1.707/0.1343 | 6 | 12.89 | 0.061 |
| | | Dairy sheep 57/1.22 | 10 | 12.89 | 0.028 |
| | Maize silage | Dairy cow 623/25.82 | 21.23 | 9.40 | 0.083 |
| | TOTAL | Dairy cow 623/25.82 | 58.13 | NA | 0.133 |
| | | Finishing pig 121.6/2.875 | 80.51 | NA | 0.038 |
| | | Broiler 1.707/0.1343 | 36 | NA | 0.103 |
| | | Dairy sheep 57/1.22 | 61 | NA | 0.053 |
| **Cry2Ab2** | Maize | Dairy cow 623/25.82 | 18.09 | 3.00 | 0.022 |
| | | Finishing pig 121.6/2.875 | 75.51 | 3.00 | 0.054 |
| | | Broiler 1.707/0.1343 | 30 | 3.00 | 0.071 |
| | | Dairy sheep 57/1.22 | 41 | 3.00 | 0.026 |
| | Maize gluten feed | Dairy cow 623/25.82 | 18.81 | 7.77 | 0.061 |
| | | Finishing pig 121.6/2.875 | 5 | 7.77 | 0.009 |
| | | Dairy sheep 57/1.22 | 10 | 7.77 | 0.017 |
| | Maize gluten | Broiler 1.707/0.1343 | 6 | 21.48 | 0.101 |
| | | Dairy sheep 57/1.22 | 10 | 21.48 | 0.046 |
| | Maize silage | Dairy cow 623/25.82 | 21.23 | 53.00 | 0.466 |
| | TOTAL | Dairy cow 623/25.82 | 58.13 | NA | 0.549 |
| | | Finishing pig 121.6/2.875 | 80.51 | NA | 0.063 |
| | | Broiler 1.707/0.1343 | 36 | NA | 0.172 |

| | | | | | |
|---|---|---|---|---|---|
| | | Dairy sheep 57/1.22 | 61 | NA | 0.089 |
| **Cry3Bb1** | **Maize** | Dairy cow 623/25.82 | 18.09 | 4.50 | 0.034 |
| | | Finishing pig 121.6/2.875 | 75.51 | 4.50 | 0.080 |
| | | Broiler 1.707/0.1343 | 30 | 4.50 | 0.106 |
| | | Dairy sheep 57/1.22 | 41 | 4.50 | 0.039 |
| | **Maize gluten feed** | Dairy cow 623/25.82 | 18.81 | 11.66 | 0.091 |
| | | Finishing pig 121.6/2.875 | 5 | 11.66 | 0.014 |
| | | Dairy sheep 57/1.22 | 10 | 11.66 | 0.025 |
| | **Maize gluten** | Broiler 1.707/0.1343 | 6 | 32.22 | 0.152 |
| | | Dairy sheep 57/1.22 | 10 | 32.22 | 0.069 |
| | **Maize silage** | Dairy cow 623/25.82 | 21.23 | 55.00 | 0.484 |
| | **TOTAL** | Dairy cow 623/25.82 | 58.13 | NA | 0.609 |
| | | Finishing pig 121.6/2.875 | 80.51 | NA | 0.094 |
| | | Broiler 1.707/0.1343 | 36 | NA | 0.258 |
| | | Dairy sheep 57/1.22 | 61 | NA | 0.133 |
| **CP4 EPSPS** | **Maize** | Dairy cow 623/25.82 | 18.09 | 2.90 | 0.022 |
| | | Finishing pig 121.6/2.875 | 75.51 | 2.90 | 0.052 |
| | | Broiler 1.707/0.1343 | 30 | 2.90 | 0.068 |
| | | Dairy sheep 57/1.22 | 41 | 2.90 | 0.025 |
| | **Maize gluten feed** | Dairy cow 623/25.82 | 18.81 | 7.51 | 0.059 |
| | | Finishing pig 121.6/2.875 | 5 | 7.51 | 0.009 |
| | | Dairy sheep 57/1.22 | 10 | 7.51 | 0.016 |
| | **Maize gluten** | Broiler 1.707/0.1343 | 6 | 20.76 | 0.098 |

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|---|---|---|---|---|---|
| | | Dairy sheep 57/1.22 | 10 | 20.76 | 0.044 |
| | **Maize silage** | Dairy cow 623/25.82 | 21.23 | 16.00 | 0.141 |
| | **TOTAL** | Dairy cow 623/25.82 | 58.13 | NA | 0.221 |
| | | Finishing pig 121.6/2.875 | 80.51 | NA | 0.061 |
| | | Broiler 1.707/0.1343 | 36 | NA | 0.166 |
| | | Dairy sheep 57/1.22 | 61 | NA | 0.086 |
| **AAD-1** | **Maize** | Dairy cow 623/25.82 | 18.09 | 5.00 | 0.037 |
| | | Finishing pig 121.6/2.875 | 75.51 | 5.00 | 0.089 |
| | | Broiler 1.707/0.1343 | 30 | 5.00 | 0.118 |
| | | Dairy sheep 57/1.22 | 41 | 5.00 | 0.044 |
| | **Maize gluten feed** | Dairy cow 623/25.82 | 18.81 | 12.95 | 0.101 |
| | | Finishing pig 121.6/2.875 | 5 | 12.95 | 0.015 |
| | | Dairy sheep 57/1.22 | 10 | 12.95 | 0.028 |
| | **Maize gluten** | Broiler 1.707/0.1343 | 6 | 35.80 | 0.169 |
| | | Dairy sheep 57/1.22 | 10 | 35.80 | 0.077 |
| | **Maize silage** | Dairy cow 623/25.82 | 21.23 | 11.00 | 0.097 |
| | **TOTAL** | Dairy cow 623/25.82 | 58.13 | NA | 0.235 |
| | | Finishing pig 121.6/2.875 | 80.51 | NA | 0.105 |
| | | Broiler 1.707/0.1343 | 36 | NA | 0.287 |
| | | Dairy sheep 57/1.22 | 61 | NA | 0.148 |

NA indicates that an inclusion rate or NEP levels were not provided and therefore no exposures were calculated.

