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Highlights

- Contamination of plant-based meat alternatives is understudied or not studied at all
- Mycotoxin and alkaloid contamination was found in plant-based meat alternatives
- Mycotoxin mixtures were reported in soy-based food
- Data in the literature do not return a full picture of the EU countries
- Data from this review can be used in future risk assessment studies

A systematic review of natural toxins occurrence in plant commodities used for plant-based meat alternatives production

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Abstract

The on-going shift from traditional diets to plant-based meat alternatives is governed by the friendly-character related to consumers' health and environment. However, the beneficial aspects of meat alternatives overshadow the possible adverse effects that accompany them. The present systematic review shows that the contamination of the most common plant-based meat alternatives, soybean, chickpea, pea and seitan with mycotoxins is understudied or not studied at all. Even though they are toxic and were found in soy-based food, tropane and β-carboline alkaloids contamination data in plant-based meat alternatives is also lacking. Mycotoxin mixtures that can have additive or synergistic toxic effects have been found in multiple soy-based food, revealing the high risk that consumers expose themselves to. To better understand the risks that come along with the shift to plant-based meat diets, future research is needed regarding contamination data of plant-based meat alternatives with natural toxins. Maximum limits for contaminants found in plant-based meat alternatives need to be established by the European Commission in order to ensure consumers' food safety.

Keywords: plant-based meat alternative, mycotoxin, alkaloid, natural toxin, contamination

1. Introduction

Over the last 50 years worldwide meat consumption rose from 23.1 kg/person/year in 1961 to 42.2 kg/person/year (Sans & Combris, 2015). Even though the EAT-Lancet Commission 'planetary healthy' diet recommends a maximum intake of red meat of 28g a day (~10kg/person/year) (Willett et al., 2019), in developed countries, red and processed meat consumption is almost four times higher (FAO, 2019). The projected world growth population is 10 billion people by 2050 indicating that the current sources of animal protein (i.e., livestock and poultry) will not be sufficient in the near future (Willet et al., 2019).

Multiple variables play a key role in the on-going shift of dietary patterns from animal-based food to meat alternative diets such as, i) the often reported association of red and processed meat consumption with increased risks of different chronic illnesses (Micha et al., 2012; Kaluza et al., 2021) and increased death rates (Larsson & Wolk, 2006; Norat et al., 2005; Pan et al., 2012; van Dooren et al., 2014; Wolk, 2016), ii) the high environmental impact, overuse of land and water resources, as well as high Green House Gas (GHG) emissions caused by animal meat production (Clune et al., 2017; Davis et al., 2016). In this context, it has been suggested that a shift toward plant-based meat alternatives may play a role in mitigating the effects of animal production on climate change and consumers' health, and fit with the recommendations made for the new alternative dietary patterns (Van Vliet, Kronberg, & Provenza, 2020; Willet et al., 2019).

~~Although meat alternatives based on proteins from fungi (Sha & Xiong, 2020), insects (Megido et al., 2016) and microalgae (Percival, 2019) have recently entered the market, and cultured-meat is becoming of growing interest for the research community, the research and innovation efforts of~~

the agri-food industry is nowadays mainly focused on plant-based meat analogues (Santo et al. 2020; He et al. 2020). For a comprehensive overview about structural and technological features, production, and consumers' acceptance of meat analogues, the readers may refer to the recent reviews from (Boukid, 2021; He et al. 2020, Onwezen et al., 2021).

The consumption of processed plant-based protein products can trace back since ancient times in countries such as China and India, and have become over time well-accepted protein sources in developed countries vegetarian diets, especially with products such as tofu, tempeh and seitan. In particular, tofu and tempeh are obtained from soymilk and fermented soybeans, respectively, while wheat gluten is used for seitan production.

Currently, a wide variety of plant-based protein are used for production of meat analogues, but legumes like soy and peas are considered the primary source due the superior technological properties of their protein fractions (Kyriakopoulou et al., 2018). On the contrary, insects and gluten may represent cost-effective options for the market (Jones,2016).

Legumes are part of the *Leguminosae* family and include seeds such as soybean, and pulses, i.e., peas, chickpeas, lentils, lupine, and beans (Kyriakopoulou et al., 2018). Among legume-based proteins, soybeans (*Glycine max*) are the most widely used due to their availability, high nutritional quality, functional properties, and high protein content (Alcorta et al., 2021, Kyriakopoulou et al., 2018). Considering that soy is becoming a highly controversial commodity due to its environmental and social sustainability (Smetana et al., 2015), there's a growing interest around the use of pea proteins for plant-based meat analogues due to its functional properties, nutritional characteristics and low potential for allergic responses (Osen et al., 2014, 2015).

Besides legume-based alternatives, the meat analogues obtained from wheat gluten are commonly sold on the market. Wheat gluten is traditionally used in the Eastern Asia cuisine, and has entered over the past decades the western markets with the trade name of seitan (Malav et al., 2015), a chewy mass of proteinaceous gluten that results as a byproduct during the isolation of starch from wheat flour (Kumar et al., 2017).

Nowadays, the large majority of studies related to meat alternatives are focused on their environmental impact (i.e., insect and soy-based burgers have a lower impact than cultured meat and mycoprotein) (Smetana et al., 2015), nutritional aspects (Rubio et al., 2020), and the determinants of consumers' preferences and acceptance in regard to plant-based meat (Davitt et al., 2021; He et al., 2020; Profeta et al., 2020; Weinrich, 2019; Onwezen et al., 2021). However, the nutritional qualities and environmental-friendly character of the plant-based meat alternatives seem to prevail and overshadow any potential food safety issue related to the raw materials.

While several studies have addressed the microbiological stability of plant-based meat analogues (Wild et al., 2014), little is known about the occurrence of chemical contaminants and natural toxins. It is generally considered that the use of high-quality ingredients and the application of common HACCP measures may represent an effective strategy to ensure the safety of plant-based meat analogues.

It should be noticed, however, that the launch of over 4400 plant-based meat imitates in the last six years (Mintel, 2019) coupled with a higher intake of plant-based food in the alternative dietary patterns, may have altered the dietary exposure considered for the risk assessment studies at the basis of the current regulatory framework. Also, in consideration of the climate change scenario, this could be particularly relevant for natural contaminants (i.e., mycotoxins and plant alkaloids) not fully regulated in plant commodities used for meat alternatives.

It is known that mycotoxins may accumulate in a wide range of crops at both pre- and post-harvest, among them grains, seeds and beans. More than 400 mycotoxins have been identified so far but, as a consequence of gaps in toxicological and occurrence data, only a few are regulated in crops, among them aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FBs), deoxynivalenol (DON) and zearalenone (ZEN). Other mycotoxins often referred as "emerging" have been found in legumes and grains, such as enniatins (ENs), beauvericin (BEA), and moniliformin (MON) as well those produced by *Alternaria* spp. (EFSA, 2018a; Rodríguez-Carrasco et al., 2019; Schollenberger et al., 2007; Tolosa et al., 2017; Uhlig et al., 2013).

Besides mycotoxins, plant alkaloids are a wide group of natural toxins synthesized in plants as secondary metabolites (Jing et al., 2014). They have demonstrated over centuries a wide range of biological activities of pharmacological importance (Debnath et al., 2018). However, the uncontrolled exposure of animals or humans to plant alkaloids through the diet can be of toxicological relevance, especially for pyrrolizidine, tropane, and β-carboline alkaloids (Allen & Holmstedt, 1980; Airaksinen & Kari, 1981; Diaz, 2015; EFSA, 2017a; EFSA 2017b; EFSA, 2018b). While some alkaloids may inherently occur in legumes, such as quinolizidine alkaloids in lupins (EFSA, 2019), usually plant toxins are found in seeds and pulses following a cross contamination at harvesting or along the production chain (EFSA, 2013a).

It is known indeed that both mycotoxins and plant alkaloids may occur in grains and legumes, and therefore their co-occurrence in plant meat alternatives cannot be ruled out. Since both mycotoxins and plant alkaloids may occur in grains and legumes, their co-occurrence in plant meat alternatives cannot be ruled out. Furthermore, while the co-occurrence of mycotoxins in crops has been largely reported (Smith et al., 2016 for review), their combined effects with other phytocompounds

~~occurring in plants meant to be source of meat alternative ingredients that might magnify their toxic effects have been recently discussed (Crudo et al., 2019).~~

Noticeably, the level of mycotoxins and plant alkaloids in the most used legume-based meat alternatives (i.e., soy, pea, chick pea) are not regulated in the European Union so far, neither is seitan although wheat is widely monitored as raw material.

Given the continuously increased frequency consumption of plant-based meat alternatives, the substitution of meat-based foods with plant-based products is likely to significantly increase the dietary intake of mycotoxins and plant alkaloids being the formers a less relevant, although not negligible, source of exposure (Carballo et al., 2019).

