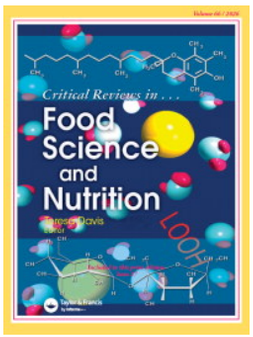

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REVIEW ARTICLE

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Toward human-relevant toxicology: a decision-oriented roadmap integrating modern tools for the safety evaluation of novel foods, functional ingredients, and nutraceuticals

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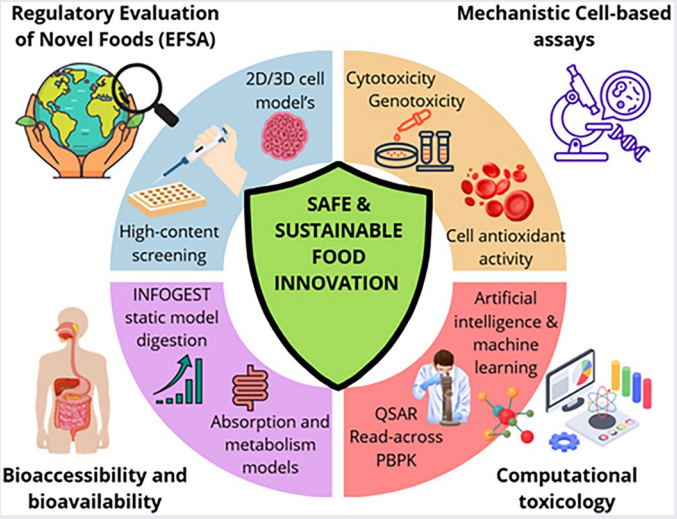
ABSTRACT

Ensuring the safety of novel foods, functional ingredients, and nutraceuticals is increasingly challenging as global food systems shift toward sustainable, health-aligned innovations. This review synthesizes conventional and next-generation approaches in food toxicology to outline a mechanistic, human-relevant, and decision-oriented framework for evaluating novel foods, nutraceuticals, and ingredients. Emphasis is placed on integrating multidisciplinary tools, including regulatory benchmarks, state-of-the-art analytical protocols, and *in vitro* and in silico systems, which are reshaping safety assessment pipelines. The dynamic regulatory context is examined, with a focus on the European Food Safety Authority's updated requirements for novel foods and its tiered toxicological strategy for assessing genotoxicity, toxicity, allergenicity, and chemical risk. Emerging scientific and technological trends are highlighted as the main drivers of a more predictive and ethically aligned toxicological paradigm. These include high-throughput cellular assays, omics-enabled molecular profiling, physiologically relevant *in vitro* models, computational prediction tools, and technologies for chemical and bioactivity characterization. Together, these advances support a modern, mechanism-based approach to safety evaluation. The review demonstrates how integrating these tools can strengthen science-based decision-making, enhance regulatory confidence, and promote responsible innovation in the advancement of next-generation foods and functional ingredients.

KEYWORDS

Computational toxicology; mechanism-based risk evaluation; next-generation *in vitro* models; novel foods safety assessment

GRAPHICAL ABSTRACT



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Introduction

Zero hunger is a key Sustainable Development Goal proposed by the United Nations for 2030, calling for worldwide cooperation to eradicate hunger and improve nutrition (United Nations 2015). Yet, the mass production of monocultures and animal products brings environmental challenges, stressing the need for new food products to meet global dietary needs (Milião et al. 2022; Rahayu et al. 2024). Ongoing scientific and technological advances in food science and nutrition have led to a steady increase in the development of new foods and food ingredients worldwide. As a result, regulatory authorities play a crucial role in ensuring that these novel products are safe for human consumption before they are marketed. Within the European Union, the European Food Safety Authority (EFSA) defines "novel foods" (NF) as foods or food ingredients that were not consumed to a significant extent in the EU before 15th May 1997, the date on which the first EU novel food regulation entered into force. This category encompasses new foods and ingredients (e.g., proteins and peptides sourced from insects), foods and ingredients obtained from previously unused sources, chemical compounds isolated from food materials and deliberately added to foods, such as phytosterols incorporated into margarine, and foods produced using innovative production methods or processing technologies, including approaches such as ultrasound-assisted extraction or ultraviolet radiation treatment. There is a common understanding of NF definitions and safety assessment requirements across countries. For example, Standards 1.1.1 and 1.5.1 of the Australia - New Zealand Food Standards Code stipulate that NFs must undergo a risk-based public health and safety assessment and specify conditions of use, including permitted use levels, use restrictions, and labeling requirements (Novel Foods 2022). In this scenario, researchers have made breakthroughs in food science and technology to explore previously unused sources or macro and micronutrients, such as carbohydrates, proteins, and lipids, to develop not only foods and ingredients, but also nutraceuticals, concentrated sources of nutrients or other substances (e.g., vitamins, minerals, amino acids, or plant extracts), presented in dose form (e.g., capsules, tablets, or powders), and intended to supplement the normal diet (Zahir et al. 2026). For example, rapeseed protein isolate is considered a novel food because, although rapeseed oil was widely consumed, isolated rapeseed protein had no prior history of use in the EU. Rapeseed protein isolate has now been tested at lab scale in high-protein food formulations, such as pasta (Axentii et al. 2025). Similarly, cricket and grasshopper protein isolates, which had no traditional use in the EU before 15th May 1997, have also recently been characterized as novel food ingredients (Sánchez-Velázquez et al. 2025). Another example of novel carbohydrates is human-identical milk oligosaccharides (HiMOs, e.g., lacto-N-tetraose, 3′-fucosyllactose, and lacto-N-neotetraose), which are structurally identical to human milk components but were not present in the EU food supply before 15th May 1997, except via breastfeeding. Industrial production (via microbial fermentation or enzymatic synthesis) makes them novel ingredients when added to foods such as infant formula and supplements. HiMOs are listed as novel foods following EFSA's safety assessment, and clinical trials are underway to assess their functional effects in humans (Phipps et al. 2018; Scheuchzer et al. 2024). For clarity, "functional ingredients" are intentionally added or retained to provide a specific physiological benefit beyond basic nutrition and remain within the food matrix rather than being presented as medicines.

While novel foods provide adequate nutrition, their safety must also be rigorously established before market introduction. This leads to a critical toxicological question: at what point does a beneficial nutrient or bioactive compound become harmful? For example, alkaloids, naturally present in coffee, cacao, and tea, can exert beneficial physiological effects at low or moderate intakes but may pose toxicological risks at higher concentrations. Although alkaloids have health effects such as antioxidant and anti-HIV activities, specific doses can cause toxicity and adverse health outcomes (Thawabteh et al. 2021; Akinboye et al. 2023). Similarly, terpenes, unsaturated hydrocarbons present in various foods, can have beneficial effects, including anticancer and anti-inflammatory properties, but can also pose risks, such as neurotoxicity and genotoxicity (Ninkuu et al. 2021; Wojtunik-Kulesza 2022). Therefore, assessing both nutritional value and dose-related safety is essential in the development and regulation of NF. Currently, the evaluation of NF toxicity relies on *in vitro*, *in vivo*, and in silico methods. Still, these approaches can be strengthened by developing more predictive assays, elucidating mechanisms of toxicity, and accounting for bioaccessibility and bioavailability.

To ensure a decision-oriented perspective, this review addresses four central questions: (i) how current regulatory frameworks and conventional toxicological approaches support hazard identification and safety evaluation of novel foods; (ii) how emerging mechanistic and *in vitro* methodologies, including digestion and cellular models, improve the understanding of compound transformation, bioaccessibility, and bioavailability; (iii) how these experimental outputs can be translated into internal dose metrics through computational approaches such as physiologically based pharmacokinetic modeling; and (iv) how the integration of new approach methodologies, omics technologies, and *in silico* tools contributes to next-generation risk assessment and regulatory decision-making. Overall, the manuscript is structured to reflect the progression from data generation to decision-making in food safety assessment. Section 2 examines traditional and novel toxicological evaluation strategies within regulatory frameworks, with emphasis on EFSA requirements and hazard identification. Subsequent sections explore emerging experimental models, including *in vitro* digestion systems and their integration with cellular assays, to characterize bioaccessibility, absorption, and metabolism of food-related compounds. Building on this, computational toxicology approaches, such as QSAR, network toxicology, and PBPK modeling, are presented as key tools to translate external exposure into internal dose and mechanistic understanding. Finally, the review discusses how these combined approaches support NGRA paradigms, integrating mechanistic evidence,

quantitative modeling, and regulatory requirements to enable more predictive, human-relevant, and transparent risk assessment.

Review methodology

This narrative review was undertaken to provide a structured and critical synthesis of current advances in food toxicology, with a particular focus on human-relevant *in vitro* approaches and next-generation methodologies. The literature search was conducted using major scientific databases, including Web of Science, Scopus, and PubMed. The search primarily covered publications from approximately 2010 to 2025, while earlier seminal studies were included when necessary to provide historical context or foundational concepts. Search strategies employed combinations of keywords related to the scope of the review, including "food toxicology," "novel foods," "new approach methodologies (NAMs)," "*in vitro* models," "in silico toxicology," "EFSA," and "risk assessment." Article selection was guided by relevance to the aims of the review, scientific robustness, and contribution to methodological innovation, mechanistic insight, or regulatory application. Priority was given to peer-reviewed original research articles, authoritative reviews, and regulatory or guidance documents from recognized bodies. The selected evidence was synthesized using a narrative approach, emphasizing conceptual integration across disciplines, recent methodological developments, and implications for regulatory risk assessment, rather than quantitative meta-analysis.