Table 48: Summary of DDE results to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1A.105, Cry2Ab2, Cry3Bb1, CP4 EPSPS and AAD-1 proteins (mg/kg BW per day) reported in the EFSA scientific opinion (EFSA GMO

Panel 2022b), results obtained using the Excel calculator (EFSA GMO Panel 2023) and results obtained using input data from the model database

| Protein | Feed Material | Animal species | DDE scientific opinion | DDE Excel calculator | DDE model database |
|---|---|---|---|---|---|
| | | Category | mg^NEP/kg^BW | mg^NEP/kg^BW | mg^NEP/kg^BW |
| Cry1F | Maize | Dairy cow | 0.020* | 0.020 | 0.013 |
| | | Finishing pig | 0.036 | 0.036 | 0.030 |
| | | Broiler | 0.084 | 0.094 | 0.040 |
| | | Dairy Sheep | 0.017 | 0.015 | 0.015 |
| | Maize gluten feed | Dairy cow | NA | 0.051 | 0.034 |
| | | Finishing pig | NA | 0.026 | 0.005 |
| | | Broiler | NA | 0.035 | NA |
| | | Dairy Sheep | NA | 0.015 | 0.009 |
| | Maize gluten | Dairy cow | NA | 0.094 | NA |
| | | Finishing pig | NA | 0.037 | NA |
| | | Broiler | NA | 0.096 | 0.057 |
| | | Dairy Sheep | NA | 0.043 | 0.026 |
| | Maize silage | Dairy cow | 0.120* | 0.125 | 0.048 |
| | | Finishing pig | NA | NA | NA |
| | | Broiler | NA | NA | NA |
| | | Dairy Sheep | NA | 0.151 | NA |
| | Maize milled by-products | Dairy cow | NA | 0.020 | NA |
| | | Finishing pig | NA | 0.038 | NA |
| | | Broiler | NA | 0.081 | NA |
| | | Dairy Sheep | NA | NA | NA |
| | TOTAL | Dairy cow | 0.140* | 0.308 | 0.095 |
| | | Finishing pig | 0.036 | 0.137 | 0.036 |
| | | Broiler | 0.084 | 0.306 | 0.098 |
| | | Dairy Sheep | 0.017 | 0.224 | 0.050 |
| Cry34Ab1 | Maize | Dairy cow | 0.220* | 0.219 | 0.142 |
| | | Finishing pig | 0.400* | 0.399 | 0.339 |

| | | | | | |
|---|---|---|---|---|---|
| | | Broiler | 0.940* | 1.051 | 0.448 |
| | | Dairy Sheep | 0.190* | 0.166 | 0.167 |
| | **Maize gluten feed** | Dairy cow | NA | 0.568 | 0.384 |
| | | Finishing pig | NA | 0.295 | 0.058 |
| | | Broiler | NA | 0.389 | NA |
| | | Dairy Sheep | NA | 0.172 | 0.105 |
| | **Maize gluten** | Dairy cow | NA | 1.046 | NA |
| | | Finishing pig | NA | 0.408 | NA |
| | | Broiler | NA | 1.075 | 0.642 |
| | | Dairy Sheep | NA | 0.476 | 0.291 |
| | **Maize silage** | Dairy cow | 1.800* | 1.800 | 0.686 |
| | | Finishing pig | NA | NA | NA |
| | | Broiler | NA | NA | NA |
| | | Dairy Sheep | NA | 2.184 | NA |
| | **Maize milled by-products** | Dairy cow | NA | 0.219 | NA |
| | | Finishing pig | NA | 0.428 | NA |
| | | Broiler | NA | 0.901 | NA |
| | | Dairy Sheep | NA | NA | NA |
| | **TOTAL** | Dairy cow | 2.000* | 3.853 | 1.212 |
| | | Finishing pig | 0.400* | 1.530 | 0.397 |
| | | Broiler | 0.940* | 3.415 | 1.091 |
| | | Dairy Sheep | 0.190* | 2.999 | 0.563 |
| **Cry35Ab1** | **Maize** | Dairy cow | 0.009 | 0.009 | 0.006 |
| | | Finishing pig | 0.016 | 0.016 | 0.014 |
| | | Broiler | 0.039 | 0.043 | 0.018 |
| | | Dairy Sheep | 0.008* | 0.007 | 0.007 |
| | **Maize gluten feed** | Dairy cow | NA | 0.023 | 0.016 |
| | | Finishing pig | NA | 0.012 | 0.002 |
| | | Broiler | NA | 0.016 | NA |
| | | Dairy Sheep | NA | 0.007 | 0.004 |

| | | | | | |
|---|---|---|---|---|---|
| | Maize gluten | Dairy cow | NA | 0.043 | NA |
| | | Finishing pig | NA | 0.017 | NA |
| | | Broiler | NA | 0.044 | 0.026 |
| | | Dairy Sheep | NA | 0.020 | 0.012 |
| | Maize silage | Dairy cow | 0.480* | 0.485 | 0.185 |
| | | Finishing pig | NA | NA | NA |
| | | Broiler | NA | NA | NA |
| | | Dairy Sheep | NA | 0.588 | NA |
| | Maize milled by-products | Dairy cow | NA | 0.009 | NA |
| | | Finishing pig | NA | 0.018 | NA |
| | | Broiler | NA | 0.037 | NA |
| | | Dairy Sheep | NA | NA | NA |
| | TOTAL | Dairy cow | 0.490* | 0.569 | 0.206 |
| | | Finishing pig | 0.016* | 0.063 | 0.016 |
| | | Broiler | 0.039* | 0.140 | 0.045 |
| | | Dairy Sheep | 0.009* | 0.621 | 0.023 |
| PAT | Maize | Dairy cow | 0.00035 | 0.00035 | 0.00022 |
| | | Finishing pig | 0.00063 | 0.00063 | 0.00054 |
| | | Broiler | 0.00150* | 0.00166 | 0.00071 |
| | | Dairy Sheep | 0.00030 | 0.00026 | 0.00026 |
| | Maize gluten feed | Dairy cow | NA | 0.00090 | 0.00061 |
| | | Finishing pig | NA | 0.00047 | 0.00009 |
| | | Broiler | NA | 0.00061 | NA |
| | | Dairy Sheep | NA | 0.00027 | 0.00017 |
| | Maize gluten | Dairy cow | NA | 0.00165 | NA |
| | | Finishing pig | NA | 0.00064 | NA |
| | | Broiler | NA | 0.00170 | 0.00101 |
| | | Dairy Sheep | NA | 0.00075 | 0.00046 |
| | Maize silage | Dairy cow | 0.04200* | 0.04154 | 0.01584 |
| | | Finishing pig | NA | NA | NA |