Thus, focusing the analysis on the European scenario, the objectives of this review were twofold:

- To highlight the occurrence and co-occurrence of mycotoxins and plant alkaloids in the most common meat substitutes.
- To summarize the state of the art with regards to current mycotoxin and plant alkaloids regulations, the chemical risks of plant toxins associated with the consumption of plant-based meat alternatives, and to potentially underline fields lacking data related to this problem.

2. Materials and methods

2.1. Search strategy

For the screening of titles, abstracts, full texts and in order to assure the scientific quality of this review and to minimise the risk of bias, the Preferred Reporting Item for Systematic Reviews and Meta-analysis (PRISMA) statement protocol was followed (Page et al., 2021).

A systematic literature review was performed in November – December 2021 in three databases (Scopus, WebofScience, and PubMed) within the timeframe of January 2000 – December 2021. The review was conducted using the following search strings: ("soy*" OR “soy” OR "s*food" OR "bean*" OR “pea” OR "peas*" OR "wheat" OR "gluten" OR "seitan" OR "plant-based meat" OR "pulses") AND ("mycotoxin*" OR "aflatoxin*" OR "total aflatoxin*" OR "deoxynivalenol" OR {OTA} OR "zearalenone" OR "nivalenol" OR "fumonisin*" OR "citrinin" OR "alternariol" OR "alternariol monomethyl ether" OR "tentoxin" OR "tenuazonic acid" OR “trichothecene*” OR “enniatin*” OR “emerging mycotoxin*” OR "plant toxin*" OR "alkaloid*" OR "pyrrolizidine" OR "tropane" OR "β-carboline" OR "ergot") AND ("contamin*" OR "occurr*" OR "co-occurr*" OR "preval*" OR "concentration*" OR {risk assessment} OR {safety assessment} OR {chemical hazard} OR {chemical safety} OR "dietary exposure") combined or alone with “AND and/or “OR”. In order to get a more comprehensive overview, the reference list of the selected articles was also checked for any additional relevant studies.

2.2. Inclusions/exclusion criteria

A publication was included in the systematic review when it met the following inclusion criteria: a) full-text downloadable paper written in English, b) articles published in peer-reviewed journals, c) concentration and prevalence of mycotoxins and alkaloids was measured, d) analytical methods mentioned, and e) samples of products intended for human consumption.

Papers that were not written in English, with not available full-text, did not provide concentration values for the contaminants, theses (because of lack of peer review process), and reviews were excluded.

Only papers from the European geographical area were considered.

2.3.Data extraction

The collected data from each publication included: the name of the first author, the year of the study, type of natural contaminants analysed, the number of total/positive samples, mean/median concentration, range of levels of concentration, method of detection, limit of quantification/detection, and country. The searches were handled with EndNote x20 (Niles Software, Clarivate). After removing duplicates, the records were exported into an online reference management platform (https://www.rayyan.ai/) for the screening of the titles and abstracts.

2.4.Statistical analysis

The data from this review were analysed with SPSS Statistics 26 (IBM Software Group, Chicago, IL). Data normality was assessed with Shapiro-Wilk test which revealed that the data were not normally distributed. Hence, the Mann-Whitney test was used to compare the number of studies regarding wheat-, soy, chickpea, pea, and seitan-based food.

The results were displayed as Sankey diagrams using Tableau Software 2020.1 (Salesforce, Seattle, WA).

3. Results and discussions

3.1. Systematic review process

Among the 3,486 articles published from 2000 to 2021, 1,212 were retrieved from Scopus, 1,530 from WoS (Web of Science), and 744 from PubMed. After the initial assessment, 802 duplicates were removed and based on the screening of the titles and abstracts another 2,334 records were also removed from the review because the studies investigated non-European scenarios, the studies were conducted on feed and not food, and/or because of the underreported details regarding the data. After assessing the eligibility of 350 records, 109~~39~~ were included in the present systematic review (Figure 1).

Insert Figure 1 here

Since data regarding commodities used for the production of meat alternatives are scarce, we decided to include EFSA reports with data submitted from Member States and the Rapid Alert System for Food and Feed (RASFF) notifications regarding contamination of legume-based food items with natural toxins.

Although the systematic review aims at returning an occurrence scenario for natural toxins in plant commodities used to produce plant-based meat alternatives in the EU, the search was enlarged to studies from Europe as a continent. Arguments for this choice are the economic area of influence of the EU market, and the food safety requirements set for pre-accession countries. Considering that the occurrence scenario, once matched with consumption data based on alternatives diets, may give rise to a reconsideration of the current EU regulatory framework, a larger geographical picture may be of interest.

The sampling year, country of origin, food item, contaminants, number of total samples, incidence, mean/median (µg/kg), range (µg/kg), limit of detection (LOD), limit of quantification (LOQ), method of measurement and associated reference are presented in Tables S1 and S2.

3.2. Characteristics of the studies included in the review

The research contained three sources of data, including a total of 100~~32~~ articles for scientific monitoring in papers from 29~~31~~ countries from Europe continental area, eight EFSA reports with official data submitted by MS, with 17~~2~~ findings containing pooled results from multiple European countries, and one RASFF non-compliance notification from Germany (see Figure 2).

Insert Figure 2 here

Most of the articles were from Poland (12/100; 12%), Italy (10/100; 10%), Spain (8/100; 8%), and Croatia (8/100; 8%), while the least were provided by Albania (1/100; 1%), Bosnia and Herzegovina (1/100; 1%), Kosovo (1/100; 1%), and Latvia (1/100; 1%). More than ninety percent of the studies emphasized on the contamination of wheat grains (91/100; 91%), while less than ten percent provided data for the contamination of soybean and/or soy-based food (8/100; 8%) along with eight EFSA reports and one RASFF notification. Contamination data for peas was found in three research articles and three EFSA reports and for chickpeas in two research articles and three EFSA reports.

~~It should be noticed that the only data for soy-, pea-, and chickpea-based food were provided by eight EFSA reports and from studies conducted in the following countries: Belgium, Croatia, France, Germany, Italy, Poland, Russia, Spain, and Sweden.~~

~~No studies have been retrieved from Ireland, Malta and Estonia, among the EU member states, and from Iceland, Montenegro, North Macedonia, Turkey and Lichtenstein among the pre-accession countries and the European Economic Area.~~

Occurrence data from different areas are useful to point out possible bias due to a different implementation of monitoring plans or issues at the border, and therefore data gaps in the European area should be considered in the future.

Data retrieved by the literature search pinpointed the large number of reports addressing mycotoxins in wheat compared to legumes, clearly a consequence of the regulatory framework implemented in Europe for mycotoxins in cereals. Similarly, plant alkaloids have been included in the EU risk assessment only recently, and this can explain the lower number of studies reported in the scientific literature.

Out of the 91 studies regarding wheat/wheat-based food contamination, 90 (98.9%) studies revolved around mycotoxin contamination and only one (1.1%) analysed the content of plant alkaloids (Figure 3). The difference between the number of studies analysing the content of mycotoxins and plant alkaloids in soy/soy-based food was not as large as that for the wheat-studies but nonetheless almost double (10/16, 62.5%, respectively 6/16, 37.5%). Regarding pea and chickpea contamination, the data that were found revolved only around mycotoxin contamination (Figure 3).

Insert Figure 3 here

An increasing trend in published articles can be seen since the early 2000s, showing a sevenfold increase from 2001 (2 articles) to 2021 (12 articles) (see also Supplementary Material, Figure S1). When comparing the number of articles published on wheat grain with soybean/soy-based food, pea/pea-based food, chickpea/chickpea-based food, and wheat gluten-based food we can see that soy, pea, and chickpea are understudied with one article for soy, one for pea, respectively none for chickpea or other non-wheat meat alternatives published in 2021, while the former was studied in

12 articles published in just 2021 (see also Supplementary Material, Figure S2). The fact that legumes are understudied is also supported by the significant difference between the number of wheat-based published articles and other plant-based meat alternatives articles ($p < 0.05$).

No data have been found regarding the contamination of wheat gluten/seitan with natural toxins. Seitan mostly undergoes nutritional quality studies and not contamination research (Ortolan & Steel, 2017; Švarc et al., 2022). This is worrisome as seitan is one of the most common plant-based meat alternatives on the market. The lack of proper studies is probably since raw wheat is already regulated, and the need to monitor the final product is likely perceived as lower. However, the fate of mycotoxins along the food production chain is strongly affected by processing, causing dilution or concentration (Schaarschmidt & Fauhl-Hassek, 2018). This should be carefully considered when the maximum limits (ML) of mycotoxins set by the EC are based on the raw material. Mycotoxins that are less water-soluble can be actually concentrated in the gluten and germ fraction (Collins & Rosen, 1981; Lauren & Ringrose, 1997). Although a decrease in mycotoxin content is expected by dilution with other ingredients, some of them like oilseeds and spices might be a source of contamination themselves (Schanufer, 2013). Therefore, the lack of data regarding natural toxins contamination on seitan food is concerning as a proper estimation of dietary exposure in the EU population cannot be performed.