Traditional and novel methods for toxicological safety evaluation of novel foods and ingredients

Novel foods and ingredients safety evaluation: a review of EFSA's regulatory framework

Interest in novel food categories is spurring the development of alternative raw materials and NF technologies, thereby necessitating that these products meet stringent safety assessment standards established by national authorities (Heo et al. 2024). EFSA is the primary body responsible for the scientific appraisal of NF, reviewing submissions on their composition, nutritional characteristics, toxicological data, and potential allergenic risks (Turck et al. 2021; Le Bloch et al. 2025). Before 15th May 1997, foods not previously consumed in Europe were classified as NF, regulated under (EC) 258/97. The expansion of global markets and rapid technological progress prompted a revision of the framework under Regulation (EU) 2015/2283, which entered into force in January 2018. Under this updated legislation, the definition of food is anchored in the established categories of novel foods, encompassing materials derived from plants, animals, microorganisms, cell cultures, and minerals, as well as products produced using emerging technological processes (Heo et al. 2024). Recently, EFSA has updated its scientific guidance on NF applications, introducing more stringent safety assessment requirements (Le Bloch et al. 2025).

To qualify for evaluation by EFSA (Turck et al. 2021), a NF or ingredient must be comprehensively characterized across multiple dimensions. This encompasses clarification of its origin, whether it is a chemical entity, polymer, cell culture extract, or a material obtained from animals, plants, microorganisms, fungi, or algae, together with a detailed description of the manufacturing process. Applicants are required to submit compositional information, including data on stability, as well as a clear explanation of the intended function or application of the NF or its source material. They must also outline proposed uses, expected use levels, and estimated intake, considering the target consumer group, potential exposure to undesirable compounds, and any relevant precautions or limitations. Additional documentation must address aspects of digestion, absorption, distribution, metabolism, and excretion; nutritional characteristics; toxicological evidence such as genotoxicity and subchronic or chronic toxicity; and any indications of allergenic potential. For toxicological evaluation, the chemical identity, molecular structure, and physicochemical properties of the novel food or ingredient must be clearly defined. Consequently, chromatographic techniques are fundamental for identifying and quantifying individual chemical constituents, enabling a solid chemical risk assessment in accordance with EFSA regulatory criteria (Gao et al. 2011). However, EFSA's NF framework (Regulation EU 2015/2283) encompasses several regulatory routes, including traditional foods from third countries and assessments informed by prior history of use or existing evidence, such that data requirements—including toxicology—are tailored on a case-by-case basis rather than uniformly extensive. For example, (i) traditional foods from third countries are assessed based on a documented history of safe use, (ii) cases where a history of consumption or prior authorization can substantially reduce data requirements, and (iii) situations where equivalence or similarity considerations can be used to tailor the assessment.

When toxicological data need to be assessed, a) the initial stage involves evaluating genotoxic potential through approaches aligned with current regulatory frameworks, particularly the ICH S2(R1) "Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use" which replaced and combined the ICH S2A and S2B guidelines. This guideline is based on the standard 3-test battery, retaining the bacterial reverse mutation (Ames) assay as a core component, combined either with *in vitro* assessment of chromosomal damage followed by *in vivo* confirmation, or with integrated *in vivo* testing across two tissues, addressing complementary endpoints such as micronucleus formation and DNA strand breaks. The corresponding OECD test guidelines for these assays are detailed in Section 2.3. This integrated testing strategy reflects the need to combine complementary assays, as no single method can capture the full spectrum of genotoxic mechanisms and to enable the detection of substances capable of inducing heritable genetic damage (Baldrick 2021; Hayashi 2022); (b) Subsequent tiers include subchronic toxicity investigations, typically 28-day/90-day oral studies in rodents, designed to assess systemic toxicity, alterations in organ weights, histopathological changes, and clinical chemistry parameters

following repeated oral exposure, where 90-day repeated-dose studies are the typical "subchronic" anchor, while 28-day is "range-finding," depending on the context (Ajiboye et al. 2024); (c) Reproductive and developmental toxicity should be performed to assess if the novel food/ingredient can trigger any harmful effects on reproductive organs or parameters (El-Hak et al. 2025); (d) Chronic toxicity and carcinogenicity studies are required if long-term exposure is expected or if there exist concerns from subchronic or genotoxicity studies (Ma et al. 2025); (e) Allergenicity assessment of protein-containing foods and ingredients is based on protein structure, homology to known allergens, and history of safe use (Pi et al. 2025); (f) Toxicokinetic study of novel chemical compounds with unclear metabolic fate to investigate absorption profile, distribution, metabolization, and excretion (Mutlu et al. 2020; Paula et al. 2025); and (g) human data, if available, from clinical studies.

Although the existing framework places a strong emphasis on consumer protection, there is no thorough evaluation of the timelines for NF application procedures. Toxicological assessments continue to rely heavily on standard methodologies, particularly animal-based testing. As a result, existing food toxicology frameworks are not yet fully equipped to incorporate next-generation approaches, including organ-on-a-chip technologies and artificial intelligence (AI)-based toxicological models. This limitation is not only conceptual but also operational, reflecting specific regulatory barriers related to validation requirements, defined contexts of use, uncertainty characterization, and standardized reporting formats. From a regulatory perspective, agencies such as the EFSA require the systematic identification of uncertainties and, wherever possible, validation against relevant data; unverified models or poorly documented performance are not acceptable. EFSA expects clear statements of model performance (e.g., reproducibility, sensitivity, and biological relevance), error margins or analogous quantitative indicators, and justification that performance is adequate for the intended regulatory decision (Benford et al. 2018; Cattaneo et al. 2023). Acceptance depends critically on the problem formulation and the context of use (Agent/Pathway/Receptor/Intervention/Output) and on aligning evidence and methods with these questions. For NF, EFSA defines domain-specific contexts of use (e.g., safety under proposed intake levels; decontamination efficiency; full molecular characterization) and expects methods to answer these specific questions (Hardy et al., 2017).

To enhance human relevance and reduce reliance on animal testing, future regulatory frameworks must embrace New Approach Methodologies (NAMs). NAMs are a broad set of non-animal, human-relevant methods used to generate data on biological effects, hazards, or mechanisms of action. They include *in vitro* systems, *in silico* models, organoid technologies, omics technologies, and other alternative approaches and are intended to improve the efficiency, relevance, and ethical basis of safety and risk assessment (Hinterberger and Stelmach 2026). In a recent study, Veltman et al. (2025) introduced a harmonized, scientifically validated workflow for evaluating the regulatory applicability of NAMs. Using craniofacial malformations induced by in utero exposure to triazoles as a case study, the authors demonstrated that the proposed workflow enables transparent and scientifically robust assessment of human-relevant toxicological pathways. Nonetheless, the use of NAMs in food toxicology remains in its early stages and warrants further attention to ensure their practical and reliable use in food science and technology.

Cellular (2D and 3D) and animal testing methods – pros and cons

Experimental animals have historically served as the "gold standard" for toxicological assessment in drug development, chemical safety, cosmetics, and food ingredients, given their capacity for modeling systemic metabolism and long-term health outcomes (Reddy et al. 2023). Their use is supported by ethical codes of conduct, which stipulate that animal testing should precede human experimentation (Poh and Stanslas 2024). Nevertheless, despite decades of use, animal models show notable drawbacks, including interspecies variability, high costs, ethical issues, and limited predictive value and direct translatability to human health (Gao et al. 2025).

In response to these challenges, the principle of the "3Rs"—Replacement, Reduction, and Refinement—has become a keystone of modern toxicology, promoting the advancement of more human-relevant approaches (Gao et al. 2025). The European Partnership for Alternative Approaches to Animal Testing (EPAA), established in 2005, illustrates this shift by supporting collaboration between regulatory bodies and industry to accelerate the adoption of alternative methods (Tarazona et al. 2025). Moreover, increasing regulatory restrictions on animal testing for cosmetics in specific jurisdictions, together with broader societal and policy-driven efforts to reduce animal use in safety assessment, underscore the growing demand for alternative approaches (Poh and Stanslas 2024). This has spurred a worldwide movement toward the development and adoption of NAMs, which encompass *in vitro*, *in silico*, *in chemico*, and *in vivo* animal surrogates that offer the potential for more human-relevant, ethically sound, and cost-effective safety assessments (Wojtunik-Kulesza 2022).

Among these alternatives, cellular models have gained prominence. Traditional two-dimensional (2D) cell cultures remain widely used because of their simplicity, reproducibility, and suitability for rapid screening. However, 2D systems do not reproduce tissue complexity, cell–cell interactions, and microenvironmental cues, which limits their physiological relevance. To overcome these limitations, three-dimensional (3D) models, including spheroids, organoids, and microfluidic "organ-on-chip" systems, have been developed. These models more closely replicate *in vivo* tissue architecture, function, and metabolic gradients, thereby greatly enhancing the predictive value of toxicological studies. Nonetheless, their application is constrained by technical challenges, cost, and standardization issues, which currently limit their widespread regulatory adoption. In particular, the translation of 3D models into regulatory contexts requires robust validation frameworks, including demonstrating inter-laboratory reproducibility, using appropriate positive and negative controls, and benchmarking against well-characterized reference

compounds. Moreover, harmonized protocols for culture conditions, endpoints, and data reporting are essential to ensure comparability across studies. Without these elements, variability between platforms and laboratories may compromise confidence in the generated data, highlighting the need for coordinated efforts toward standardization and fit-for-purpose validation before these models can be routinely integrated into risk assessment frameworks (İpek et al. 2023).

Computational and *in silico* approaches, including quantitative structure–activity relationship (QSAR) models, read-across methods, and artificial intelligence (AI)-driven simulations, complement *in vitro* systems by predicting toxicological endpoints based on biological data and chemical structure (Reddy et al. 2023). Integrating these computational tools with 3D culture models offers a powerful approach to improving safety assessments while minimizing animal use. In conclusion, while no single method currently provides a complete replacement for animal testing, integrating various *in vitro* systems together with computational models is progressively redefining the toxicological toolbox. Such approaches are consistent with the 3Rs principle, increase predictive accuracy for human outcomes, and show promise to reduce, and eventually replace substantially, animal experimentation in food safety and toxicology.