| | | | | | |
|---|---|---|---|---|---|
| | | Broiler | NA | NA | NA |
| | | Dairy Sheep | NA | 0.05040 | NA |
| | **Maize milled by-products** | Dairy cow | NA | 0.00035 | NA |
| | | Finishing pig | NA | 0.00068 | NA |
| | | Broiler | NA | 0.00142 | NA |
| | | Dairy Sheep | NA | NA | NA |
| | **TOTAL** | Dairy cow | 0.04200* | 0.04478 | 0.01667 |
| | | Finishing pig | 0.00063* | 0.00242 | 0.00063 |
| | | Broiler | 0.00150* | 0.00539 | 0.00172 |
| | | Dairy Sheep | 0.00030* | 0.05169 | 0.00089 |
| **Cry1A.105** | **Maize** | Dairy cow | 0.021 | 0.021 | 0.013 |
| | | Finishing pig | 0.038 | 0.038 | 0.032 |
| | | Broiler | 0.089 | 0.100 | 0.042 |
| | | Dairy Sheep | 0.018 | 0.016 | 0.016 |
| | **Maize gluten feed** | Dairy cow | NA | 0.054 | 0.036 |
| | | Finishing pig | NA | 0.028 | 0.006 |
| | | Broiler | NA | 0.037 | NA |
| | | Dairy Sheep | NA | 0.016 | 0.010 |
| | **Maize gluten** | Dairy cow | NA | 0.099 | NA |
| | | Finishing pig | NA | 0.039 | NA |
| | | Broiler | NA | 0.102 | 0.061 |
| | | Dairy Sheep | NA | 0.045 | 0.028 |
| | **Maize silage** | Dairy cow | 0.220* | 0.217 | 0.083 |
| | | Finishing pig | NA | NA | NA |
| | | Broiler | NA | NA | NA |
| | | Dairy Sheep | NA | 0.263 | NA |
| | **Maize milled by-products** | Dairy cow | NA | 0.021 | NA |
| | | Finishing pig | NA | 0.041 | NA |
| | | Broiler | NA | 0.085 | NA |
| | | Dairy Sheep | NA | NA | NA |

| | | | | | |
|---|---|---|---|---|---|
| | **TOTAL** | Dairy cow | 0.240* | 0.411 | 0.133 |
| | | Finishing pig | 0.038* | 0.145 | 0.038 |
| | | Broiler | 0.089* | 0.324 | 0.103 |
| | | Dairy Sheep | 0.018* | 0.340 | 0.053 |
| **Cry2Ab2** | **Maize** | Dairy cow | 0.035 | 0.035 | 0.022 |
| | | Finishing pig | 0.060* | 0.063 | 0.054 |
| | | Broiler | 0.150* | 0.166 | 0.071 |
| | | Dairy Sheep | 0.030 | 0.026 | 0.026 |
| | **Maize gluten feed** | Dairy cow | NA | 0.090 | 0.061 |
| | | Finishing pig | NA | 0.047 | 0.009 |
| | | Broiler | NA | 0.061 | NA |
| | | Dairy Sheep | NA | 0.027 | 0.017 |
| | **Maize gluten** | Dairy cow | NA | 0.165 | NA |
| | | Finishing pig | NA | 0.064 | NA |
| | | Broiler | NA | 0.170 | 0.101 |
| | | Dairy Sheep | NA | 0.075 | 0.046 |
| | **Maize silage** | Dairy cow | 1.200* | 1.223 | 0.466 |
| | | Finishing pig | NA | NA | NA |
| | | Broiler | NA | NA | NA |
| | | Dairy Sheep | NA | 1.484 | NA |
| | **Maize milled by-products** | Dairy cow | NA | 0.035 | NA |
| | | Finishing pig | NA | 0.068 | NA |
| | | Broiler | NA | 0.142 | NA |
| | | Dairy Sheep | NA | NA | NA |
| | **TOTAL** | Dairy cow | 1.235* | 1.547 | 0.549 |
| | | Finishing pig | 0.060* | 0.242 | 0.063 |
| | | Broiler | 0.150* | 0.539 | 0.172 |
| | | Dairy Sheep | 0.030 | 1.613 | 0.089 |
| **Cry3Bb1** | **Maize** | Dairy cow | 0.052 | 0.052 | 0.034 |
| | | Finishing pig | 0.095 | 0.095 | 0.080 |

| | | | | | |
|---|---|---|---|---|---|
| | | Broiler | 0.220* | 0.249 | 0.106 |
| | | Dairy Sheep | 0.045 | 0.039 | 0.039 |
| | Maize gluten feed | Dairy cow | NA | 0.134 | 0.091 |
| | | Finishing pig | NA | 0.070 | 0.014 |
| | | Broiler | NA | 0.092 | NA |
| | | Dairy Sheep | NA | 0.041 | 0.025 |
| | Maize gluten | Dairy cow | NA | 0.248 | NA |
| | | Finishing pig | NA | 0.097 | NA |
| | | Broiler | NA | 0.255 | 0.152 |
| | | Dairy Sheep | NA | 0.113 | 0.069 |
| | Maize silage | Dairy cow | 1.270* | 1.269 | 0.484 |
| | | Finishing pig | NA | NA | NA |
| | | Broiler | NA | NA | NA |
| | | Dairy Sheep | NA | 1.540 | NA |
| | Maize milled by-products | Dairy cow | NA | 0.052 | NA |
| | | Finishing pig | NA | 0.101 | NA |
| | | Broiler | NA | 0.213 | NA |
| | | Dairy Sheep | NA | NA | NA |
| | TOTAL | Dairy cow | 1.300* | 1.755 | 0.609 |
| | | Finishing pig | 0.095 | 0.362 | 0.094 |
| | | Broiler | 0.220* | 0.809 | 0.258 |
| | | Dairy Sheep | 0.045 | 1.733 | 0.133 |
| CP4 EPSPS | Maize | Dairy cow | 0.033 | 0.033 | 0.022 |
| | | Finishing pig | 0.061 | 0.061 | 0.052 |
| | | Broiler | 0.140* | 0.160 | 0.068 |
| | | Dairy Sheep | 0.029 | 0.025 | 0.025 |
| | Maize gluten feed | Dairy cow | NA | 0.087 | 0.059 |
| | | Finishing pig | NA | 0.045 | 0.009 |
| | | Broiler | NA | 0.059 | NA |
| | | Dairy Sheep | NA | 0.026 | 0.016 |