The number of studied mycotoxins in legumes from the scientific literature is broken down in Figure 4.

Insert Figure 4 here

The large majority is focused on soy-based food, while only a few of them have considered pea or chickpea containing food. A total of 25~~2~~ mycotoxins were monitored across the studies, AFB_1

(nine times), OTA (eight times), and ZEN (seven times) being the most frequently included in the screening.

Overall, available data are not enough to provide a reliable picture of mycotoxins occurrence in legume-based products, especially for pea/chickpea.

As for plant alkaloids contamination data, only 6 records have considered their occurrence in legume-based food in Europe between 2000 – 2021, and all for soybean/soy-based products (Figure 5).

Insert Figure 5 here

Differently from what was described for mycotoxins, data related to contamination with TAs were retrieved from two EFSA reports (EFSA, 2013a; EFSA, 2018b) and one RASFF notification (RASFF 2020.0366), while no studies in the scientific literature were identified. Additional data regarding the contamination of peas and chickpea flour with atropine and scopolamine have been reported by EFSA, (2018b), however 99% of the data is left-censored and the values range from 0 μg/kg to 0.22 μg/kg.

Three research articles, two from Spain and one from Germany, presented results of soy-based food contamination with β-carboline alkaloids (Diem & Herderich, 2001; Herraiz, 2004; Herraiz & Vera, 2021).

3.3. Occurrence of mycotoxins and plant alkaloids in plant-based meat alternative reported in European studies between 2000 – 2021

Starting from the papers included in the systematic review and described in the previous paragraph, the contamination ranges (as mean values) for mycotoxins and alkaloids reported in plant-based meat alternatives were extracted and reported in Table 1. Figures S3 and S4 show the level of incidence for some mycotoxins occurring both in wheat grains and plant-based meat alternatives and mixtures of mycotoxins found in wheat/wheat-based food.

Insert Table 1 here

The highest concentration of AFB_1 was found in soy-based burgers in Italy (10.1 μg/kg) (Rodríguez-Carrasco et al., 2019) which is higher than the highest maximum limit set by the EC for AFB_1 in hazelnuts and nuts (8 μg/kg). Regarding soy as a raw material, one study reported mean value concentrations between 1 – 5 μg/kg AFB_1 in soybeans from Russia (Oleynikova et al., 2020). AFB_1 incidence varied from 10% in soy-based burgers to 100% in soybean seeds. No data was found for the other aflatoxins in plant-based meat alternatives (soybean, pea, etc.).

Based on this data, the reports on AFB_1 occurrence in soy-based burgers indicates that they may contribute to the overall exposure to aflatoxins in the general population. Considering the toxicological concern related to aflatoxins exposure (EFSA, 2020a), the inclusion of soy-based meat alternatives in a proper exposure assessment is therefore advisable.

The highest mean contamination value for OTA was found for peas from Germany (49.4 μg/kg) (Kunz et al., 2020) while an average value of 2.26 μg/kg OTA was reported in soybeans (results pooled from 30 European countries) (EFSA, 2020b). While the incidence for soybean/soy-based food is not mentioned, the incidence of OTA in peas from Germany was 9.1% (Kunz et al.,2020).

As for aflatoxins, the contamination values found in plant-based meat alternatives for OTA are in the range of, when not higher than, the maximum permitted limit set in wheat for this mycotoxin, further suggesting a potential health concern due to the lack of a specific regulation for mycotoxins in legume-based products, especially in view of the OTA genotoxic potential recently underlined by EFSA (EFSA, 2020b).

Among *Fusarium* mycotoxins, type A trichothecenes have been often reported in legumes. Regarding the highest mean value of HT-2 in soybean/soy-based food, Schollenberger et al., (2007) found a mean concentration of 11 µg/kg in soy flour from Germany, while T-2 was found in high concentrations in soybean seeds from Russia (251.3 µg/kg) (Oleynikova et al., 2020). The latter value indicates that T-2 toxin can be found in soybeans in contents higher than the maximum limits set for T-2 + HT-2 in grains and cereal products for human consumption (15 – 200 µg/kg) (EC, 2013). For chickpea, the only available data was pooled from 20 countries where the mean upper bound (UB) was 3 µg/kg T-2 (EFSA, 2017).

One of the T-2 toxin metabolites, T-2 tetraol was also found in partially defatted soy products from Germany (32 µg/kg) (Schollenberger et al., 2007). In soy-based products, HT-2 occurred in 10% of soy flour samples and 20% of textured soy protein and partially defatted and products (Schollenberger et al., 2007).

T-2 incidence varied from 5% in soy-based burgers from Italy (Rodríguez-Carrasco et al., 2019) up to 100% in soybean seeds from Croatia (Oleynikova et al., 2020), while T-2 tetraol was found in 20% of partially defatted soy products (Schollenberger et al., 2007). For chickpea the incidence was not reported (EFSA, 2017). Scirpentriol (STO), diacetoxyscirpenol (DAS) and monoacetoxyscirpenol (MAS) were also reported in soy flour (mean value of 25 µg/kg), soy kernels (mean value of 21 µg/kg) and partially defatted soy products (mean value of 19.5 µg/kg) from Germany, respectively (Schollenberger et al., 2007). More in details, in soy-based products, the incidence for MAS and STO was 10% in soy flour, while DAS occurred in 20% of roasted soy kernels from Germany (Schollenberger et al., 2007). Although there are no regulations established for the above-mentioned mycotoxins, EFSA, (2018c) proposed a TDI for 4,5-DAS of 0.65 µg/kg b.w. which can be used for STO, DAS, and MAS as well.

DON, the most predominant type B trichothecene mycotoxin in grains worldwide, was less frequently reported in legumes compared to type A trichothecenes. Only one study reported its occurrence in soy-based burgers from Italy, along with its acetylated forms (367.5 µg/kg, 154.7 µg/kg, 757.4 µg/kg for DON, 3-AcDON and 15-AcDON, respectively) (Rodríguez-Carrasco et al., 2019). Based on this report, DON and 3-AcDON incidence was 5% in soy-based burgers from Italy (Rodríguez-Carrasco et al., 2019). DON and 15-AcDON were also reported in soy sauce and roasted soy kernels from Germany, with an incidence of 25% and 20% respectively (Schollenberger et al., 2007).

Regarding ZEN contamination in plant-based meat alternatives, the highest mean level was found again in soy flour from Germany (214 µg/kg) (Schollenberger et al., 2007). The incidence of ZEN in soybean/soy-based products was as low as 10% in soy flour and as high as 100% in soybean seeds. ZEN metabolites, α – ZEL and β – ZEL were reported in soybean seeds, soy flour and soy protein concentrate with values ranging from 2 µg/kg to 100 µg/kg; their incidence was 10% and 20% in soy flour samples and textured soy protein from Germany, respectively (Schollenberger et al., 2007).

Fumonisins have been found in legumes as well, with the highest mean value of FB_1 reported in soy-based burgers from Italy (260.5 µg/kg) (Rodríguez-Carrasco et al., 2019), and lower concentrations found in peas from Poland (0.02 – 0.9 µg/kg for FB_1, FB_2 and FB_3) (Waśkiewicz, Stępień, Wilman, & Kachlicki, 2013).

As for emerging mycotoxins, enniatins (ENA, ENA_1, ENB, ENB_1) were found in soy-based burgers with an incidence ranging from 31% to 84% (Rodríguez-Carrasco et al., 2019). *Alternaria* mycotoxins such as AOH and AME were also detected in soy-based burgers from Italy (mean content: 184.4 µg/kg, 207.5 µg/kg respectively) with a frequency range of 5 – 9% (Rodríguez-Carrasco et al., 2019). Occurrence of TEN in soybean and peas was reported by EFSA high as 1.6 µg/kg in soybean and 3.8 µg/kg in peas (pooled from three European countries) (EFSA, 2016), with an incidence in peas from Latvia of 100%.

Plant toxins from the class of TAs such as atropine and scopolamine were reported in soy-based food. The highest contamination values regarding TAs in soybean/soy-based food comes from a RASFF notification from Germany with organic soy flakes imported from Austria that had contents of 19 µg/kg atropine and 6.4 µg/kg scopolamine (RASFF 2020.0366). Atropine and scopolamine have also been reported in soybean flour (results pooled from 16 European countries with a mean middle bound of 1.54 µg/kg, respectively 0.77 µg/kg) (EFSA, 2018b).

Although tropane alkaloids have been recently regulated in a range of food commodities by the European Union (EC, 2021), no regulation is currently set or planned to be set for soybeans or other plant-based meat alternatives regardless of their reported occurrence.

Another type of alkaloids, precisely β-carboline norharman and β-carboline harman were found in soy sauce from Spain (0.044 µg/kg, 0.18 µg/kg respectively) (Herraiz, 2004). A more recent study found higher contents of β-carbolines (1a, 1b, 2, and 3) in soy sauce from Spain with mean values between 0.22 – 1,050 µg/kg (Herraiz & Vera, 2021). Soy sauce from Germany was also found to be contaminated with carbohydrate-derived β-carbolines with mean values from 643.5 µg/kg to 1,819 µg/kg (Diem & Herderich, 2001).