Cutting-edge cytotoxicity assays for screening novel foods, ingredients, and nutraceuticals

The food matrix, a complex assembly of natural products, represents one of the richest and least-explored chemical ecosystems on Earth. Bioactive compounds found in foods, such as polyphenols, carotenoids, vitamins, terpenoids, and alkaloids, act as natural defence molecules and constitute an immense reservoir of bioactive compounds with potential health benefits. Yet, of the estimated 49,000 secondary metabolites, only about 26,000 have been characterized and are known to have bioactivities, leaving a vast number of food-derived compounds unstudied (Barabási et al. 2019). This chemical diversity delivers promising scaffolds for functional ingredients, nutraceuticals, and novel foods, but also demands systematic toxicological evaluation to ensure safety. The pharmaceutical sector offers a useful comparison: from 1981 to 2014, more than half of all authorized small-molecule therapeutics originated from natural compounds or were structurally modeled on them (Azevedo et al. 2023). Currently, this same inspiration drives both industries, in which natural compounds and their synthetic analogues are designed and modified from the laboratory bench through *in silico* and *in vitro* simulations. In this transforming landscape, establishing high-throughput, predictive toxicological screening frameworks is key to bridging innovation and safety, ensuring that the next generation of food prototypes is both successful and reliable.

In this innovation process, toxicological screening functions as a critical control point, connecting the development of food-related products to practical applications. In this workflow, toxicity assessment occupies a central position, typically following the identification and synthesis of promising candidate chemical compounds with alleged health benefits and preceding large-scale production and human testing. Its primary role is to rapidly filter out unsafe or unstable molecules, reducing attrition rates and ensuring that only compounds with acceptable safety profiles advance to later stages. Toxicological evaluation of novel foods, functional ingredients, and nutraceuticals relies on a suite of cell-based assays that may share principles and biological targets, including assessments of cell viability, metabolic activity, membrane integrity, cell death pathways, DNA damage, and other metabolic endpoints (Table 1). These methods provide a detailed safety profile by detecting metabolic, structural, and genotoxic effects while accounting for the intrinsic complexity of food toxicology, which is shaped by the widespread, continuous consumption of foods across diverse populations.

In cell viability assays, changes in cell number during *in vitro* toxicity screening depend primarily on cell proliferation and death, thereby determining whether cell populations expand or shrink over time (Sabirova et al. 2025). Cell death, however, is closely connected with metabolic networks, which can differentially affect biomarkers commonly used in viability assays. In addition, particular attention must be given to assay interference when evaluating complex food matrices. Bioactive compounds such as polyphenols, pigments, and reducing agents (e.g., ascorbic acid, vitamin E) may directly interact with tetrazolium salts, reducing them to formazan in the absence of cells, instantly generating a dark blue product and producing false signals of cytotoxicity or protection. These artifacts are common and assay- and compound-dependent, so cell-free interference controls and complementary or non-spectroscopic viability methods are essential when testing such agents (Somayaji and Shastry 2021). Therefore, careful interpretation is required to distinguish true metabolic alterations from those arising from accidental cell death, regulated cell death pathways, autophagy, senescence, or other cellular perturbations (Zein-Sabatto et al. 2025). Advances in analytical and cellular-based metabolic methodologies have increased their relevance, making them central tools in toxicological evaluation. Techniques such as extracellular flux analysis (Seahorse) allow real-time quantification of glycolysis, mitochondrial respiration, and fatty acid oxidation, providing a dynamic view of how xenobiotics or bioactive compounds alter cellular energy metabolism. Complementary assays, including lactate dehydrogenase (LDH) release, serve as sensitive markers of loss of cell membrane integrity/necrosis, linking cytotoxic outcomes to underlying metabolic dysfunction. Together, these approaches form a comprehensive system for assessing cellular bioenergetics and detecting early toxic responses, thereby linking metabolic pathway analysis to mechanistic toxicology and next-generation risk assessment (Yoo et al. 2024).

In line with this perspective, Zein-Sabatto et al. (2025) emphasize multi-assay strategies to capture the complex and overlapping mechanisms of cell death, acknowledging that no single endpoint can fully represent cellular viability. The authors integrated multimodal cytotoxicity data using a multivariate framework based on linear mixed-effects regression

Table 1. Integrated summary of cell-based assays applied in food toxicology.

Assay type/example	Measured endpoint or principle	Key features/mechanism	Applications in food toxicology	Typical references
Dye Exclusion (e.g., Trypan Blue, VVBlue)	Membrane integrity (live/dead discrimination)	Dead cells uptake dye; live cells exclude it	Simple and direct viability count; used for high-throughput screening of cytotoxicity	(Kamiloglu et al., 2020) (Guidelines for cell viability assays); (Vitipon, et al., 2025)
Colourimetric (e.g., MTT, WST-1, NRU, LDH)	Metabolic activity, mitochondrial function, enzyme activity	Reduction-based colour change indicating metabolic competence	Detects cytotoxicity, metabolic inhibition, and membrane damage from food ingredients or contaminants	(Kamiloglu, et al., 2020) (Guidelines for cell viability assays); (Vitipon, et al., 2025)
Fluorometric (e.g., Resazurin, GelRed, SYTO 9, Annexin V, caspase activation, and DNA fragmentation assays (e.g., TUNEL)	Fluorescent indicator of live/dead status or DNA content	High sensitivity allows multiplexing and flow cytometric quantification, providing kinetic and mechanistic detail.	Used for evaluating oxidative stress and viability. Apoptosis and Necrosis Detection:	(Kumar et al., 2015); (Zheng, et al., 2025); (Sabirova, et al., 2025)
Luminometric (e.g., ATP-based assays)	ATP quantification as a viability marker	Highly sensitive, rapid, and compatible with automation	Detects early metabolic stress and cytotoxicity in food prototype screening	(Kamiloglu et al., 2020) (Guidelines for cell viability assays); (Desousa et al., 2023)
LDH Assay	Extracellular LDH activity (membrane leakage)	Indicates necrosis and late-stage apoptosis	Used to differentiate between necrotic and apoptotic cell death induced by food compounds	(Tihăuan et al., 2023); (Marzi et al., 2024)
Seahorse Assay	Oxygen consumption and extracellular acidification	Measures mitochondrial and glycolytic function	Evaluates mitochondrial toxicity and metabolic disruption by bioactive food components	(Desousa et al., 2023)
Multiparametric/Combined	ATP, esterase, DNA integrity, and other endpoints	Captures multiple cellular injury mechanisms	Increases accuracy and reduces false negatives in the toxicological screening of novel food ingredients	(Zein-Sabatto et al., 2025); (Sabirova et al., 2025)
Advanced/Label-Free (e.g., THz spectroscopy, membrane potential)	Real-time cell property analysis without staining	Non-destructive and continuous monitoring of living cells	Allows dynamic assessment of cellular responses to complex food matrices or nanomaterials	(Madorran et al., 2022)

Note: Summary of the most used assay platforms for evaluating cytotoxicity, metabolic activity, and membrane integrity in food toxicology. Multiparametric, label-free assays are increasingly used to capture multiple endpoints, thereby improving predictive accuracy and compliance with non-animal testing frameworks.

and principal component analysis (PCA), enabling dimensionality reduction and correlation of multiple assay outputs. Applied among distinct culture models (self-aggregating 3D tumor microtissues and primary cortical neurospheroids), this approach revealed various mechanisms of action not captured by single-assay designs. In line with the growing use of multi-assay strategies for cytotoxicity assessment, Sabirova et al. (2025) proposed an integrated flow cytometry workflow that simultaneously quantifies cell death and proliferation through multiparametric analysis. The protocol combines propidium iodide, annexin V, JC-1 staining, bromodeoxyuridine, and Cell Trace Violet within a single experiment, facilitating concurrent evaluation of apoptosis, cell cycle progression, membrane integrity, and mitochondrial potential.

In addition to multiparametric cytotoxicity assays, genotoxicity and mutagenicity testing are regulatory cornerstones in food toxicology, providing the scientific basis for evaluating food additives, contact materials, and novel foods or ingredients. Genotoxicity and mutagenicity testing are mandated by agencies such as the U.S. Food and Drug Administration (FDA), the EFSA, and the Organization for Economic Cooperation and Development (OECD), for the safety assessment of substances that may enter the food chain. This is due to the potential of genotoxic agents to cause DNA damage, mutations, and ultimately increase cancer risk or heritable genetic disorders (Hayashi 2022).

The terms "genotoxicity" and "mutagenicity" are not well defined; however, genotoxicity often refers to the ability of agents (chemical, physical, or biological) to damage cellular genetic material (DNA, RNA, and chromosomes), leading to a variety of genetic alterations. In contrast, mutagenicity is a specific, narrower subset of genotoxicity and denotes the induction of permanent, heritable changes (mutations) in the DNA sequence of genes or chromosomes. Mutagenic agents (mutagens) cause stable genetic alterations that can be transmitted to daughter cells or offspring. This includes direct DNA damage, chromosomal breaks, or interference with the cellular machinery which preserves genome integrity (Soloneski and Larramendy 2021). Based on the correlation between chemical carcinogenicity and mutagenicity, mutation assays have been established as a gold standard biomarker of potential carcinogenicity and, together with extensive genotoxic evaluations, have been applied in regulatory toxicology (Hayashi 2022). These testing frameworks, encompassing both *in vitro* and *in vivo* approaches, provide a harmonized basis for genetic hazard assessment (Doak et al. 2023; Burgum et al. 2024).

Considering these assays, the core *in vitro* genotoxicity battery includes the bacterial reverse mutation assay (OECD 2020) for point mutations and the mammalian cell micronucleus assay (OECD 2023) for chromosomal damage. Positive findings trigger *in vivo* follow-up testing, typically using assays such as the rodent micronucleus test (OECD 2016a) or the transgenic rodent mutation assay (OECD 2025). In

this context, the *in vivo* Comet assay (OECD 2016b) may be used to assess DNA strand-break endpoints in relevant target tissues and to provide complementary mechanistic information.