| | | | | | |
|---|---|---|---|---|---|
| | Maize gluten | Dairy cow | NA | 0.160 | NA |
| | | Finishing pig | NA | 0.062 | NA |
| | | Broiler | NA | 0.164 | 0.098 |
| | | Dairy Sheep | NA | 0.073 | 0.044 |
| | Maize silage | Dairy cow | 0.370* | 0.369 | 0.141 |
| | | Finishing pig | NA | NA | NA |
| | | Broiler | NA | NA | NA |
| | | Dairy Sheep | NA | 0.448 | NA |
| | Maize milled by-products | Dairy cow | NA | 0.033 | NA |
| | | Finishing pig | NA | 0.065 | NA |
| | | Broiler | NA | 0.137 | NA |
| | | Dairy Sheep | NA | NA | NA |
| | TOTAL | Dairy cow | 0.400* | 0.683 | 0.221 |
| | | Finishing pig | 0.061 | 0.234 | 0.061 |
| | | Broiler | 0.140* | 0.521 | 0.166 |
| | | Dairy Sheep | 0.029 | 0.572 | 0.086 |
| AAD-1 | Maize | Dairy cow | 0.058 | 0.058 | 0.037 |
| | | Finishing pig | 0.110* | 0.105 | 0.089 |
| | | Broiler | 0.250* | 0.277 | 0.118 |
| | | Dairy Sheep | 0.050* | 0.044 | 0.044 |
| | Maize gluten feed | Dairy cow | NA | 0.149 | 0.101 |
| | | Finishing pig | NA | 0.078 | 0.015 |
| | | Broiler | NA | 0.102 | NA |
| | | Dairy Sheep | NA | 0.045 | 0.028 |
| | Maize gluten | Dairy cow | NA | 0.275 | NA |
| | | Finishing pig | NA | 0.107 | NA |
| | | Broiler | NA | 0.283 | 0.169 |
| | | Dairy Sheep | NA | 0.125 | 0.077 |
| | Maize silage | Dairy cow | 0.250* | 0.254 | 0.097 |
| | | Finishing pig | NA | NA | NA |

| | | | | |
|---|---|---|---|---|
| | Broiler | NA | NA | NA |
| | Dairy Sheep | NA | 0.308 | NA |
| **Maize milled by-products** | Dairy cow | NA | 0.058 | NA |
| | Finishing pig | NA | 0.113 | NA |
| | Broiler | NA | 0.237 | NA |
| | Dairy Sheep | NA | NA | NA |
| **TOTAL** | Dairy cow | 0.310* | 0.794 | 0.235 |
| | Finishing pig | 0.110* | 0.403 | 0.105 |
| | Broiler | 0.250* | 0.899 | 0.287 |
| | Dairy Sheep | 0.050* | 0.522 | 0.148 |

NA indicates that an inclusion rate or NEP levels were not provided and therefore no exposures were calculated.
*Indicates that zero digits were added to the original value to obtain three digits after the decimal point. In the case of PAT protein, the zero digits were added to obtain five digits after the decimal point.

### B.2.2. Second Case study: Feed Contaminant (OTA)

Dairy Cow

Table 49: Default values for live weight and feed intake as reported in the model database.

| Animal category | Live weight (kg) | Feed intake (kg/day) | Reference |
|---|---|---|---|
| | | **Dry Matter (DM)** | |
| Dairy Cow | 623 | 25.82 | Darabighane et al., 2020 |

Table 50: Dairy cow diet as reported in the model database.

| Feed Material | Composition (%) |
|---|---|
| Maize silage | 21.23 |
| Maize gluten feed (20% CP) | 18.81 |
| Maize grain, ground | 18.09 |
| Barley grain, ground | 14.57 |
| Soybean meal (44%) | 14.50 |
| Canola meal | 5.00 |

| | |
|---|---|
| Mineral & Vitamin Mix | 2.86 |
| Beet pulp | 2.46 |
| Fat powder | 2.30 |

## Dairy Sheep

Table 51: Default values for live weight and feed intake as reported in the model database.

| Animal category | Live weight (kg) | Feed intake (kg/day) | Reference |
|---|---|---|---|
| | | Dry Matter (DM) | |
| Dairy sheep | 57.00 | 1.22 | Milis et al., 2005 |

Table 52: Dairy sheep diet as reported in the model database.

| Feed Material | Composition (%) |
|---|---|
| Maize grain | 41.00 |
| Alfalfa hay | 30.00 |
| Maize gluten meal (60% CP) | 10.00 |
| Maize gluten feed (20% CP) | 10.00 |
| Wheat straw | 5.00 |
| Mineral and vitamin mix | 4.00 |

## Fattening Pig

Table 53: Default values for live weight and feed intake as reported in the model database.

| Animal category | Live weight (kg) | Feed intake (kg/day) | Reference |
|---|---|---|---|
| | | Dry Matter (DM) | |
| Fattening pig | 43.5 | 2.187 | Sevillano et al., 2018 |

Table 54: Fattening pig diet as reported in the model database.

| Feed Material | Composition (%) |
|---|---|
| Maize | 69.84 |
| Soybean meal (48% CP) | 18.5 |
| Molasses cane | 5.00 |
| Maize gluten feed (20% CP) | 2.5 |
| Vitamin and mineral mix | 4.61 |

Broilers

Table 55: Default values for live weight and feed intake as reported in the model database.

| Animal category | Live weight (kg) | Feed intake (kg/day) | Reference |
|---|---|---|---|
| | | Dry Matter (DM) | |
| Broilers | 1.70 | 0.134 | Abdel-Wareth et al., 2019 |

Table 56: Broiler diet as reported in the model database.

| Feed Material | Composition (%) |
|---|---|
| Maize, ground | 30.00 |
| Sorghum, ground | 30.00 |
| Soybean meal (44%) | 25.00 |
| Maize Gluten Meal (60% CP) | 6.00 |
| Sunflower Oil | 5.52 |
| Mineral & vitamin mix | 3.48 |

Appendix C

# GM feed Example 3

## Appendix C – GM feed Example 3

This appendix describes a third example (Example 3) related to genetically modified (GM) feed for the proof of concept of the developed model database. The focus is on Atlantic salmon and dogs, representing the fish and companion animal categories, respectively. Data on animals (body weight, feed intake) and animal diets (feed material inclusion rates) were adapted from the model database.

The selected scientific opinion (EFSA GMO Panel 2024) for GM feed Example 3 concerns the risk assessment of the GM maize DP202216: Assessment of genetically modified maize DP202216 for food and feed uses, under Regulation (EC) No 1829/2003 (EFSA GMO Panel 2024).

As reported in the scientific opinion (EFSA GMO Panel 2024), dietary exposure to ZMM28 and PAT proteins was estimated via the consumption of maize grain and forage. Eleven animal categories were considered: fattening chickens, laying hens, fattening turkey, fattening pigs, lactating sows, fattening cattle, dairy cows, sheep/goats, salmon, dogs, cats.

The consumption of maize grain was reported for all eleven species. The consumption of forage was reported only for laying hens, lactating sows, fattening cattle, dairy cows and a forage inclusion rate was provided only for these species. The estimations of dietary exposure to newly expressed proteins were based on default values for animal body weight, daily feed intake, and inclusion rates (percentage) of maize grain and forage in diets/rations (OECD, 2013, FAO recommendations[6]). A conservative scenario with 100% replacement of conventional maize products by the maize DP202216 products was considered.