In consideration of the toxicological effects of these compounds, the lack of comprehensive data about the occurrence of TAs and β-carboline alkaloids in soy-based food clearly indicates the need of a more thorough research regarding the contamination of plant-based meat alternatives with alkaloids. Thoroughly investigations and further clinical studies are needed, especially for β-carboline alkaloids to draw attention and help establish health-based guidance values. Currently, no maximum limits are set for β-carboline alkaloids in food in the European Union.

3.4. Co-occurrence of mycotoxins in plant-based meat alternative reported in European studies between 2000 – 2021

Mycotoxins are known to frequently co-occur in food commodities, and their combined toxic effects are of growing interest for risk assessment. Mycotoxin co-occurrence may cause synergistic or additive interactions which may enhance the adverse effects on consumers' health (EFSA, 2013b; Tan et al., 2011; Zhao et al., 2015). While mycotoxins co-occurrence is largely studied in wheat and other cereals for human and animal consumption, very little is known about legumes.

Figure 6 displays the incidence of the co-occurring mycotoxins in soy-based food and the country from which the studies were conducted.

Insert Figure 6 here

Fourteen different combinations of toxins were reported mainly from three articles from Germany, Italy, and Russia. Although little is known so far about the overall effect of mycotoxin mixture *in vivo*, their potential combined action *in vitro* has been recently discussed in the literature with additive/synergistic effects reported in most cases. Of note, combined effects have been described for many key mechanisms of action deemed at the basis of the toxicological outcomes of mycotoxins. As an example, myelotoxicity has been described for trichothecenes and enniatins, and such an activity may be at the basis of the hematological disorders observed in humans intoxicated with these mycotoxins (Le Dréan et al., 2005). The co-exposure of human granulo-monocytic hematopoietic progenitors (cells present in the blood and bone marrow) to DON + T-2 results in additive/synergistic myelotoxic effects while DON + ZEN, T-2 + ZEN, and BEA + ENB show additive myelotoxic effects (Ficheux et al., 2012). The combined toxic effect of DON + T-2 is known to be more potent than the individual effect of these mycotoxins. DON + T-2 reduce the viability of hepatic cells (HepG2) up to more than 48% and 63% than DON and T-2 alone. The mixture further reduces the viability of HepG2 cells up to 44% more when compared with DON alone (Fernández-Blanco et al., 2018). The mixture of DON + T-2 has also antagonistic, additive, and synergic cytotoxic effects on human chondrocyte cell line (C28/I2) and rat primary chondrocytes (, which are cell systems used for studying cartilage repair mechanisms, depending on their concentration and combination ratio (Lin et al., 2019). Mixtures of AME + ENs ranged from 5% to 16% in soy-based burgers, while in soybean seed the incidence was 20% for combinations of AFB_1 with FB_1 and T-2 (Oleynikova et al., 2020; Rodríguez-Carrasco et al., 2019). The *in vitro* studies on emerging *Fusarium* and *Alternaria* toxins, which have been found in soy-based food, indicate that these mycotoxins have a wide spectrum of toxicity including cytotoxic, genotoxic, xenoestrogenic and endocrine-modulating effects (Fraeyman et al., 2017; Crudo et al., 2019). As an example, combinations of enniatins (ENs), ENA, ENA_1, ENB, and ENB_1, have additive effects which lead to significant reduction of the intestinal barrier integrity of IPEC-J2 cells (cell line derived from the small intestine) (Springler et al., 2016) and cytotoxic synergistic effects on CHO-K1 cells (cell line derived from Chinese hamster ovary) at high concentrations (Lu et al., 2013). AFB_1 + FB_1 mixtures, which have been found in soybeans, induce oxidative stress in spleen mononuclear cells and target immune cells (Mary et al., 2012).

Schollenberger et al., (2007) found toxin mixtures of A- and B-type trichothecenes together with toxins of the estrogenic group, ZEN, α-ZEL, and β-ZEL in soy food. Hence, SCIRP + MAS + HT-2 + DON + ZEN + α-ZEL + β-ZEL were found in one soy flour sample (10%), SCIRP + MAS + T-2 Tetraol + DON + ZEN + α-ZEL and MAS + HT-2 + DON + ZEN + α-ZEL were found in partially defatted soy products (40%), while a combination of MAS + HT-2 + DON + ZEN + α-ZOL was found in a soy textured protein sample (20%). Their study indicates that ZEN/α-ZEL and ZEN/β-ZEL were found in most samples, suggesting an increased prevalence of ZEN in soy food. ZEN and its metabolites have estrogenic effects (Tatay et al. 2018; Yang et al. 2017) and hepatotoxic effects (Marin et al., 2019). Mixtures of ZEN and its metabolites, α-ZOL+ β-ZOL show antagonistic effects on liver cancer cells (HepG2) at low concentrations, while at high concentrations synergistic cytotoxicity effects take place on HepG2 cells (Marin et al., 2019). The combined estrogenic or toxic effects of ZEN with its metabolites, AOH, or GEN are mainly additive or synergetic (Balázs et al., 2021) highlighting the risk consumption of soy-based food.

The cytotoxic effects of DON, ENB, and AOH were studied in human adenocarcinoma (Caco-2) cells. The mixtures decrease cell viability from highest to lowest in the following order: (DON + ENB) > (ENB + AOH) > (DON + AOH) > (DON + AOH + ENB) (Fernández-Blanco et al., 2016).

It should be noticed that the main mode of action of ZEN is through estrogenic activity. The enzymatic reduction of ZEN leads to the production of the metabolite α – ZEL which has even greater affinity for the estrogen receptors than the parent compounds, and β – ZEL, which has a lower affinity than the parent compounds. Since ZEN was found in soybean, soy flour, and soy protein concentrate, the combined exposure to other estrogenic substances (i.e., isoflavones in soya) could exert an additive, synergistic or antagonistic effect as already reported *in vitro* (Balázs et al., 2021). The interactions of genistein (GEN), an isoflavone found in soy-based food, with ZEN and AOH range from antagonistic, anti-estrogenic effects at very low concentrations to synergistic effects at 1:1 ratio (Vejdovszky et al., 2017). GEN is believed to show protective actions against estrogen-dependents cancers; however, it can also act as a mildly pro-carcinogenic compound (Adlercreutz, 2002).

The current dietary shift from traditional diets to plant-based meat alternatives is mainly drove by the beneficial aspects of the alternative diets for the environment and the human health (Micha et al., 2012; Kaluza et al., 2012). However, multiple gaps in knowledge related to the occurrence of natural toxins in plant-based meat analogues have been identified in this review. While current risk assessment for natural toxins in cereals is based on a wide base of data collected over years from multiple countries and using thoroughly validated analytical methods, information about mycotoxins and plant alkaloids contamination in plant-based meat analogues is still scant and jeopardized. Among other, the total lack of data for seitan/wheat gluten is of particular concern. Although toxic and found in high contents in soy-based food, there is also a deficiency regarding the study of TAs and β-carboline alkaloids in plant-based meat alternatives.

From a regulatory point of view, maximum limits are likely to be established for plant-based meat alternatives, especially those based on soy. In this regard, further studies are needed to clarify the potential additive/synergistic interactions between toxins and phytoestrogens occurring in soy.

Thus, future research regarding contamination data of plant-based meat alternatives with natural toxins could help to better understand the risks that come along with the shift to alternative diets, overall suggesting a need for a reconsideration of the exposure assessment to natural toxins within new dietary patterns.

4. Conclusions

This systematic review was the first attempt at assessing the chemical risk of plant-based meat alternatives related to the occurrence of natural toxins from studies conducted in Europe between 2000 – 2021. While wheat/wheat-based food are commonly carefully monitored for mycotoxin occurrence, there's a gap regarding data for semifinished materials used for seitan, and nothing is known about the fate of mycotoxins along its production chain. Of utmost relevance, the most common plant-based meat alternatives such as soybeans, chickpeas, peas, and seitan are understudied or not studied at all regarding mycotoxin and plant alkaloids occurrence.

Overall, it can be said that a shift in dietary pattern should be accompanied by a shift in the exposure scenario to ensure a proper risk assessment in regard of the actual lifestyle of the EU population.

The data from this systematic review can serve as a starting point for conducting future studies related to the contamination and chemical risk assessment of plant-based meat alternatives.

5. Limitations

This systematic review has a couple of limitations such as: i) publication bias, which is one of the most common limitation in systematic reviews; publication bias increases the publication frequency of effective studies when compared to ineffective studies (Rothstein et al., 2005), and ii) due lack of data or no data at all for the specific component of plant-based meat alternatives (i.e., soy protein concentrate, pea protein concentrate etc.) we used contamination data that we found for the plant-based meat alternatives as a raw material and any other food based on the meat alternative ingredient (i.e., soy sauce, soy products etc.).