In this context, genotoxicity assessment supports risk assessment, regulatory decision-making, and disease understanding and based on its underlying mechanisms, is differentiated into primary (direct and indirect) and secondary genotoxicity. Direct primary genotoxicity occurs when a compound or its reactive metabolite interacts with DNA, causing adducts, strand breaks, or crosslinks. In contrast, indirect primary genotoxicity arises without direct DNA interaction, through disruption of DNA replication, repair processes, topoisomerase activity, or via intracellular oxidative stress (OS). Both are considered primary, as they result from the intrinsic properties of the substance and are independent of inflammation (Antunović et al. 2011; Doak et al. 2023; Sharma et al. 2025). A battery of complementary assays is employed to capture distinct genotoxic endpoints, including gene mutation (e.g., Ames test), chromosomal damage (micronucleus and chromosomal aberration assays), and DNA strand breaks (comet assay). The mammalian micronucleus test is a validated method for detecting damage to the chromosomal and mitotic apparatuses, enabling the simultaneous identification of clastogenic and aneugenic effects (Doak et al. 2023). In cases of uncertainty, follow-up studies are required to clarify the underlying mode of action, often involving *in vivo* assays such as the micronucleus test and the comet assay, in accordance with OECD Test Guidelines. Current evidence, however, reveals a methodological bias in primary genotoxicity assessment, largely dominated by the comet assay, which primarily detects DNA strand breaks and oxidative lesions. While suitable for identifying OS-mediated mechanisms, this approach may underrepresent other relevant pathways, such as DNA adduct formation, mutagenicity, and chromosomal alterations, typically captured by mutation and cytogenetic assays. This imbalance highlights a limitation of relying on a single predominant endpoint and underscores the need for integrated multi-assay strategies to capture the full spectrum of primary genotoxic mechanisms (Di Ianni et al. 2022). Addressing these limitations requires a shift toward more mechanistically informed approaches that integrate multiple biological levels of the DNA damage response. In this context, emerging methodologies, including toxicogenomics (RNA-seq, microarrays), epigenomics (DNA methylation profiling), γH2AX assay, high-content imaging, and 3D *in vitro* models, provide enhanced insight into molecular and cellular responses to genotoxic stress, supporting a more comprehensive identification of genotoxic modes of action (Turkez et al. 2017). In contrast, secondary genotoxicity arises indirectly through inflammation-mediated mechanisms that activate and recruit phagocytes, such as macrophages and neutrophils. This process generates reactive oxygen and nitrogen species (ROS/RNS) and releases cytokines and chemokines, which can induce DNA damage in neighboring target cells. To investigate these mechanisms, advanced *in vitro* approaches have been developed to better mimic inflammatory conditions, including conditioned media, co-culture systems that enable cell–cell interactions, and complex 3D microtissue models (e.g., spheroids and organoids) that recapitulate tissue architecture (Vallabani and Karlsson 2022). These strategies complement conventional inflammation-based systems by incorporating cytotoxicity-related endpoints and support weight-of-evidence approaches for genotoxicity assessment, as recommended by EFSA (Antunović et al., 2011).

Building on these standardized assays, regulatory structures have evolved to incorporate more integrative and evidence-based approaches to chemical and food safety assessment. The EFSA Scientific Committee has established detailed guidance on the weight-of-evidence (WoE) approach, defining it as a formal process for assembling, evaluating, and integrating data to determine relative support for scientific conclusions. The model emphasizes reliability, relevance, and consistency as key criteria for weighing evidence across several data streams. In parallel, the traditional animal-based paradigm in toxicological evaluation has been progressively questioned due to concerns regarding reproducibility, ethical constraints, and translational value. Consequently, NAMs encompassing *in vitro*, *in silico*, and omics-based tools are being advanced as complementary or alternative strategies that provide mechanistic, high-throughput information on toxic responses (Miccoli et al. 2022).

(Lin et al. 2023) applied an integrated methodological structure to evaluate the cytotoxicity, viability, and genotoxicity of co-occurring Alternaria toxins in human gastric epithelial (GES-1) cells. This multiparametric experiment integrates viability, apoptotic, and genotoxic endpoints within a quantitative interaction model, in accordance with EFSA's Next-Generation Risk Assessment (NGRA) framework. NGRA is a structured risk assessment approach that integrates NAMs with exposure assessment, mechanistic understanding, and weight-of-evidence evaluation to support human health decision-making. NGRA aims to move away from routine reliance on animal testing by focusing on biological plausibility, human relevance, and problem-driven assessment, while explicitly addressing uncertainty (Leso et al. 2025; Zhong et al. 2026). The author used a colourimetric assay to assess cell viability, measuring mitochondrial dehydrogenase activity and expressing viability as the percentage of live cells relative to untreated controls. Another multiparametric *in vitro* screening was performed by Recoules et al. (2025), which assayed 32 food additives and six mixtures from the NutriNet-Santé cohort using a γH2AX/pH3 dual-biomarker assay to simultaneously measure cytotoxicity and genotoxicity. Cytotoxicity was determined by relative cell count (%RCC) from total DNA fluorescence, and genotoxicity was quantified by the ratio of γH2AX and pH3 fluorescence intensity between treated and control cells. The evaluation applied EFSA's NAMs guidance, adopting a 1.5-fold induction threshold and $a < 50\%$ cytotoxicity cutoff to classify responses as positive.

In toxicological research, quantitative analysis enables researchers to derive, compare, and classify key cytotoxicity endpoints and parameters. These metrics serve as essential thresholds and screening criteria for evaluating the toxicological potential of new compounds and food-derived extracts during product and ingredient development. In the quantitative assessment of cytotoxicity (do Carmo et al. 2021), key

parameters commonly used to evaluate cell viability in toxicological studies were outlined, including the 50% inhibitory concentration (IC_{50}), the growth inhibition concentration (GI_{50}), and the lethal concentration (LC_{50}) (Table 2). (Lin et al. 2023) presented the half-maximum inhibitory concentration (IC_{50}) and related parameters, such as median effect dose (Dm), slope (m), and correlation coefficient (r), derived from the median effect equation of Chou and Talalay ($fa/fu = (D/Dm)^m$), where fa and fu denote the affected and unaffected cell fractions, respectively. Furthermore, combined toxicity was analyzed using the Combination Index (CI) and Dose Reduction Index (DRI) equations. Interactions were classified as synergistic (CI < 0.9), additive (0.9–1.1), or antagonistic (CI > 1.1) (Coecke et al. 2005; Krewski et al. 2010; Hardy et al, 2017; OECD 2018).

In this context, particular attention should be given to the interpretation of cytotoxicity data derived from *in vitro* cell-based assays, which are widely used in anticancer drug screening frameworks, such as those proposed by the National Cancer Institute (NCI). However, in a food toxicology context, the use of such classification thresholds requires careful consideration, as IC_{50} values should be interpreted as comparative indicators of cytotoxic potential within a broader hazard identification framework. Although these models play a key role in screening-level hazard identification by enabling the detection of cytotoxic effects, they also serve as screening tools for product evaluation, help reduce animal use, and provide mechanistic insight (Krewski et al. 2010; Coecke et al. 2005). For descriptive purposes, lower IC_{50} values may indicate higher cytotoxic potential (e.g., ≤ 20–30 µg/mL), intermediate values (e.g., 21–200 µg/mL) suggest moderate cytotoxicity, and higher values (e.g., > 200 µg/mL) reflect lower cytotoxic potential under the tested conditions (Canga et al. 2022; Femina et al. 2023). The NCI also sets an IC_{50} upper limit of 30 µg/mL as a criterion for prioritizing crude extracts for further investigation. In comparison, plant extracts with IC_{50} values up to 100 µg/mL have been reported in the literature as exhibiting detectable cytotoxic effects within this range (Canga et al. 2022). For instance, Prayong et al. (2008) also suggest that lower IC_{50} values (e.g., < 100 µg/mL) may indicate greater cytotoxic potential. In contrast, intermediate ranges (e.g., 100–1000 µg/mL) indicate moderate effects, and higher values (>1000 µg/mL) are generally associated with low cytotoxicity under the tested conditions. These ranges are not intended as fixed classification criteria, but rather as general guidance to support data interpretation, given that they do not fully capture the complexity of whole-organism responses and should therefore be harmonized with *in vivo* and other complementary approaches to achieve a comprehensive toxicological assessment (Antunović et al. 2011; EFSA, 2017; OECD 2018).

Table 2. Cytotoxicity equations for evaluating toxicological parameters.

Toxicological parameter		
Abbreviation	Concept	Equation
IC_{50}	The concentration of the compound that inhibits cell growth by 50% after treatment	$(T/C) \times 100 = 50$
GI_{50}	The concentration of the compound that inhibits cell growth by 50%, considering the control cells and the number of cells placed at the beginning of treatment	$([T\text{-}T0]/[C\text{-}T0]) \times 100 = 50$
LC_{50}	The concentration of the compound that results in a net loss of 50% of the cells, relative to the number of cells added at the start of treatment	$([T\text{-}T0]/T0) \times 100 = -50$

Note: T = number of cells after the treatment time; C = control cells at the same treatment time, under the same conditions, except for the compound tested; T0 is the number of cells at time zero and T and C are the number of treated and control cells, respectively, after treatment time T, with T > T0 (GI_{50}) or T < T0 (LC_{50}) (do Carmo et al. 2018).