Mean protein levels (dry weight) of ZMM28 and PAT analytically determined in grains and forage from the maize DP202216 treated with the intended herbicide used for dietary exposure were used for the animal dietary exposure assessment. Some of the grain and forage samples analysed in maize DP202216 for the presence of ZMM28 protein were below the limit of quantification (LOQ) (respectively, 0.0069 and 0.036 ng/mg dry weight). To estimate dietary exposure, a value equal to half the LOQ value was assigned to those samples to calculate the mean.

As this additional GM feed Example 3 represents fish and companion animals for the proof of concept of the feed consumption model database, we first reproduced the animal dietary exposure of maize DP202216 for Atlantic salmon and dogs and reported the animal exposure assessment as in the scientific opinion (EFSA GMO Panel 2024) (Table 57).

6 https://www.fao.org/fishery/affris/species-profiles/atlantic-salmon/tables/en/–Atlantic salmon–table 3. Due to the absence of corn grain inclusion values, the corn gluten meal values were used, resulting in a conservative estimate for salmon.

Table 57: Animal dietary exposure to PAT and ZMM28 proteins as reported on the scientific opinion (EFSA GMO Panel 2024), based on the consumption of maize grain and forage.

| Animal dietary exposure (mg/kg BW per day) | | | | | | |
|---|---|---|---|---|---|---|
| | ZMM28 | | | PAT | | |
| | Grain (G) | Forage (F) | G + F | Grain (G) | Forage (F) | G + F |
| Chicken for Fattening | 0.00066 | NA | NA | 0.88 | NA | NA |
| Laying hen | 0.00045 | 0.00035 | 0.00080 | 0.59 | 0.20 | 0.79 |
| Turkey for fattening | 0.00035 | – | – | 0.47 | – | – |
| Pig for fattening | 0.00031 | – | – | 0.41 | – | – |
| Sow lactating | 0.00025 | 0.00040 | 0.00065 | 0.34 | 0.23 | 0.56 |
| Cattle for fattening | 0.00019 | 0.000106 | 0.00125[a] | 0.26 | 0.61 | 0.86[a] |
| Dairy cow | 0.00011 | 0.00123 | 0.00134 | 0.15 | 0.71 | 0.86 |
| Sheep/goat | 0.00007 | – | – | 0.10 | – | – |
| Salmon | 0.00002* | – | – | 0.03* | – | – |
| Dog | 0.00009 | – | – | 0.12 | – | – |
| Cat | 0.00006 | – | – | 0.08 | – | – |

Abbreviation: –, forage not included in the daily ration.
[a]For DP202216 maize grain+forage combination replacement scenario, the inclusion rate for cattle for fattening would be 160% of the diet; therefore, the exposure reported to each protein is an overestimation.

*Inclusion rates of maize grains in salmon diets (10%) were based on FAO recommendations[6].

We then calculated the animal dietary exposure to ZMM28 and PAT proteins using the Excel calculator proposed in the EFSA statement of 2023 (EFSA GMO Panel 2023). The Excel calculator was employed to calculate the daily dietary exposure (DDE) (Table 58).

Table 58: Animal daily dietary exposure to ZMM28 and PAT proteins (mg/kg BW per day) performed with the Excel calculator (EFSA GMO Panel 2023).

| Protein | Feed material | Animal Species | Inclusion rate | NEP level | Daily dietary exposure to NEP |
|---|---|---|---|---|---|
| | | Category | IR% | $\mu g^{NEP}/g^{DM}$ | $mg^{NEP}/kg^{BW}$ |
| ZMM28 | Grain | Salmon<br>5/0.03 | NA | NA | NA |
| | | Dog<br>25/0.36 | 50 | 0.01200 | 0.00009 |

| | | | | | |
|---|---|---|---|---|---|
| | Gluten feed | Salmon 5/0.03 | 10 | 0.03108 | 0.00002 |
| | | Dog 25/0.36 | 30 | 0.03108 | 0.00013 |
| | Gluten meal | Salmon 5/0.03 | 10 | 0.08592 | 0.00005 |
| | | Dog 25/0.36 | 32 | 0.08592 | 0.00040 |
| | Forage/silage | Salmon 5/0.03 | NA | NA | NA |
| | | Dog 25/0.36 | NA | NA | NA |
| | Milled by-products | Salmon 5/0.03 | NA | NA | NA |
| | | Dog 25/0.36 | NA | NA | NA |
| | TOTAL | Salmon 5/0.03 | 20 | NA | 0.00007 |
| | | Dog 25/0.36 | 112 | NA | 0.00062 |
| PAT | Grain | Salmon 5/0.03 | NA | NA | NA |
| | | Dog 25/0.36 | 50 | 16.000 | 0.11520 |
| | Gluten feed | Salmon 5/0.03 | 10 | 41.440 | 0.02486 |
| | | Dog 25/0.36 | 30 | 41.440 | 0.17902 |
| | Gluten meal | Salmon 5/0.03 | 10 | 114.560 | 0.06874 |
| | | Dog 25/0.36 | 32 | 114.560 | 0.52789 |
| | Forage/silage | Salmon 5/0.03 | NA | NA | NA |
| | | Dog 25/0.36 | NA | NA | NA |
| | Milled by-products | Salmon 5/0.03 | NA | NA | NA |
| | | Dog 25/0.36 | NA | NA | NA |
| | TOTAL | Salmon 5/0.03 | 20 | NA | 0.09360 |
| | | Dog 25/0.36 | 112 | NA | 0.82211 |

NA indicates that an inclusion rate or NEP levels were not provided and therefore no exposures were calculated.

Lastly, the animal dietary exposure to ZMM28 and PAT proteins was assessed by replacing the default animal and consumption data with the extracted values from the model database (Table 59).

Table 59: Animal daily dietary exposure to ZMM28 and PAT proteins (mg/kg BW per day) performed with the model database input data.

| Protein | Feed material | Animal categories BW (kg)/total diet intake (kg dw) | Inclusion rate | NEP level | Daily dietary exposure to NEP |
|---|---|---|---|---|---|
| | | Category | IR% | $\mu g^{NEP}/g^{DM}$ | $mg^{NEP}/kg^{BW}$ |
| ZMM28 | Maize | Dog<br>10/0.2 | 26* | 0.012 | 0.00006 |
| | Maize gluten | Salmon<br>2/0.04 | 5.5 | 0.086 | 0.00009 |
| PAT | Maize | Dog<br>10/0.2 | 26* | 16.000 | 0.08320 |
| | Maize gluten | Salmon<br>2/0.04 | 5.5 | 114.560 | 0.12602 |

*The inclusion rate of maize (26%) was adapted from the inclusion rate of rice due to the lack of data regarding maize in the dog diet reported in the model database.

Table 60 shows the DDE results from the EFSA scientific opinion (EFSA GMO Panel 2024), results obtained using the Excel calculator (EFSA GMO Panel 2023) and the results obtained using the model database.