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Figure captions

Figure 1 - Flowchart of the exclusion and inclusion studies based on the PRISMA guideline.

Figure 2 - Sankey diagram showing the country of origin of the European articles that published contamination data with mycotoxins and/or alkaloids on raw commodities used for the production of plant-based meat alternatives (wheat, soy, pea, chickpea) between 2000 – 2021; wider nodes indicate a higher number of published articles

Figure 3 - Sankey diagram displaying the number of articles on wheat, soy, pea, chickpea and the contaminants analysed in European articles between 2000 – 2021; wider nodes indicate a higher number of analysed contaminants

Figure 4 - Sankey diagram displaying the analysed mycotoxins in legumes in European articles between 2000 – 2021; wider nodes indicate a higher number of analysed contaminants

Figure 5 - Sankey diagram displaying the type of alkaloids studied in the European articles regarding wheat/wheat-based food and soybean/soy-based food between 2000 – 2021; wider nodes indicate a higher number of analysed contaminants

Figure 6 - Sankey diagram displaying the incidence of co-occurring mycotoxins in soy-based food from European studies between 2000 – 2021; wider nodes represent a higher incidence

Figure 1

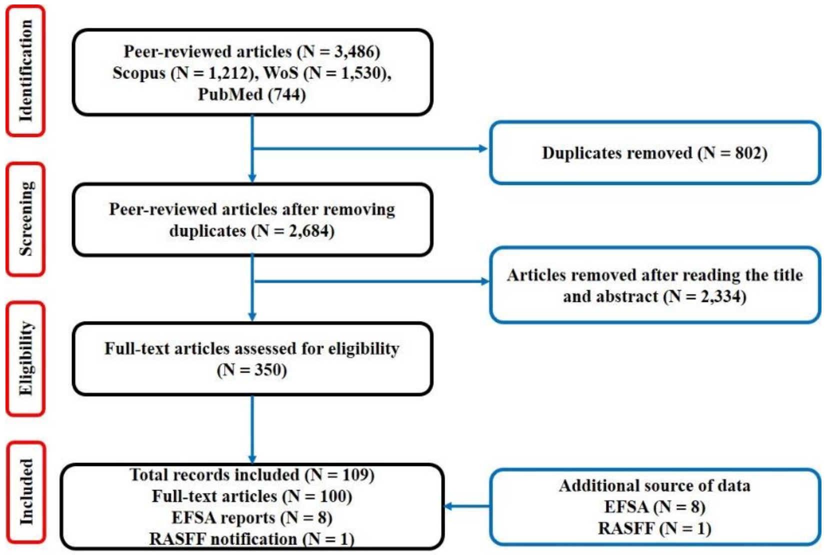
Identification
Peer-reviewed articles (N = 3,486)
Scopus (N = 1,212), WoS (N = 1,530),
PubMed (744)
Duplicates removed (N = 802)
Screening
Peer-reviewed articles after removing duplicates (N = 2,684)
Articles removed after reading the title and abstract (N = 2,334)
Eligibility
Full-text articles assessed for eligibility (N = 350)
Included
Total records included (N = 109)
Full-text articles (N = 100)
EFSA reports (N = 8)
RASFF notification (N = 1)
Additional source of data
EFSA (N = 8)
RASFF (N = 1)

Figure 2

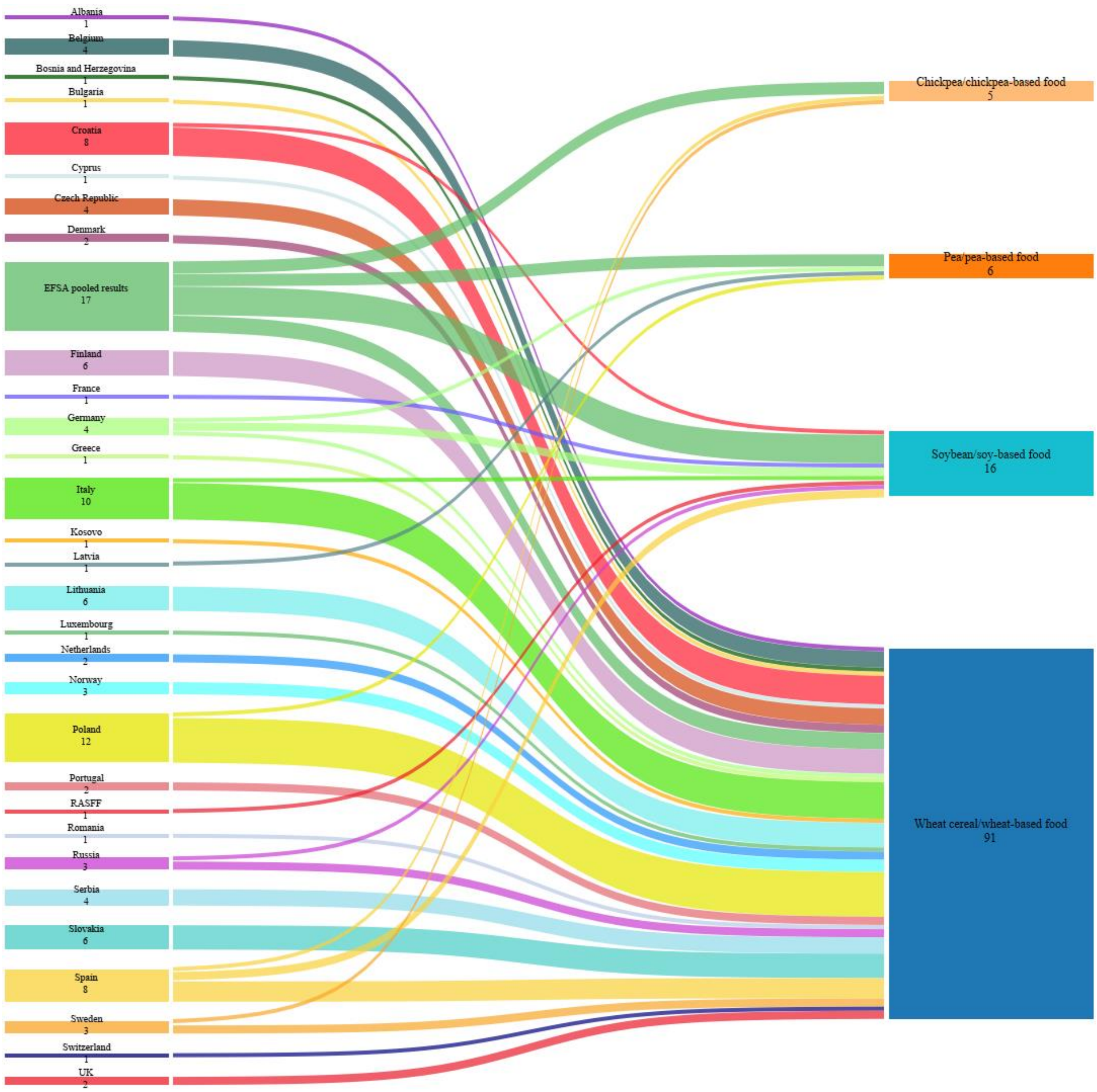
Albania 1
Belgium 4
Bosnia and Herzegovina 1
Bulgaria 1
Croatia 8
Cyprus 1
Czech Republic 4
Denmark 2
EFSA pooled results 17
Finland 6
France 1
Germany 4
Greece 1
Italy 10
Kosovo 1
Latvia 1
Lithuania 6
Luxembourg 1
Netherlands 2
Norway 3
Poland 12
Portugal 2
RASFF 1
Romania 1
Russia 3
Serbia 4
Slovakia 6
Spain 8
Sweden 3
Switzerland 1
UK 2
Chickpea/chickpea-based food 5
Pea/pea-based food 6
Soybean/soy-based food 16
Wheat cereal/wheat-based food 91