High-throughput screening (HTS) in vitro models and "omics" approaches in toxicological evaluation

HTS *in vitro* approaches are rapidly becoming a bedrock of toxicological risk assessment in food science. What was formerly regarded as a specialized toolset has evolved into a driving force behind next-generation toxicity testing, expanding the possibilities for evaluating food safety with superior precision, speed, and scientific depth. In practice, HTS enables testing a variety of compounds against whole organisms or selected molecular or cellular targets, and this approach has been used to estimate toxicity and to understand the mechanisms of action of these substances (Mezencev and Subramaniam 2019). Modern HTS pipelines combine automated liquid handling, plate-based assays, and high-content imaging with both 2D monolayers and increasingly HTS-compatible 3D constructs to generate dense dose–response and time-course datasets that characterize potency, kinetics, and modes of action at scale (Lynch et al. 2024). Moreover, recent advances comprise automated 3D bioprinting and microfluidic platforms toward high-throughput compound screening and mechanistic studies (Bircsak et al. 2021; Kang et al. 2021), demonstrating that automation is a key component of high-throughput screening. The NRC's vision for toxicity testing in the twenty first century explicitly recommended moving away from isolated apical endpoints toward pathway-focused assays in human cells using robotic screening, quantitative measures of *in vitro* perturbation, and *in silico* methodologies, including genomic and post-genomic approaches (National Research Council, 2007).

A major advance was the combination of HTS with mechanistic understanding, precise dose- and time-dependent exposures, and global molecular readouts (genomics, transcriptomics, proteomics, metabolomics), collectively recognized as toxicogenomic (Mezencev and Subramaniam 2019; Štampar and Žegura 2024). Multiparametric "omics" technologies allow the simultaneous assessment of numerous biological parameters and provide systems-level fingerprints of cellular responses that uncover molecular mechanisms beyond simple viability loss, map pathways, and provide an integrated approach (Ramirez-Hincapie et al. 2023). The wealth of information associated with HTS data and toxicogenomics can support the assessment of human health risks, mainly through (i) evaluating the dose-response relationship for toxicity values, (ii) grasping the potential mechanism of action, and (iii) predicting adverse effects of exposure (Mezencev and Subramaniam 2019).

Programs such as Tox21 and ToxCast have pioneered the use of HTS and toxicogenomics, creating substantial datasets that inform risk assessment, regulatory decisions, and mechanistic understanding of toxicity. Platforms now routinely probe multiple concentrations and exposure durations to derive benchmark dose metrics and temporal activation profiles of stress and adaptive reactions—information that is critical when assessing short-lived exposures, typical of dietary intake, versus chronic low-dose exposure scenarios (Lynch et al. 2024). Examples of Tox21 and ToxCast include prioritizing food additives & contaminants, case studies on indirect food additives, endocrine disruptor screening, toxicity profiling of natural compounds, safety assessment of food contact materials, and predicting chemical toxicity (read-Across) for application areas such as food packaging and plastics, pesticide residues, and flavoring agents and nutrients. Targeted and broad-spectrum metabolomics assays adapted to 96-well formats now permit multiplexed, time-resolved interrogation of metabolic pathways (e.g., energy metabolism, redox status, xenobiotic biotransformation), enabling classification of modes of action and informing point-of-departure derivation for risk assessment (Ramirez-Hincapie et al. 2023; Lynch et al. 2024). Such multiparametric datasets allow rapid evaluation of thousands of chemicals in relevant biological systems, consequently altering how we assess the safety of food-related compounds, contaminants, and nanomaterials.

Toxicogenomics can be divided into three types: mechanistic (that seeks to understand the mechanisms of adverse effects induced by toxic substances using functional genomics data, particularly transcriptomics), predictive (that uses class prediction approaches to predict adverse outcomes, such as carcinogenicity or hepatotoxicity, from toxicogenomic data), and quantitative (which analyzes dose-dependent changes in gene expression at the level of individual genes, metabolic pathways, and other biologically defined gene groups) (Mezencev and Subramaniam 2019). Ramirez-Hincapie et al. (2023) developed a highly reproducible, targeted *in vitro* metabolomics platform in 96-well plates and optimized critical experimental parameters to enable rapid, cost-effective hepatotoxicity screening using 2D HepG2 cell culture. They demonstrated the applicability of the assay to reproduce dose-response effects in metabolomics, distinguish between different mechanisms of action of liver toxicity, and detect key metabolites and patterns representative of general and specific hepatotoxicity. In conclusion, these approaches can determine the exposure levels at which specific "omics" responses become abnormal, and these levels can be used to estimate starting points for deriving toxicity values.

Despite these advantages, some challenges limit the routine adoption of HTS + omics in early food safety processes, since these methods are still comparatively expensive, require significant materials and labor, and demand substantial bioinformatics capacity, especially since data harmonization, reproducibility, and validation throughout several platforms are areas of ongoing development (Ramirez-Hincapie et al. 2023; Lynch et al. 2024). Critically, bridging HTS/omics outputs to human-relevant exposure requires a quantitative framework that translates pathway-level perturbations into internal dose metrics. This can be operationalized through transcriptomic benchmark concentration (BMC) modeling to derive points of departure from gene expression changes, followed by quantitative *in vitro* to *in vivo* extrapolation (qIVIVE) to estimate equivalent human plasma concentrations. These concentrations can then be integrated into physiologically based pharmacokinetic (PBPK/PBK) models to simulate absorption, distribution, metabolism, and excretion, enabling the prediction of internal doses under realistic dietary exposure scenarios. Such an approach allows the comparison of bioactivity thresholds with estimated human exposure levels, thereby contextualizing the magnitude of cellular perturbations. Thus, to ensure the continuous production and development of novel and safe food products, ingredients and nutraceuticals, the combination of "omics" technologies with high-throughput methods (anchored in quantitative IVIVE and Physiologically based pharmacokinetic (PBPK) modeling) is fundamental for advancing beyond simple cytotoxicity endpoints toward a mechanistic, dose-informed toxicology framework that supports risk assessment and regulatory decision-making. toward a comprehensive, mechanism-based toxicology.

Cell-based assays to assess the cellular antioxidant activity of novel foods, ingredients, and nutraceuticals

Antioxidants are chemical species that, at relatively low concentrations, can slow or inhibit chain reactions initiated by reactive species and free radicals. There are three accepted mechanisms for preventive antioxidant activity: (1) Chain-breaking (radical-scavenging) activity, in which antioxidants donate a hydrogen atom (HAT mechanism) or an electron (SET mechanism) to reactive radicals, thereby terminating radical propagation reactions; (2) Preventive antioxidant activity through metal chelation, whereby transition metal ions such as Fe^{2+} and Cu^{2+} are bound, reducing their ability to catalyze radical generation; and (3) Peroxide decomposition, typically through enzymatic catalysis (e.g., catalase [CAT], glutathione peroxidase [GPx], and peroxiredoxins), which converts hydrogen peroxide and lipid hydroperoxides into less reactive products (Oroian and Escriche 2015).

Antioxidants may be classified as enzymatic and non-enzymatic. Non-enzymatic antioxidants comprise a diverse group of hydrophilic and lipophilic compounds, including ascorbic acid, glutathione, flavonoids, phenolic acids, and carotenoids, which are widely distributed in foods. These compounds may attenuate OS by scavenging reactive species, interrupting radical chain reactions, chelating transition metal ions, and, in some cases, modulating the activity or expression of endogenous antioxidant enzymes (Rudenko et al. 2023). Enzymatic antioxidants are proteins that catalyze the conversion of reactive oxygen species and their oxidation products into less harmful molecules. The principal antioxidant enzymes responsible for maintaining redox homeostasis in human tissues are CAT, superoxide dismutase (SOD), and GPx.

CAT is an antioxidant enzyme composed of four protein subunits, each containing a heme group as a prosthetic

group, and NADPH molecules associated with it, which act as protective agents, preventing catalase inactivation. The reaction catalyzed by CAT is the conversion of hydrogen peroxide into water and O_2, and it exhibits one of the highest catalytic efficiencies among enzymes (Anwar et al. 2024). SOD is a group of antioxidant metalloenzymes that dismutate the superoxide radical, producing O_2 and H_2O_2, the latter being decomposed by catalase and glutathione peroxidase (Rosa et al. 2021). SOD activity is relevant since the superoxide anion ($O_2\bullet^-$) is a free radical produced during cellular respiration and is more reactive in organic than aqueous environments, making membrane lipids its primary target. The various types of SOD are distinguished by their metal cofactors and cellular localization: these enzymes may be found in the nucleus, cytoplasm, lysosomes, mitochondrial matrix, and other cellular compartments (Zheng et al. 2023). Another antioxidant metalloenzyme is GPx. Selenocysteine is the catalytic site of GPx, and these enzymes use glutathione (GSH) as a reducing agent to catalyze the reduction of H_2O_2 or organic hydrogen peroxide to water or the corresponding alcohol. In mammals, there are eight different types of GPx, each with a distinct location in the cell (cytoplasm, mitochondria, or cell membrane) and in the body (plasma, gastrointestinal system, nose, or liver) (Pei et al. 2023).

CAT, SODs, and GPx together constitute the human enzymatic arsenal for ROS capture, playing a vital role in the removal and reduction of $O_2\bullet^-$, H_2O_2, and •OH (Zheng et al. 2023), as well as other ROS. In physiological environments, ROS are crucial for maintaining cellular homeostasis, as they participate in processes such as signaling regulation, proliferation, differentiation, and even programmed cell death. When ROS levels are below the optimal range, signaling mechanisms are disrupted, impairing the control of homeostatic functions. Conversely, excessive ROS production may create an imbalance between this antioxidant defence and reactive species, favoring oxidants, leading to OS (Masenga et al. 2023). OS constitutes a central mechanism underlying toxicity and, therefore, a critical endpoint in food toxicology. Its importance lies in the fact that OS can act both as a cause and a consequence of several chronic diseases, including diabetes mellitus (Zhang et al. 2020), cancer (do Carmo et al. 2021), neurodegenerative diseases (Teleanu et al. 2022), and cardiovascular diseases (Granato 2022).

Methods to assess the ROS levels in biological systems

Although accurately measuring ROS remains challenging, various analytical methods are available. These include the use of fluorescent and chemiluminescent probes, spectrophotometric and spectrometric techniques, chromatographic analyses, and assays employing genetically encoded fluorescent proteins, which together enable the detection and quantification of oxidative processes at multiple biological levels (Table 3).