Table 60: DDE results from the EFSA scientific opinion (EFSA GMO Panel 2024), results obtained using the Excel calculator (EFSA GMO Panel 2023) and the results obtained using the model database

| Protein | Feed material | Animal species | DDE Scientific Opinion | DDE Excel calculator | DDE model database |
|---|---|---|---|---|---|
| | | Category | $mg^{NEP}/kg^{BW}$ | $mg^{NEP}/kg^{BW}$ | $mg^{NEP}/kg^{BW}$ |
| ZMM28 | Maize | Atlantic Salmon | 0.00002 | NA | NA |
| | | Dog | 0.00009 | 0.00009 | 0.00006 |
| | Maize gluten feed | Atlantic Salmon | NA | 0.00002 | NA |
| | | Dog | NA | 0.00013 | NA |
| | Maize gluten | Atlantic Salmon | NA | 0.00005 | 0.00009 |
| | | Dog | NA | 0.00040 | NA |
| | Maize silage | Atlantic Salmon | NA | NA | NA |
| | | Dog | NA | NA | NA |

| | | | | | |
|---|---|---|---|---|---|
| | **Maize milled by-products** | Atlantic Salmon | NA | NA | NA |
| | | Dog | NA | NA | NA |
| | **TOTAL** | Atlantic Salmon | 0.00002 | 0.00007 | 0.00009 |
| | | Dog | 0.00009 | 0.00062 | 0.00006 |
| **PAT** | **Maize** | Atlantic Salmon | 0.03000* | NA | NA |
| | | Dog | 0.12000* | 0.11520 | 0.08320 |
| | **Maize gluten feed** | Atlantic Salmon | NA | 0.02486 | NA |
| | | Dog | NA | 0.17902 | NA |
| | **Maize gluten** | Atlantic Salmon | NA | 0.06874 | 0.12602 |
| | | Dog | NA | 0.52789 | NA |
| | **Maize silage** | Atlantic Salmon | NA | NA | NA |
| | | Dog | NA | NA | NA |
| | **Maize milled by-products** | Atlantic Salmon | NA | NA | NA |
| | | Dog | NA | NA | NA |
| | **TOTAL** | Atlantic Salmon | 0.03000* | 0.09360 | 0.12602 |
| | | Dog | 0.12000* | 0.82211 | 0.08320 |

NA indicates that an inclusion rate was not provided and therefore no exposure calculations were performed

*Indicates that zero digits were added to the original value to obtain three digits after the decimal point.

Table 60 shows an increase in the DDE results in obtained with the input data from the model database for Atlantic salmon consuming maize gluten and thus, for the total animal dietary exposure. In this case, the DDE increase can be explained by the different body weight and total daily intake. According to the Excel calculator, Atlantic salmon has a body weight of 5 kg and a total daily intake of 0.03 kg$^{DM}$/animal. In the model database, Atlantic salmon has a body weight of 2 kg and a total daily intake of 0.04 kg$^{DM}$/animal, leading to a higher daily dietary intake and dietary exposure to the NEPs. Moreover, different feed materials were considered in the diet of Atlantic salmon.

In the scientific opinion (EFSA GMO Panel 2024), only maize grain has a reported inclusion rate. In the Excel calculator (EFSA GMO Panel, 2023), the EFSA GMO Panel reports inclusion rates for maize gluten feed and maize gluten. In the model database, only maize gluten has a reported inclusion rate.

On the other hand, a decrease in DDE results was found with the input data from the model database for dogs consuming maize grain. In this case, the DDE difference can be explained by the different inclusion rates of maize grain. In the Excel calculator, maize grain has an inclusion rate of 50%, while in the model database, the inclusion rate is 26%, leading to a lower daily dietary intake and dietary exposure to the NEPs. As with Atlantic salmon, different feed materials were considered in the diet of dogs. In the scientific opinion (EFSA GMO Panel 2024), only maize grain has a reported inclusion rate. In the Excel calculator, the EFSA GMO Panel (EFSA GMO Panel, 2023) reports inclusion rates for maize grain, maize gluten feed and maize gluten. In the model database, only maize grain (adapted from the inclusion rate of rice) has a reported inclusion rate, contributing to the lower total DDE in the results obtained with the input data from the model database.

Example 3 showed some disparities compared to Examples 1 and 2. In fact, eleven animal categories (fattening chickens, laying hens, fattening turkeys, fattening pigs, lactating sows, fattening cattle, dairy cows, sheep/goat, salmon, dogs, and cats) were considered, with the inclusion of salmon, dogs and cats which were not considered in the other two Examples. Moreover, as with Example 2, in Example 3 the focus was also exclusively on the exposure to maize grains and forage and not on other maize products (e.g. maize gluten feed, maize gluten) or maize by-products (e.g. maize milled by-products) considered in Example 1.

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# GENERAL CONCLUSION

The aim of this thesis was to contribute to the development of sustainable and innovative strategies in the agri-food and feed sectors by valorising co-/by-products of the food supply chain and former food products (FFPs) within a circular economy framework. To achieve this, the research focused on the toxicological, nutritional, and functional characterisation of cocoa hulls (CHs), FFPs, and hemp co-products, and addressed methodological challenges in animal exposure assessment through the development of a proposal for a harmonised feed consumption database. The findings demonstrate that CHs contain polyphenols and linked antioxidant activity, being a promising functional ingredient in animal diets. However, theobromine levels were found to be the main limiting factor for their safe use. Inclusion levels of around 5–7% of complete feed appear feasible within EU regulatory limits, though variability between batches underscores the need for case-by-case evaluation. Similarly, FFPs confirmed their nutritional potential and general compliance with EU maximum limits, but their wide variability in methylxanthine content requires careful management. Animal dietary exposure assessments revealed that while most species remain below toxicological thresholds, piglets are particularly sensitive, necessitating species and stage-specific evaluations. Hemp co-products were shown to provide appreciable phenolic content and antioxidant activity, with bioaccessibility confirmed through ex vivo digestion, highlighting their potential as functional feed ingredients, if safety aspects (e.g., cannabinoid residues) are addressed. Beyond the characterisation of individual matrices, this thesis also reported the proposal for the development of a feed consumption database. By demonstrating application through case studies, the work shows that reliable and harmonised data structures may improve risk assessments, regulatory consistency, and innovation transfer. Overall, the results confirm that the targeted valorisation of food co-/by-products and FFPs can support a more sustainable and resource-efficient agri-food system, provided that toxicological risks are rigorously monitored and managed. This work underscores that advancing circular feed practices requires not only scientific characterisation of ingredients but also robust risk assessment tools and interdisciplinary collaboration. The integration of expertise in food and feed safety, toxicology, nutrition, and data science has provided insights and methodological advances that may support the development of safer, more sustainable, and circular food and feed systems, in line with the objectives of the 2030 Agenda for Sustainable Development.