Figure 3

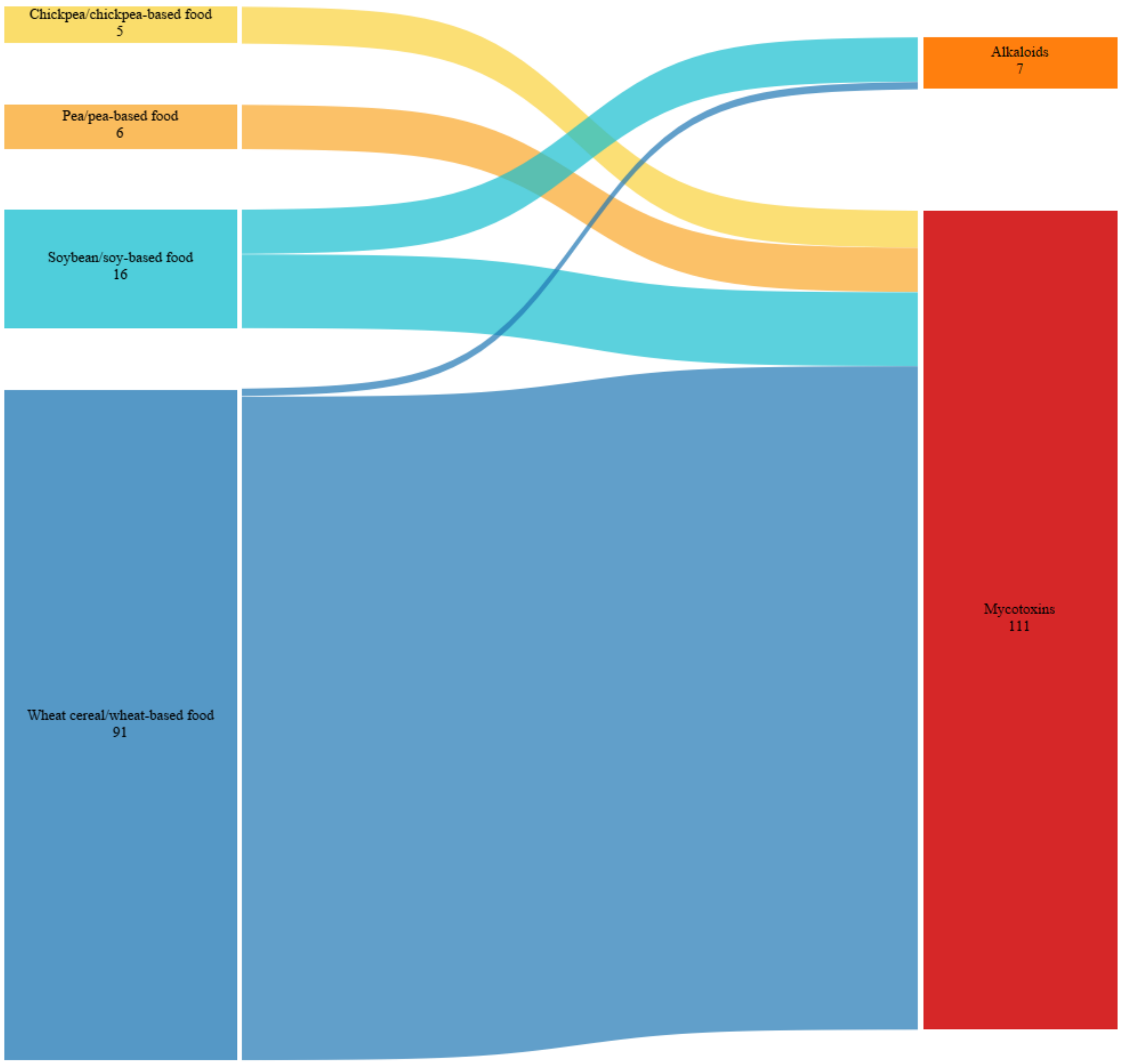
Chickpea/chickpea-based food
5
Pea/pea-based food
6
Soybean/soy-based food
16
Wheat cereal/wheat-based food
91
Alkaloids
7
Mycotoxins
111

Figure 4

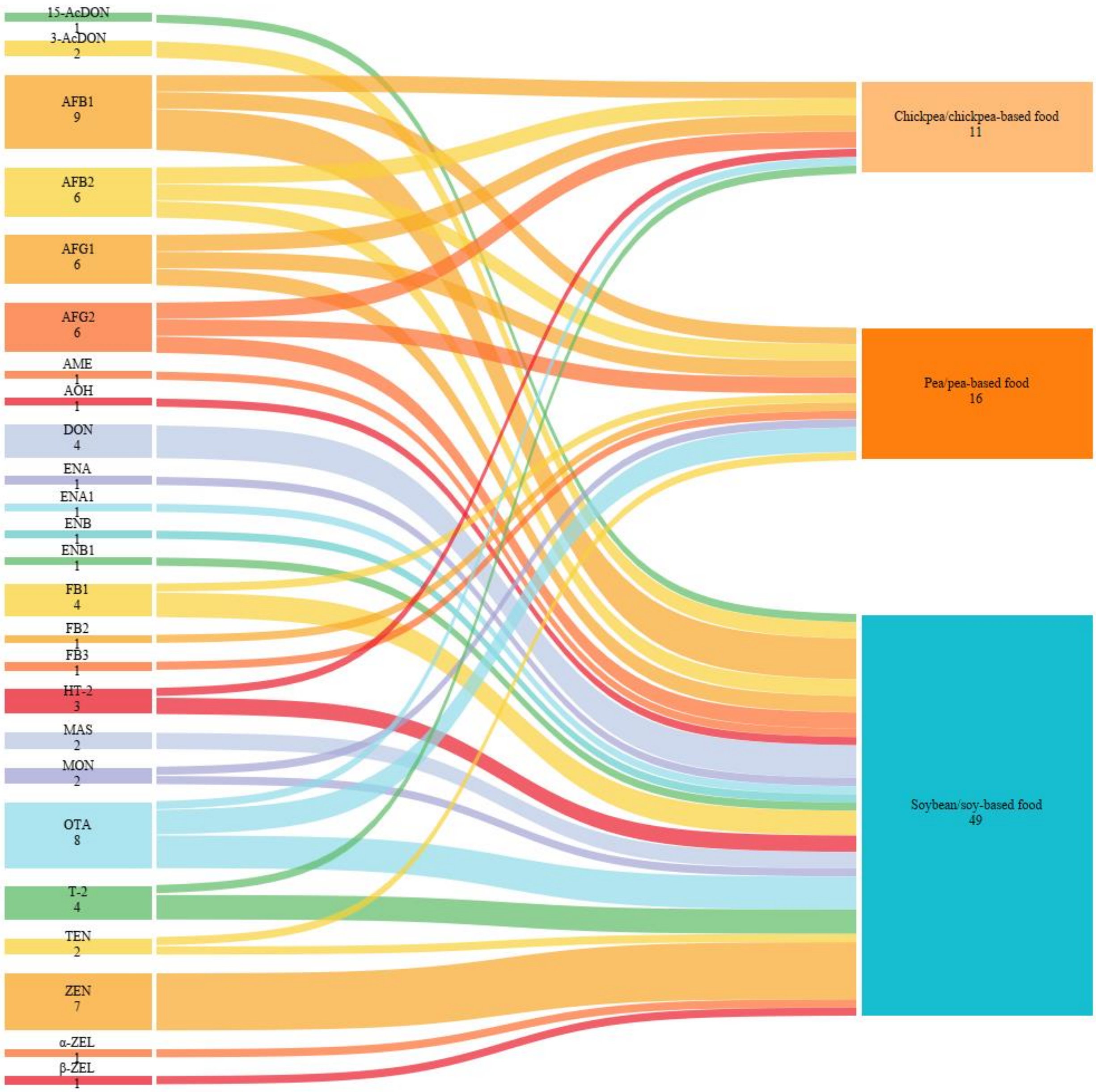
15-AcDON
1
3-AcDON
2
AFB1
9
AFB2
6
AFG1
6
AFG2
6
AME
1
AOH
1
DON
4
ENA
1
ENA1
1
ENB
1
ENB1
1
FB1
4
FB2
1
FB3
1
HT-2
3
MAS
2
MON
2
OTA
8
T-2
4
TEN
2
ZEN
7
α-ZEL
1
β-ZEL
1
Chickpea/chickpea-based food
11
Pea/pea-based food
16
Soybean/soy-based food
49