Three fluorescent probes are commonly used to measure the ROS level in biological systems: DCFH-DA, Amplex Red, and dihydroethidium (DHE). Dichlorodihydrofluorescein diacetate (DCFH-DA) is widely used, although it lacks specificity because it reacts with several intracellular oxidants and is prone to artifacts. DCFH-DA penetrates the cell and, in the cytoplasm, is hydrolyzed by intracellular esterases, generating DCFH. ROS oxidize this product to DCF, a fluorescent compound. The fluorescence intensity (485 nm excitation, 520 nm emission) is measured, and higher fluorescence indicates higher intracellular ROS levels (Cruz et al. 2024). On the other hand, Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine, AR) is a fluorescent probe used to detect extracellular H_2O_2, as it cannot penetrate cells (Akter et al. 2021). AR is oxidized by hydrogen peroxide, forming the resorufin, which is assessed by fluorescence. This reaction is catalyzed by horseradish peroxidase, and the fluorescence is measured at $\lambda_{Excitation}$ = 544 nm and $\lambda_{Emission}$ = 584 nm (Liu et al. 2025).

DHE shows a specific response to the intracellular superoxide radical ($O_2^{-}\bullet$). The reaction with the superoxide radical yields 2-hydroxyethidium, a red-fluorescent product. Nonetheless, DHE is susceptible to nonspecific oxidation by

Table 3. Characteristics of ROS detection methods.

Method type	Probes/ chromophore	Specificity	Examples of food samples in cellular antioxidant activity
Fluorometry	DCFH-DA	H_2O_2, $^{\cdot}OH$, HClO, $ONOO^-$	Normal and cancer cells culture – flour and aqueous extract of cocoa shell (Cañas et al., 2023); grapefruit seed dichloromethane and hydroethanolic extracts (Magurano et al., 2021); commercial blueberries and their bioaccessible fraction (Sánchez-Velázquez et al., 2021); sunflower seed-derived bioactive peptides (Tonolo et al., 2024). Erythrocytes – blackcurrant press cake by-product aqueous extracts (dos Santos Lima et al., 2024); Kamut® wheat bran (Razem et al., 2025); ora-pro-nobis leaf and berry hydroethanolic extracts (Cruz et al., 2024).
Fluorometry	Amplex red	H_2O_2	Normal and cancer cells culture – *Alternanthera sessilis* ethanolic extract (Mohd Razali et al., 2022); onion skin aqueous extract (Volpi et al., 2021); *Ex vivo* male mice kidney tissue culture – hydroethanolic grape pomace extract (Figueras et al., 2025).
Fluorometry	DHE	Mainly $O_2^{\cdot-}$	*Ex vivo* male mice kidney tissue culture – hydroethanolic grape pomace extract (Figueras et al., 2025); Normal and cancer cells culture – Syrian oregano ethanolic extract (Mesmar et al., 2022)
Spectrophotometry	NBT	$O_2^{\cdot-}$	Normal and cancer cells culture – lemon balm, peppermint, basil, rosemary, sage, and winter savoury extracts obtained with hydrometanol, hydroetanol, and water (Oalđe Pavlović et al., 2021)
Chemiluminescence-derived	Luminol	H_2O_2, $^{\cdot}OH$, $O_2^{\cdot-}$, $ONOO^-$	Rat colon tissue samples – *Thymus longicaulis* subsp. *chaubardii* ethanolic extract (Ozbeyli et al., 2025); Neutrophils – Brazilian red propolis (Justino et al., 2024)
Chemiluminescence-derived	Lucigenin	$O_2^{\cdot-}$	Rat colon tissue samples – *Thymus longicaulis* subsp. *chaubardii* ethanolic extract (Ozbeyli et al., 2025)

$ONOO^-$, $^{\bullet}OH$, and H_2O_2, yielding a second fluorescence product, ethidium, with a fluorescence spectrum very similar to that of ethidium. Thus, the fluorescence intensity is measured at 610 nm emission and 480 nm excitation (Kumar and Gullapalli 2024). However, to quantify ROS levels spectrophotometrically, the nitroblue tetrazolium (NBT) assay may be used. Basically, NBT is transported into the cytoplasm, where it is reduced by $O_2^{-}\bullet$, generating insoluble formazan crystals. These crystals, trapped inside the cell, must be solubilized with dimethyl sulfoxide (DMSO), and absorbance is measured at 630 nm (Tunc et al. 2010). Chemiluminescence assays are also helpful, and two probes are applied: luminol and lucigenin. Luminol is oxidized in the cytoplasm by ROS in an unspecific manner, measuring global levels of these species, whereas lucigenin reacts specifically with extracellular $O_2^{-}\bullet$. Furthermore, lucigenin can undergo redox cycling, accepting electrons and transferring them to oxygen, thereby generating artificial superoxide, which may, in turn, cause the system to generate reactive species independently of the sample. Consequently, there is an overestimation of OS and an alteration in the assessment of antioxidant activity (Kobayashi et al. 2001).

Dual cytotoxicity and antioxidant activity screening of novel foods, ingredients, and nutraceuticals using erythrocytes as a biological model

Bioactivity and potential toxicity should be evaluated not only by their magnitude but also by understanding their mechanisms of action. Accordingly, complementary approaches, ranging from cell-free and cell-based assays to *in vivo* and *in silico* protocols, are required to address key questions, including tissue specificity, affected or protected cellular components, enzyme modulation, and the types of stress induced or mitigated. In this context, mammalian erythrocytes (e.g., human, horse, and sheep red blood cells, RBC) serve as a valuable biological model for investigating cytotoxicity and bioactivity, particularly in studies of OS and antioxidant compounds (Figure 1). Because excessive ROS generation is a major driver of cytotoxicity, particularly through oxidative damage to cellular membranes and enzymes, highly sensitive biological models that are responsive to redox imbalance are essential for mechanistic toxicological assessment.

RBCs are enucleated, highly specialized cells essential for O_2 transport. They are especially useful for cytotoxicity assessment due to their ease of isolation, high abundance, and lack of culture requirements, which substantially reduce experimental time, cost, and labor. Moreover, oxidative or mechanical stress induces measurable physiological, morphological, and functional alterations in RBC, providing insight into damage mechanisms and cellular defence responses (Obeagu et al. 2024). Consequently, RBC-based assays are increasingly applied to evaluate the safety and potential toxicity of food samples, extracts, and novel foods (Rischer et al. 2022) (Remigante et al. 2022a). An important consideration when working with RBCs is that their use typically requires appropriate ethical and biosafety oversight, including approval by a research ethics committee, informed consent from human donors (or a documented exemption), and compliant procedures for donor identification and sample handling.

Although RBC possess efficient endogenous antioxidant defenses—including GSH, CAT, GPx, and SOD—that detoxify ROS such as H_2O_2 and free radicals, these systems may be overwhelmed under acute or sustained OS (Podsiedlik et al. 2020). As a result, RBC remain susceptible to oxidative injury, making them a suitable model for high-throughput screening of antioxidant efficacy and cytotoxicity of novel foods, bioactive-rich extracts, and nutraceuticals. Oxidative damage can be evaluated using multiple biomarkers tailored to specific molecular targets (Möller et al. 2022). For example, hemolysis reflects loss of membrane integrity, while intracellular ROS accumulation indicates a cytoplasmic oxidative burden. Together, these endpoints enable RBC to serve as sensitive indicators of OS and functional reporters of the protective or harmful effects of test compounds on distinct cellular compartments (Massaccesi et al. 2020; Plasencia et al. 2025).

Overall, in *in vitro* HTS protocols, exposure of RBCs to chemically induced oxidative stress—using oxidizing agents such as hydrogen peroxide or peroxyl radical generators [e.g., AAPH – 2,2′-azobis(2-methylpropionamidine) dihydrochloride]—activates multiple molecular targets and interconnected biochemical pathways. Under supraphysiological oxidative conditions, key erythrocyte components, including the lipid bilayer, cytoskeletal network, and hemoglobin, are particularly susceptible to oxidative modification. Disruption of redox homeostasis compromises membrane integrity and erythrocyte function, ultimately leading to impaired redox signaling, physiological dysfunction, and hemolysis (Chatzinikolaou et al. 2024). A primary molecular target is the polyunsaturated fatty acids (PUFAs) of the membrane phospholipid bilayer, which undergo ROS-induced lipid peroxidation, generating lipid hydroperoxides (LOOH/ROOH). These unstable intermediates rapidly decompose into secondary reactive aldehydes and ketones, such as malondialdehyde (MDA), a biomarker of oxidative damage (Mendanha et al. 2012). Both primary and secondary lipid oxidation products, including isoprostanes and 4-hydroxy-2-,3-*trans*-nonenal, can diffuse into the cytoplasm, amplifying ROS generation (Pandey and Rizvi 2010). Their accumulation disrupts membrane permeability, fluidity, and elasticity, affecting Ca^{2+} handling and redox balance, thereby weakening barrier function and increasing hemolysis. Membrane damage also promotes the release of ferrous iron (Fe^{2+}), which drives Fenton-like reactions and hemoglobin oxidation, further exacerbating OS (Remigante et al. 2022b; Yang et al. 2024).