# LIST OF PUBLICATIONS AND FURTHER ACTIVITIES

## List of publications

### Part of this thesis

**Mercogliano, F.**, Bani, C., Tretola, M., Landolfi, C., Ottoboni, M., Cheli, F., Restani, P., Pinotti, L., & Di Lorenzo, C. (2025). Preliminary Assessment of Alkaloid Content in Cocoa (*Theobroma cacao L.*) Hulls for Safe Consumption as a Feed Ingredient. *Toxins*, 17(9), 441-455. https://doi.org/10.3390/toxins17090441

**Mercogliano, F.**, Di Lorenzo, C., Tretola, M., Bani, C. & Pinotti, L. Animal dietary exposure to methylxanthines through the inclusion of Former Food Products (FFPs) in feed. (2026). *ACS Agricultural Science & Technology*. https://doi.org/10.1021/acsagscitech.5c01031

Lanzoni, D., **Mercogliano, F.**, Rebucci, R., Bani, C., Pinotti, L., Di Lorenzo, C., Savoini, G., Baldi, A., & Giromini, C. (2024). Phenolic profile and antioxidant activity of hemp co-products following green chemical extraction and ex vivo digestion. *Italian journal of animal science*, 23(1). 651-663. https://doi.org/10.1080/1828051X.2024.2348098

Pinotti, L., Pastorelli, G., Varese, G., Cheli, F., Di Lorenzo, C., Montanelli, S., Landolfi, C., Mangano, G., Gazerro, C., Perricone, V., Ottoboni, M., Moradei, A., Abbate, R., Luciano, A., & **Mercogliano, F.** (2024). A proposal for the development of a feed consumption database using a standardised feed classification system. *Efsa supporting publications. 21*(9), 1-136. https://doi.org/10.2903/sp.efsa.2024.EN-9036

### Outside of this thesis

Bani, C., Martínez-Villaluenga, C., Di Lorenzo, C., Restani, P., Bautista-Expósito, S., Pozzoli, C., **Mercogliano, F.**, & Peñas, E. (2025). Nutritional composition and antidiabetic potential of fine and coarse flours from common and tartary buckwheat. *LWT*, 232(1), 1–10. https://doi.org/10.1016/j.lwt.2025.118404

Bani, C., Restani, P., Tripodi, S., **Mercogliano, F.**, Colombo, F., & Di Lorenzo, C. (2025). Unexpected Cow's Milk Proteins in a "Vegan" Easter Egg as a Cause of Anaphylaxis. *Foods*, 14(10), 1–8. https://doi.org/10.3390/foods14101737

Bani, C., Peñas, E., Baron, G., Martínez-Villaluenga, C., **Mercogliano, F.**, Aldini, G., Piazza, S., Di Lorenzo, C., & Restani, P. (2025). Characterization of the phenolic profile and in vitro antioxidant potential of different varieties of common buckwheat (Fagopyrum esculentum Moench) and tartary buckwheat (Fagopyrum tataricum (L.) Gaertn.). *LWT*, 215(1), 1–10. https://doi.org/10.1016/j.lwt.2024.117261

Restani, P., Di Lorenzo, C., Antoce, A. O., Araujo, M., Bani, C., **Mercogliano, F.**, Ruf, J., Kosti, R. I., & Teissedre, P. (2025). Moderate Wine Consumption and Gastrointestinal Diseases. *Nutrients*, 17(10), 1–25. https://doi.org/10.3390/nu17101608

Bani, C., Cappa, C., Restani, P., Sala, M., Colombo, F., **Mercogliano, F.**, & Di Lorenzo, C. (2024). Physicochemical and nutritional quality of pigmented rice and bran: Influence of milling and cooking. *LWT*, 208(1), 1–12. https://doi.org/10.1016/j.lwt.2024.116653

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Bani, C., Di Lorenzo, C., Restani, P. A., **Mercogliano, F.**, & Colombo, F. (2023). Phenolic Profile and In Vitro Antioxidant Activity of Different Corn and Rice Varieties. *Plants*, 12(3), 1–14. https://doi.org/10.3390/plants12030448

Di Lorenzo, C., Bani, C., **Mercogliano, F.**, Bosso, A., & Restani, P. (2023a). Valorization of wine industry by-products: Characterization of phenolic profile and investigation of potential healthy properties. *Bio web of conferences*, 68(1), 1–9. https://doi.org/10.1051/bioconf/20236804016

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Restani, P. A., Colombo, F., Biella, S., Bani, C., **Mercogliano, F.**, & Di Lorenzo, C. (2022). Diet, Polyphenols, and Human Evolution. *Applied sciences*, 12(15), 1–15. https://doi.org/10.3390/app12157805

## Mobility

- Novel and sustainable food systems: understanding and driving the required change in research and communication (SUFORECO), Department of Food Science and Department of Veterinary and Animal Science, Copenhagen, Denmark, 2024 (2 weeks)
- Visiting PhD at Wageningen Food Safety Research (WFSR), Wageningen, The Netherlands, 2024-2025 (6 months)

## External collaborations

- ToxHub s.r.l. – Online collaboration on the EFSA procurement project "A proposal for the development of a feed consumption database using a standardised feed classification system."
- GlutenSens – Research and Development activities. Glutensens is an innovative startup that aims to support people with celiac disease with a high-tech device to detect gluten contamination.
- Procura di Milano (Public Prosecutor's Office of Milan), ATS Milano (Agenzia di Tutela della Salute – Health Protection Agency) and N.A.S. (Nuclei Antisofisticazioni e Sanità dell'Arma, Arma dei Carabinieri) – Analytical testing for court proceedings
- Food industry company – Online collaboration for the initial phases of an EU novel food dossier preparation

## Conferences/Workshops/Symposiums/Summer Schools

- 9th International Feed Conference "Innovating Feed - Shaping the Future"– Novi Sad, 2025

  **Mercogliano, F.**, Bani, C., Tretola, M., Pinotti, L., Di Lorenzo, C. (2025). Theobromine and caffeine in former food products intended for animal nutrition. (poster; awarded best poster presentation)

  **Mercogliano, F.**, Pastorelli, G., Cheli, F., Gazerro, C., Di Lorenzo, C., Pinotti, L. (2025). A proposal for the development of a feed consumption database using a standardised feed classification system: a proof of concept for GMO. (poster)
- Global forum for animal feed and feed regulators. *Innovations for greater availability of sustainable, safe, and quality feed* (FAO and IFIF) – Rome, 2025
- Conference on the Safety of Innovative Plant-Based Preparations (SITOX) – Milan, 2025
- 59th Congress of the European Societies of Toxicology (EUROTOX) – Athens, 2025