Figure 5

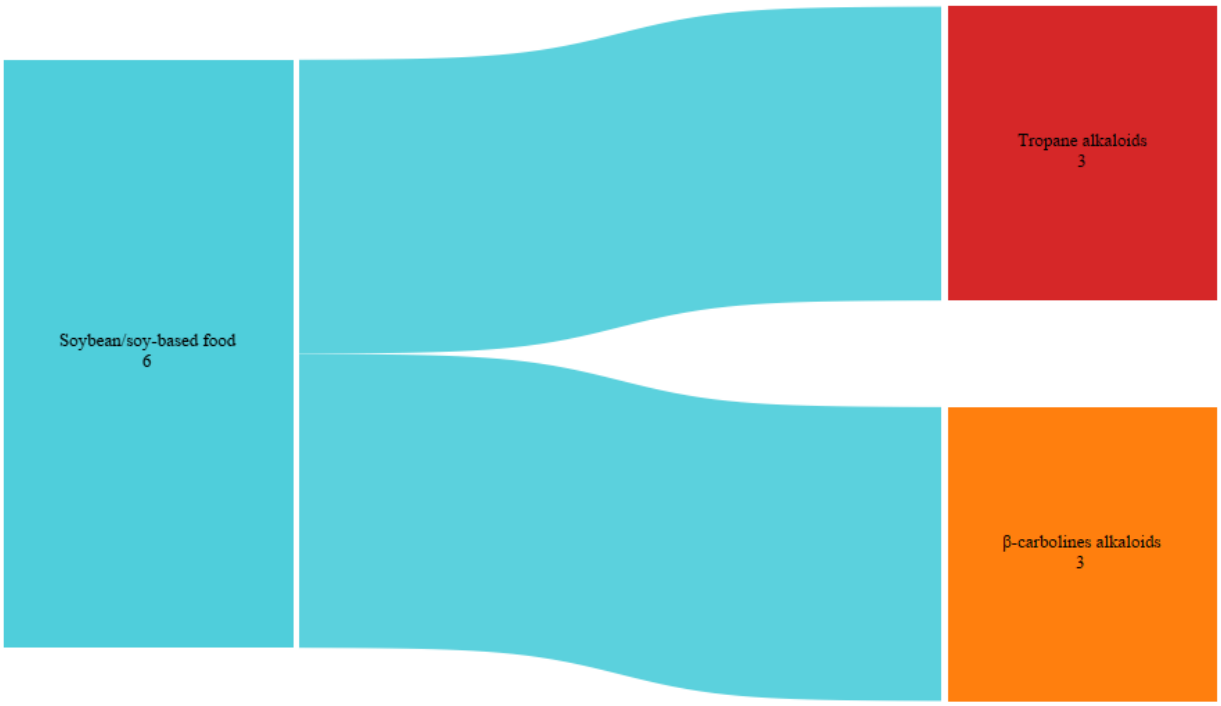
Soybean/soy-based food
6
Tropane alkaloids
3
β-carbolines alkaloids
3

Figure 6

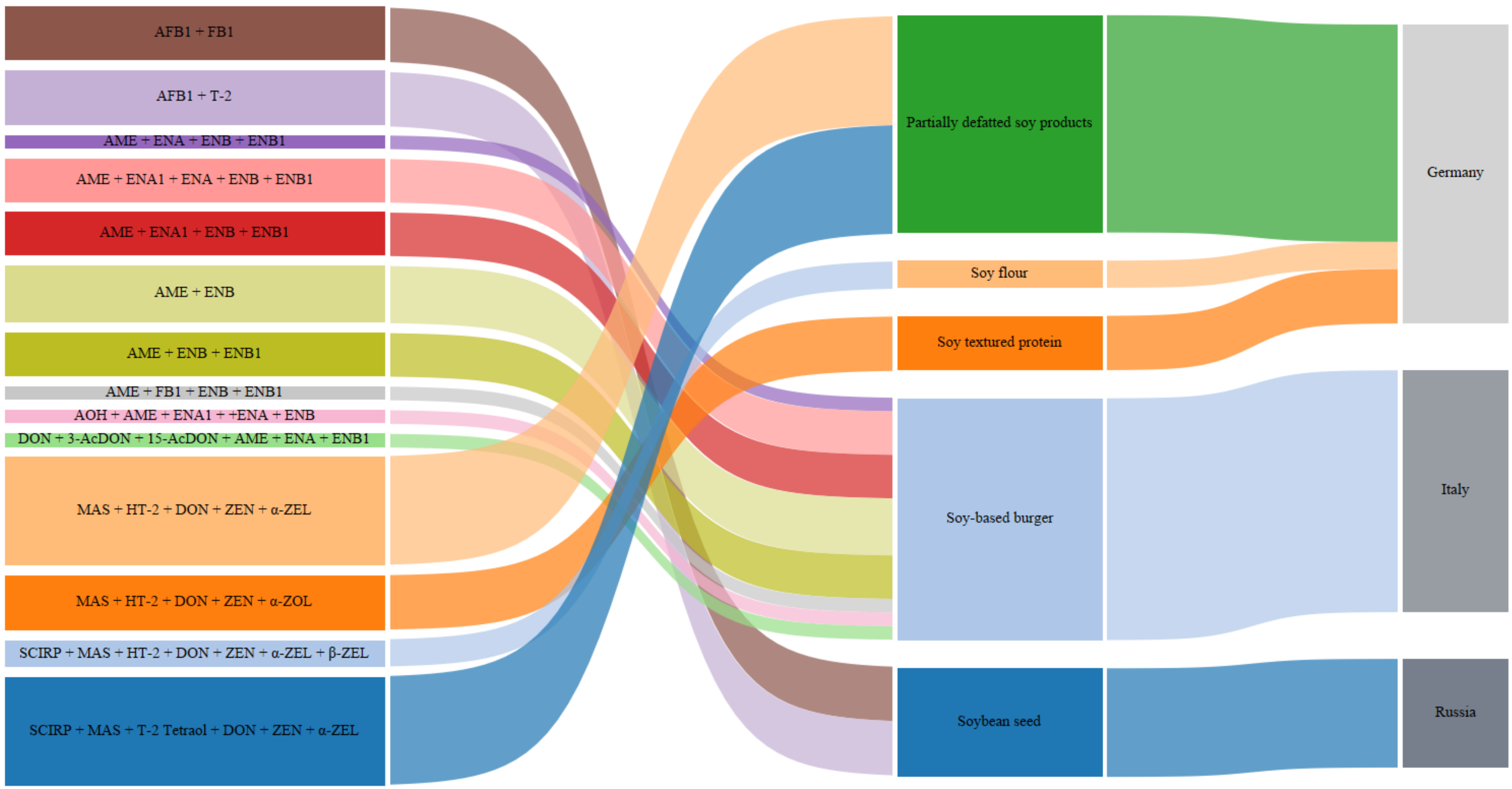
AFB1 + FB1
AFB1 + T-2
AME + ENA + ENB + ENB1
AME + ENA1 + ENA + ENB + ENB1
AME + ENA1 + ENB + ENB1
AME + ENB
AME + ENB + ENB1
AME + FB1 + ENB + ENB1
AOH + AME + ENA1 + +ENA + ENB
DON + 3-AcDON + 15-AcDON + AME + ENA + ENB1
MAS + HT-2 + DON + ZEN + α-ZEL
MAS + HT-2 + DON + ZEN + α-ZOL
SCIRP + MAS + HT-2 + DON + ZEN + α-ZEL + β-ZEL
SCIRP + MAS + T-2 Tetraol + DON + ZEN + α-ZEL
Partially defatted soy products
Soy flour
Soy textured protein
Soy-based burger
Soybean seed
Germany
Italy
Russia

Table 1. Soy/soy-based, chickpea/chickpea-based, and pea/pea-based food and the range of mean values of contamination with mycotoxins and alkaloids that were found in studies conducted in Europe between 2000 – 2021.

Food	Contaminant	Mean range (µg/kg)	Sampling year	Country	Reference
	Mycotoxins				
Soybean seeds/soy-based burgersfood	AFB_1	1.1 – 10.1	2019; 2020	Italy; Russia	Oleynikova et al., 2020; Rodríguez-Carrasco et al., 2019
Pea/pea without pods		0.00 – 0.47	2003 – 2018	Pooled from 26 European countries	EFSA, 2020a
Chickpea/chickpea flour		0.00 – 4.2			
Soybean/soybean flour	AFB_2	0.00 – 0.89			
Pea/pea without pods		0.00 – 0.2			
Chickpea/chickpea flour		0.00 – 0.4			
Soybean/soybean flour	AFG_1	0.00 – 1.76			
Pea/pea without pods		0.00 – 0.2			
Chickpea/chickpea flour		0.00 – 0.4			
Soybean/soybean flour	AFG_2	0.00 – 1.77			
Pea/pea without pods		0.00 – 0.2			
Chickpea/chickpea flour		0.00 – 0.4			
Soybean/soy-based foodsoybean flour/textured soy protein	OTA	0.95 – 2.26	2020	Pooled from 30 European countries	EFSA, 2020b

Pea/pea without pods~~-based food~~		49.4	2018	Germany	Kunz et al., 2020
Chickpea~~/chickpea-based food~~		0.1 – 5	1996 – 1998	Pooled from 30 European countries~~Sweden~~	EFSA, 2020b; Thuvander et al., 2001
~~Soybean/soy-based food~~Partially defatted soy products/textured soy protein/soy protein concentrate/soybean flour	HT-2	5 – 11	2006	Germany	Schollenberger et al., 2007
Chickpea~~/chickpea-based food~~		11.51	2016	Spain	Carballo et al., 2018
Soybean seed/soybean/soybean flour~~-based food~~	T-2	0.71 – 251.3	2011 – 2016; 2019	Results pooled from 20 countries; Russia	EFSA, 2017; Oleynikova et al., 2020
Chickpea~~/chickpea-based food~~		Mean UB: 3	2011 - 2016	Results pooled from 20 countries	EFSA, 2017
~~Soybean/soy-based food~~Partially defatted soy products	T-2 Tetraol	32	2006	Germany	Schollenberger et al., 2007
~~Soybean/soy-based food~~Roasted soy kernel	DAS	21	2006	Germany	Schollenberger et al., 2007