Under OS, membrane-associated proteins represent critical ROS targets, particularly through oxidation of sulfhydryl (–SH) groups. Thiol oxidation leads to disulfide bond formation, destabilizing the membrane–cytoskeletal network, altering erythrocyte morphology, and markedly reducing cellular deformability, which impairs microvascular transit (Yang et al. 2024). OS also induces protein carbonylation, an irreversible modification resulting from ROS-mediated oxidation of amino acid side chains, including lysine, arginine, and proline (Velásquez et al. 2019). In general, these protein

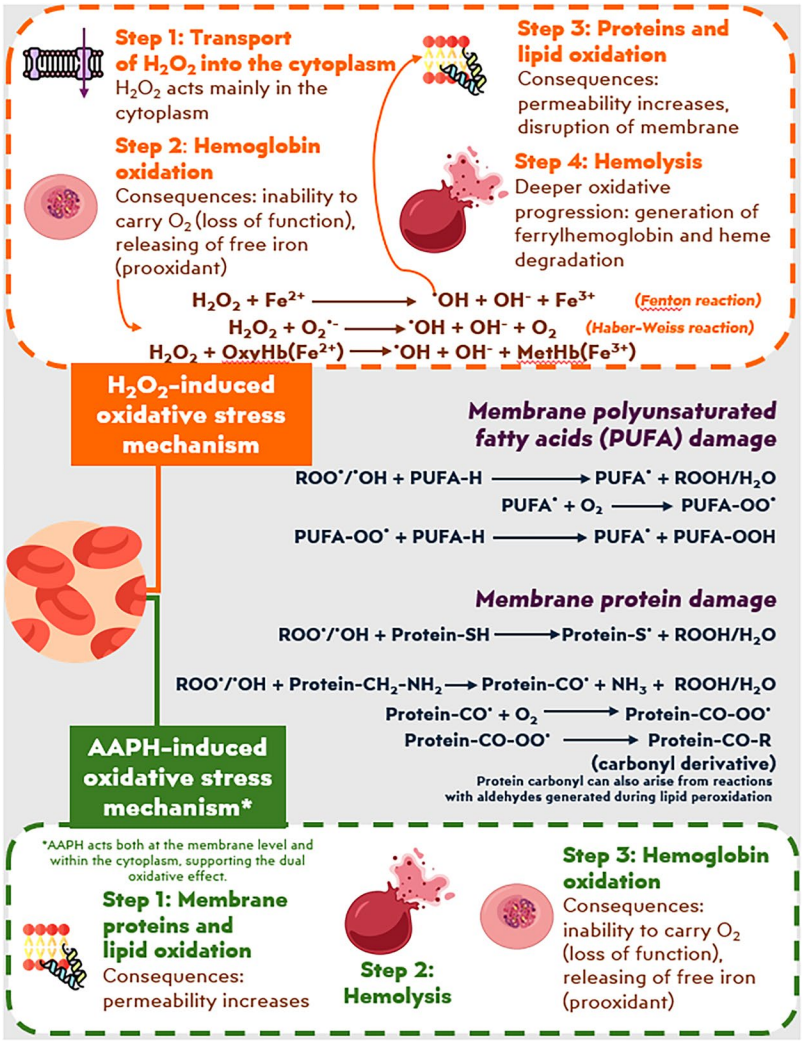

Figure 1. Mechanisms of oxidative damage in erythrocytes induced by H_2O_2 and AAPH. H_2O_2 can oxidize hemoglobin and, in the presence of redox-active iron ($Fe^{2}\rightarrow$), undergo Fenton chemistry to generate hydroxyl radicals ($^{\bullet}OH$) that initiate lipid peroxidation and protein oxidation. AAPH decomposes thermally to generate alkyl radicals, which react with oxygen to form peroxyl radicals ($ROO^{\bullet}$), thereby providing a continuous source of peroxyl-mediated lipid oxidation. Propagation of lipid peroxidation (RH → $R^{\bullet}$ → $ROO^{\bullet}$ → ROOH) and formation of reactive ferryl hemoglobin species ($HbFe^{4+}$=O) damage membrane lipids and proteins, cause heme loss and free iron release, and ultimately increase membrane permeability and hemolysis. Antioxidant defenses (SOD, catalase, GPx/GSH) can limit the accumulation of free radicals; markers of damage include methemoglobin, lipid hydroperoxides (LOOH/ROOH), TBARS/MDA, and protein carbonyls. Created in Microsoft PowerPoint using icons from Flaticon under a paid subscription.

modifications compromise membrane function and promote hemolysis. In parallel, oxidative damage to hemoglobin favors methemoglobin formation and the release of free heme and pro-oxidant Fe^{2+}, both of which intensify oxidative injury and sustain ROS propagation (Fujii et al. 2021; Vallelian et al. 2022).

A critical parameter providing mechanistic insight into oxidative stress in RBCs is the choice of oxidizing agent. Among the most used are H_2O_2 and AAPH, which differ in physicochemical properties and modes of action. H_2O_2 is a cost-effective oxidant that is endogenously generated during cellular respiration and, when applied exogenously, enters RBCs mainly through aquaporin channels exhibiting peroxiporin activity, rather than via unrestricted passive diffusion across the membrane (Orrico et al. 2023). Once internalized, H_2O_2 indirectly promotes lipid peroxidation by initiating radical-mediated chain reactions and reacts with oxyhemoglobin ($HbFe^{2+}$), leading to its oxidation to methemoglobin ($HbFe^{3+}$) and, under more severe oxidative conditions, to ferryl hemoglobin ($HbFe^{4+}$=O; $HbFe^{2+}$ + H_2O_2 → $HbFe^{4+}$=O + H_2O). Ferryl Hb formation is associated with globin-centered radical production, heme degradation, and heme release. The accumulation of $HbFe^{3+}$ can be readily quantified by UV–Vis spectrophotometry, either by endpoint or kinetic measurements (Melo et al. 2024).

As OS progresses, heme degradation releases free ferrous iron (Fe^{2+}) (Shevchenko 2024), which fuels Fenton reactions with H_2O_2, generating highly reactive hydroxyl radicals ($^{\bullet}OH$) and thereby amplifying intracellular ROS levels. These radicals rapidly oxidize membrane-associated proteins such as band 3, spectrin, and ankyrin (Protein–H + $^{\bullet}OH$ → Protein$^{\bullet}$ + H_2O), forming protein-centered (Protein$^{\bullet}$) and peroxyl radicals (Protein–$OO^{\bullet}$) that propagate oxidative damage and generate protein hydroperoxides (Protein–$OO^{\bullet}$ + Protein–H → Protein–OOH + Protein$^{\bullet}$). Subsequent decomposition of these species into alkoxyl radicals induces

protein conformational changes, increases membrane permeability, and ultimately leads to hemolysis, a secondary endpoint resulting from cumulative protein and lipid oxidation (Remigante et al. 2023; Blat et al. 2024; Shevchenko 2024). This ROS-driven hemolytic response is a functional manifestation of RBC cytotoxicity. In parallel, PUFAs within the lipid bilayer undergo ROS-mediated peroxidation, further compromising membrane fluidity and integrity. Collectively, the oxidant employed dictates not only the primary molecular targets but also the downstream oxidative pathways and resulting cellular outcomes (Blat et al. 2024).

In AAPH-treated erythrocytes at 37 °C, AAPH undergoes thermal decomposition to generate alkyl radicals (AAPH → 2 R•), which rapidly react with oxygen to form peroxyl radicals (R• + O_2 → ROO•). These hydrophilic, short-lived radicals exhibit limited membrane permeability and primarily attack lipids and proteins at the outer membrane surface, initiating lipid peroxidation within the lipid bilayer. During the initiation phase, hydrogen abstraction from polyunsaturated fatty acids (PUFA–H) generates lipid radicals (PUFA•), which react with O_2 to form lipid peroxyl radicals (PUFA–OO•). These species propagate chain reactions with neighboring PUFA, resulting in the accumulation of lipid hydroperoxides (PUFA–OO• + PUFA–H → PUFA–OOH + PUFA•). Sustained lipid peroxidation, together with secondary formation of reactive ferryl hemoglobin ($HbFe^{4+}$=O), disrupts membrane lipids and proteins, compromising bilayer integrity. This damage promotes heme release and liberation of redox-active iron, ultimately increasing membrane permeability, impairing erythrocyte function, and enhancing susceptibility to hemolysis (Yang et al. 2017; Du et al. 2020).

A key distinction between OS induced by AAPH and H_2O_2 lies in cellular accessibility. AAPH-derived radicals are hydrophilic and poorly penetrate the erythrocyte membrane; therefore, oxidative damage occurs primarily at the membrane surface, targeting phospholipids and membrane proteins. In this context, membrane lipid oxidation increases cellular fragility and leads to hemolysis, while hemoglobin oxidation becomes evident only after extensive membrane damage (Zou et al. 2001). Although ROO• radicals themselves do not readily cross the membrane, secondary lipid peroxidation products can diffuse into the cytoplasm and promote intracellular OS, as consistently reported (Tedesco et al. 2000; Revin et al. 2019; Kaźmierczak et al. 2023). In contrast, H_2O_2 readily enters the cytosol, where it drives Fenton-type reactions and directly oxidizes intracellular targets, damaging RBCs from the inside out with hemoglobin as the primary target (Biltoet al. 2012).

Compared with H_2O_2-induced lipid oxidation, AAPH-mediated oxidation progresses more slowly but in a sustained manner, making it a stable model for assessing antioxidant capacity at the membrane interface. Similar to H_2O_2, AAPH-derived peroxyl radicals oxidize protein amino acid side chains, leading to thiol oxidation, formation of disulfide bonds, sulfinic and sulfonic acids, and protein carbonyl derivatives. In addition, peroxyl radicals can abstract hydrogen atoms from lipid hydroperoxides or protein backbones, thereby amplifying oxidative cascades in both membrane lipids and proteins. Accordingly, this constant radical flux model has been widely used in lipid oxidation studies involving novel foods, bioactive-rich extracts, and nutraceuticals (Yang et al. 2017; Kaźmierczak et al. 2024; Męczarska et al. 2025). The hemolytic pathways induced by H_2O_2 and AAPH further indicate that H_2O_2 is better suited for evaluating intracellular OS, whereas AAPH is more appropriate for examining membrane-directed effects of xenobiotics. These complementary models can be applied independently or in combination to investigate antioxidant and cytoprotective mechanisms of bioactive compounds, as demonstrated in studies employing H_2O_2 (Yasir et al. 2022), AAPH (El Azab and Mostafa 2022; Wang et al. 2024), or *tert*-butyl hydroperoxide (Deepika et al. 2025) as oxidizing agents. This biological model should be regarded as a mechanistic and screening tool, rather than a proxy for dietary risk assessment. Within these constraints, it remains valuable for elucidating redox-related mechanisms, comparative antioxidant activity, and early-stage hazard prioritization of NF, functional ingredients, and nutraceuticals.