  **Mercogliano, F.**, Rizzo, S., Righetti, L., Bani, C., Di Lorenzo, C., Faassen, E. J., Van Leewen, S. (2025). Transformation of PFAS precursors in *Tetradesmus Obliquus*: insights from LC-HRMS analysis. *Toxicology letters*, 441, S319-S319a. https://dx.doi.org/10.1016/j.toxlet.2025.07.743 (poster)
- XIV National Congress of Food Chemistry – Milan, 2025

  **Mercogliano, F.**, Bani, C., Tretola, M., Pinotti, L., Restani, P., Di Lorenzo, C. (2025). Former Food Products (FFPs) as alternative feed ingredients: assessment of undesirable substances from the original food matrix. (poster)
- 46th World Congress of Vine and Wine – Chișinău, 2025

  **Mercogliano, F.**, Bani, C., Di Lorenzo, C., & Restani, P. (2025). Possible toxicological risk arising from contamination of grapes and derivatives by emerging mycotoxins: patulin. *IVES Conference Series*, *46th World Congress of Vine and Wine*. https://doi.org/10.58233/pBg1rIzj (oral presentation)
- International Workshop on Feed Risk Assessment-Chemical Safety. (RIVM and FAO) – Utrecht, 2025

  **Mercogliano, F.** (2025). Interactive case studies - Impact of feed material substitution on animal dietary exposure and risk characterisation. (oral presentation)
- 22° National Congress of the Italian Society of Toxicology (SITOX) – Bologna, 2025

  **Mercogliano, F.**, Bani, C., Restani, P., & Di Lorenzo, C. (2025). Sfide per la sicurezza dei botanicals negli integratori alimentari: focus sui contaminanti. (oral presentation)
- Stakeholder workshop event - Task 6 of the EFSA Procurement OC/EFSA/GMO/2021/05 – Online, 2025

  **Mercogliano, F.** (2025). Proof of concept for the development of a feed consumption database. (oral presentation)
- Symposium "Environmental occurrence, hazard and risk of PFAS" - EnvironChemTox (MCT), Wageningen, 2024
- Workshop "Risk Assessment in Food Safety: Educational and Scientific Opportunities" – Istituto Zooprofilattico Sperimentale, Online, 2024
- International Conference "New Trends on Sensing-Monitoring-Telediagnosis for Life Sciences", NT SMT–LS – Online, 2024

**Mercogliano, F.**, Bani, C., Tretola, M., Pinotti, L., Restani, P., & Di Lorenzo, C. (2024). Utilising cocoa (*Theobroma cacao* L.) by-products for sustainable animal feed: an evaluation of polyphenol and antioxidant content. (oral presentation; awarded II$^{nd}$ prize – young scientist)

- Conference on "bio" pesticides - Italian Society of Toxicology (SITOX), Milan, 2024
- Webinar "Protein safety assessment in GMOs" – European Food Safety Authority, Online, 2023
- 8th International Feed Conference "Present and Future Challenges" – Milan, 2023

  **Mercogliano, F.**, Bani, C., Tretola, M., Pinotti, L., Restani, P., & Di Lorenzo, C. (2023). Evaluation of phenolic profile and antioxidant activity of cocoa (*Theobroma Cacao* L.) by-products to explore their potential as animal feed additives. (oral presentation)
- Food Authenticity PhD Excellence Academy – University of Parma, Parma, 2023
- International Summer School in Food Safety and Healthy Living, FSHL – Bucharest, 2022
- Workshop on Advances in alternative non-animal methods and policies – Fin3RC, Online 2023
- XIII National Congress of Food Chemistry – Marsala, 2023

  **Mercogliano, F.**, Bani, C., Tretola, M., Pinotti, L., Restani, P., & Di Lorenzo, C. (2023). Characterization of the phenolic profile of cocoa (*Theobroma cacao* L.) processing by-products for their possible use in animal feed. (poster)
- 21° National Congress of the Italian Society of Toxicology (SITOX) – Bologna, 2023
- Workshop on Sustainable Animal Nutrition – Online, 2022
- "Il contributo della valutazione Life Cycle Assessment nel settore agroalimentare" Workshop - Milan, 2022
- Autumn School in Food Chemistry -1st edition Italian School in Food Chemistry– Pavia, 2022
- International Summer School in Food Safety and Healthy Living, FSHL – Online, 2022

## Attended PhD courses

- Alimentazione animale, implicazioni per la food safety e la food security (2 ECTS credits)
- Quality and safety of food of animal origin (2 ECTS credits)
- Basi biochimiche della nutrizione (2 ECTS credits)
- Food supplements and diet therapy (2 ECTS credits)
- Microbiology in translational health science and technology (4 ECTS credits)
- Eating behavior, lifestyles and risk of chronic diseases (4 ECTS credits)
- Gastrointestinal disorders and diet (3 ECTS credits)
- New approach methodologies in toxicology (2 ECTS credits)
- Feeding future generations sustainability: scientific innovation and legal challenges of food regulation (3 ECTS credits)

## Teaching experience

- Tutorship (ex art.45) for the laboratory of Food Chemistry in the Bachelor's program in Chemical safety and Toxicological Environmental Sciences
- Tutorship for the course Analysis of Pesticides and Contaminants in Food and Environmental Matrices in the Bachelor's program in Chemical Safety and Toxicological Environmental Sciences

- Support for examination activities in the teaching course of Food Chemistry in the Bachelor's program in Chemical safety and Toxicological Environmental Sciences and of Dietetic products in the Master's program in Pharmacy

## Thesis co-supervision

- Experimental Master's Thesis in Chemistry and Pharmaceutical Technologies by Giulia Nicu "Optimization of gliadin extraction process for gluten detection: new perspectives for the development of an innovative device to ensure the safety of celiac patients"
- Experimental Master's Thesis in Chemistry and Pharmaceutical Technologies by Valentina Gregis "Economia circolare e biodiversità: valorizzazione di sottoprodotti della filiera agro-alimentare e di matrici vegetali del territorio"
- Experimental Bachelors's Thesis in Chemical safety and Toxicological Environmental Sciences by Giulia D'Amato "Ricerca e quantificazione di micotossine in sottoprodotti di cacao (Theobroma L.) tramite metodica ELISA: implicazioni per la sicurezza nella mangimistica"
- Experimental Master's Thesis in Pharmacy by Arianna D'Onofrio "Valorizzazione di sottoprodotti dell'industria alimentare per la dieta animale: aspetti di sicurezza e potenziali effetti benefici"

## Other

Member of the UNIMI University Libraries Commission, representing doctoral students (2023-2024)