~~Soybean/soy-based food~~Partially defatted soy products	MAS	6 – 19.5	2006	Germany	Schollenberger et al., 2007
~~Soybean/soy-based food~~Soybean flour	STO	25	2006	Germany	Schollenberger et al., 2007
~~Soybean/soy-based food~~Soy flour/soy-based burgers	DON	11 – 367.5	2006; 2019	Germany; Italy	Rodríguez-Carrasco et al., 2019; Schollenberger et al., 2007
~~Soybean/soy-based food~~Soy sauce/soy-based burgers	3-AcDON	14 – 154.7	2006; 2019	Germany; Italy	Rodríguez-Carrasco et al., 2019; Schollenberger et al., 2007
~~Soybean/soy-based food~~Roasted soy kernels/soy-based burgers	15-AcDON	11 – 757.4	2006; 2019	Germany; Italy	Rodríguez-Carrasco et al., 2019; Schollenberger et al., 2007
~~Soybean/soy-based food~~Soy protein concentrate/soybean flour	ZEN	2 – 214	2006	Germany	Schollenberger et al., 2007
~~Soybean/soy-based food~~Textured soy protein//soybean flour	α – ZEL	6.5 – 11	2006	Germany	Schollenberger et al., 2007
Soy flour~~Soybean/soy-based food~~	β – ZEL	5	2006	Germany	Schollenberger et al., 2007

Canned s~~S~~oybean/soy-based burgers~~food~~	FB$_1$	100 – 260.5	2000 – 2001; 2019	France; Italy	LeBlanc et al., 2005; Rodríguez-Carrasco et al., 2019
Pea~~/pea-based food~~		0.9	2011	Poland	Waśkiewicz et al., 2013
Pea~~/pea-based food~~	FB$_2$	0.08			
Pea~~/pea-based food~~	FB$_3$	0.02			
~~Soybean/soy-based food~~Soy-based burgers	ENA	323.81	2019	Italy	Rodríguez-Carrasco et al., 2019
~~Soybean/soy-based food~~Soy-based burgers	ENA$_1$	67.34	2019	Italy	Rodríguez-Carrasco et al., 2019
~~Soybean/soy-based food~~Soy-based burgers	ENB	157.55	2019	Italy	Rodríguez-Carrasco et al., 2019
~~Soybean/soy-based food~~Soy-based burgers	ENB$_1$	132.18	2019	Italy	Rodríguez-Carrasco et al., 2019
Soybean/soybean flour-~~based food~~	MON	2 – 30	2002 – 2015	Results pooled from six countries	EFSA, 2018a
Pea~~/pea-based food~~		0 – 50			
~~Soybean/soy-based food~~Soy-based burgers	AOH	184.4	2019	Italy	Rodríguez-Carrasco et al., 2019
~~Soybean/soy-based food~~Soy-based burgers	AME	207.5	2019	Italy	Rodríguez-Carrasco et al., 2019
Soybean~~/soy-based food~~	TEN	0.3 – 1.6	2010 - 2015	Germany, Netherlands, Luxembourg; UK	EFSA, 2016

Pea~~/pea-based food~~		0.69 – 3.8	2019	Latvia	Reinholds et al., 2021
Food	**Contaminant**	**Mean (µg/kg)**	**Sampling Year**	**Country**	**Reference**
	Alkaloids				
Soybean flour~~/soy-based food~~organic soy flakes	Atropine	1.47 – 19	2010 – 2018; 2020	Pooled results from 16 European countries; Germany	EFSA, 2018b; RASFF 2020.0366
Soybean flour/organic soy flakes~~/soy-based food~~	Scopolamine	0.68 – 6.4	2010 – 2018; 2020	Pooled results from 16 European countries; Germany	EFSA, 2018b; RASFF 2020.0366
Soy sauce~~bean/soy-based food~~	β-carbolines norharman	0.044	2004	Spain	Herraiz, 2004
	β-carbolines harman	0.18			
~~Soybean/soy-based food~~Soy sauce	Carbohydrate-Derived β-Carbolines (1a)	1,819	2001	Germany	Diem & Herderich, 2001
	Carbohydrate-Derived β-Carbolines (1b)	1,751.8			
	Carbohydrate-Derived β-	643.5			

	Carbolines (2a/b)				
~~Soybean/soy-based food~~Soy sauce	β-Carbolines (1a)	0.63 – 954.6	2021	Spain	Herraiz & Vera, 2021
	β-Carbolines (1b)	0.65 – 1,050			
	β-Carbolines (2)	0.22 – 968.8			
	β-Carbolines (3)	0.38			

UB = upper bound

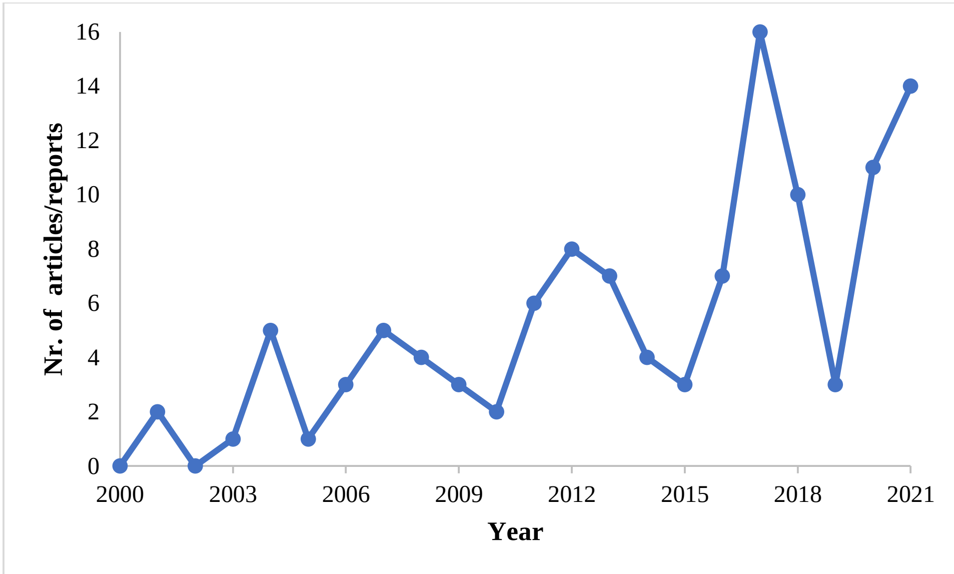

Figure S1. The number of European articles/reports published regarding mycotoxins and plant alkaloids in plant-based meat alternatives (wheat, soy, pea, chickpea) between 2000 – 2021

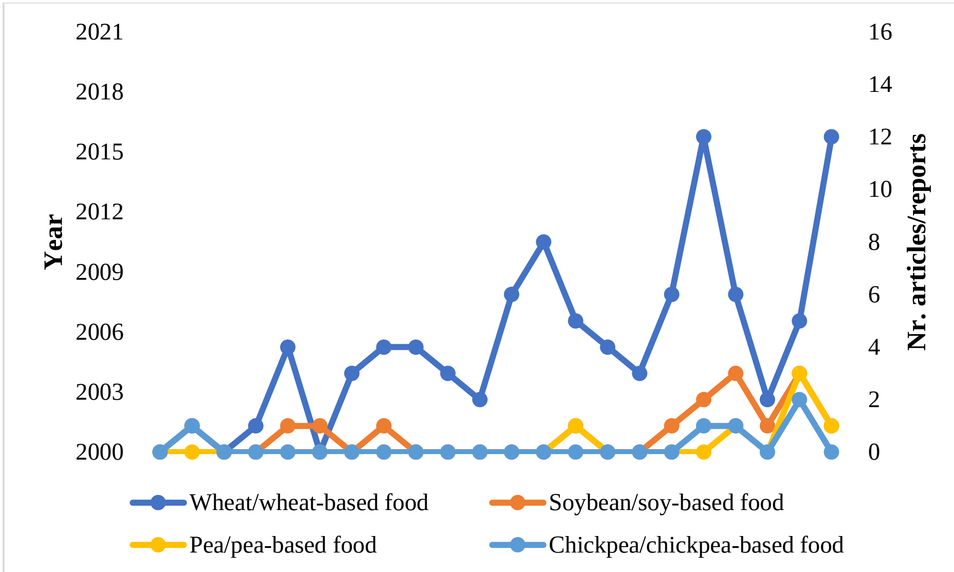

Figure S2. The number of European articles/reports published regarding mycotoxins and plant alkaloids in wheat/wheat-based food, soybean/soy-based food, pea/pea-based food, chickpea/chickpea-based food between 2000 – 2021

Graphical Abstract

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CRediT author statement

Octavian Augustin Mihalache: Conceptualization, Software, Formal analysis, Investigation, Writing - original draft, Writing – review & editing, Visualization

Luca Dellafiora: Formal analysis, Investigation, Writing – review & editing, Supervision

Chiara Dall'Asta: Conceptualization, Resources, Formal analysis, Investigation, Writing - original draft, Writing – review & editing, Project administration, Supervision