To obtain a more comprehensive understanding of interactions between food samples and human cells, several studies have refined existing protocols to evaluate protective and cytotoxic effects in RBCs. For instance, a simplified protocol for evaluating the protective effects of natural products and foods against oxidative damage (HERYCA-P) has recently been integrated. This method evaluates oxidative damage and protection at both the membrane and cytoplasmic levels, thereby showing potential mechanisms of action or toxicity of foods (Cruz et al. 2024). Through the HERYCA-P approach, researchers can synchronously measure hemolysis, intracellular ROS generation, advanced protein oxidation products, free iron content, and lipid peroxidation, providing a holistic assessment of erythrocyte oxidative status and antioxidant protection (Cruz et al. 2026).

Cutting-edge computational toxicology

This section presents an integrated, end-to-end computational toxicology workflow that links early hazard identification to decision-relevant risk characterization (Figure 2). To make this pipeline more operational, the outputs from each module are interpreted within a weight-of-evidence framework. The workflow proceeds from chemical identity and exposure-context definition to tier-1 structure-based screening using QSAR and read-across, target inference using machine-learning-based drug-target prediction, network toxicology and module detection within protein-protein interaction graphs, functional interpretation through Gene Ontology and KEGG enrichment, structure-based evaluation by molecular docking and molecular dynamics simulations, and finally physiologically based pharmacokinetic modeling to translate external exposure into internal dose metrics. In this framework, QSAR and read-across serve as initial hazard flags when their applicability is adequate; docking and MD provide supportive evidence for target plausibility; and network or omics results receive greater weight when they converge on biologically plausible and exposure-relevant pathways. When outputs are discordant, evidence with stronger applicability, mechanistic plausibility, and exposure

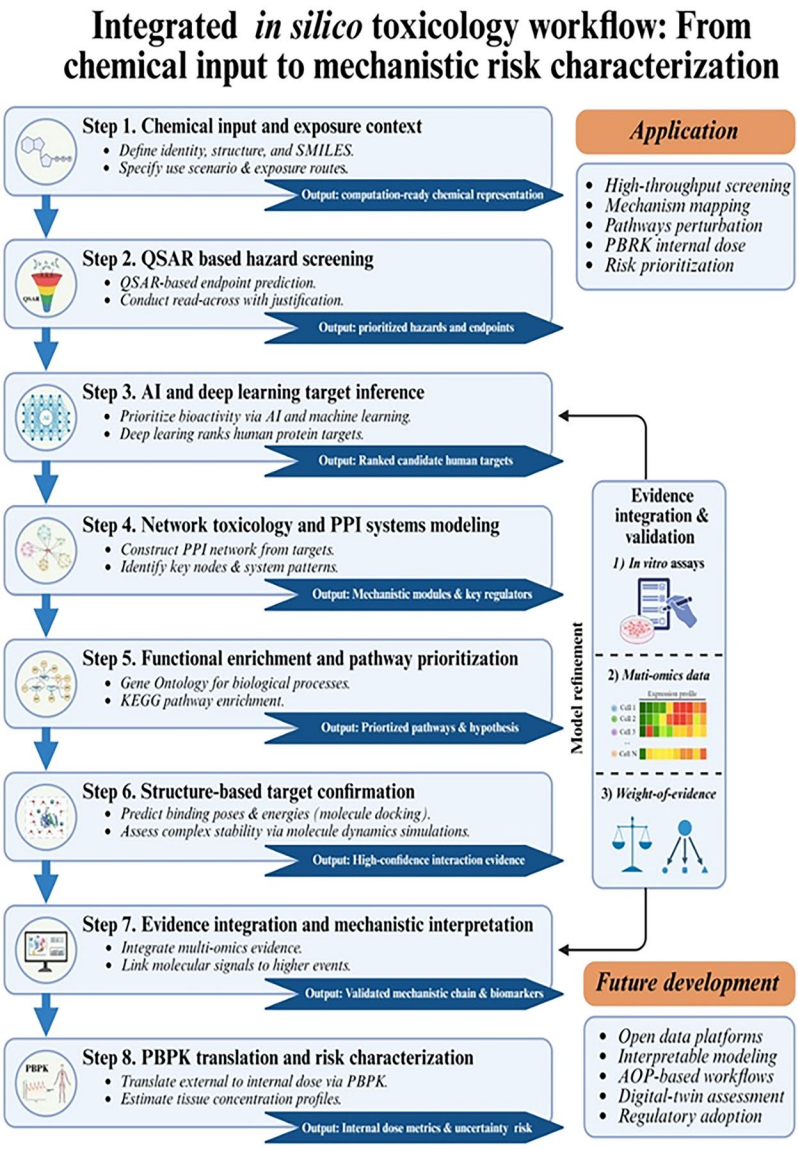

Figure 2. Computational toxicology workflow linking chemical inputs to mechanism-informed risk characterization. The workflow integrates chemical definition and exposure context with tier-1 QSAR and read-across screening to prioritize hazards and endpoints, followed by AI- and deep-learning-based drug–target prediction to rank candidate human targets. Targets are embedded in PPI networks to identify key regulators and MCODE-derived modules, which are interpreted using GO and KEGG enrichment to formulate testable mechanistic hypotheses. Structure-based confirmation is performed via molecular docking and molecular dynamics, assessing binding plausibility and interaction stability using trajectory metrics such as RMSD and contact persistence. PBPK modeling then translates external exposure into time-resolved tissue concentrations and internal dose metrics, enabling alignment of predicted bioactivity with realistic *in vivo* exposures for uncertainty-aware, mechanism-informed risk characterization. Created in BioRender. C, J. (2026) https://BioRender.com/ew44o0a.

relevance is prioritized, while unresolved differences are carried forward as uncertainty. Progression to targeted *in vitro* confirmation is considered when at least two independent evidence streams support the same hazard hypothesis, or when a single high-concern and exposure-relevant signal warrants precautionary testing. This workflow should be understood as a stepwise strategy for food safety assessment rather than a drug-discovery pipeline. After chemical identity and dietary exposure context are defined, QSAR and read-across are mainly used for early hazard screening and grouping. In contrast, protein target prediction is used to infer candidate biological targets or molecular initiating events that may be perturbed by food-related chemicals, thereby helping to prioritize pathways for downstream analysis. Network toxicology, enrichment analysis, and docking or molecular dynamics then provide complementary mechanistic information for hypothesis generation and refinement, as illustrated by food-relevant applications involving contaminants such as acrylamide or aflatoxin B1. By contrast, PBPK modeling is used at the later risk-characterization stage, rather than at the initial input stage, because its main purpose is to translate oral or dietary exposure into internal dose metrics, including plasma or tissue concentrations, that are more directly relevant to systemic exposure and target-organ risk. This later-stage application is particularly important for food contaminants and novel ingredients, for which gastrointestinal absorption, first-pass metabolism, and bioavailability can substantially influence internal exposure. In this framework, target prediction supports biological prioritization, whereas PBPK supports dose translation and risk

interpretation within an EFSA-aligned weight-of-evidence assessment.

Chemical input, exposure context, and QSAR-based hazard screening

A robust computer-based toxicology assessment begins with rigorous chemical definition and exposure-context specification, because both steps directly determine model applicability and the understandability of downstream predictions (Fourches et al. 2010; Mansouri et al. 2024). In practice, chemical structures should be curated into computation-ready formats that preserve molecular integrity (e.g., stereochemistry, tautomeric state, ionization form, and salt handling), together with harmonized identifiers such as canonical SMILES and InChI (O'Boyle 2012). For multi-constituent substances and UVCBs, transparent documentation of composition ranges and identity uncertainty is essential to avoid overconfident inferences. In parallel, the exposure context (routes, scenarios, and key assumptions) should be specified in a fit-for-purpose manner to ensure that screening endpoints remain aligned with decision-making needs prior to toxicokinetic translation.

Within this selected input space, quantitative structure–activity relationship modeling uses statistical and machine-learning algorithms to relate structural descriptors to toxicity-relevant endpoints, and it is widely used for Tier-1 hazard screening. Regulatory confidence is strengthened when models adhere to OECD validation principles (defined endpoints, unambiguous algorithms, applicability domains, predictive metrics, and mechanistic interpretation where feasible). For example, deep-learning QSAR models have been applied to large-scale endocrine disruption screening, including endpoints such as estrogenic activity and sex hormone-binding globulin binding (Heo et al. 2019). In practice, commonly used platforms and toolboxes, including VEGA and the OECD QSAR Toolbox, facilitate endpoint screening and documentation aligned with regulatory criteria (Benfenati et al. 2019). To increase decision relevance, QSAR predictions are frequently paired with read-across, in which evidence is transferred from suitable analogues, supported by a transparent rationale for similarity and uncertainty characterization (Pradeep et al. 2017). REACH is cited here only as an example of the rigor expected for read-across justification. In food safety assessment, the same general principles, including structural similarity, toxicokinetic plausibility, mechanistic consistency, and uncertainty analysis, are also relevant within an EFSA-aligned weight-of-evidence framework. Nevertheless, tier-1 QSAR/read-across are constrained by applicability-domain limits, training-set bias, and limited mechanistic granularity for complex endpoints, supporting the need for subsequent mechanism-oriented analyses and confirmatory evidence (Noga and Jurowski 2025).

Target inference using AI and machine learning

State-of-the-art computational toxicology increasingly relies on artificial intelligence and machine learning (ML) as the core engines of next generation *in silico* assessment. Machine-learning algorithms, including random forests, support vector machines, and neural networks, are now routinely applied to large toxicological datasets, such as ToxCast bioactivity profiles, to predict hazards or prioritize chemicals (Pathan et al. 2025). These models can integrate heterogeneous information by combining classical chemical descriptors with transcriptomic or other omics fingerprints to predict hepatotoxicity, endocrine disruption or other complex toxicological endpoints (Bai et al. 2025). Recent reviews emphasized that AI-driven models markedly improve toxicity prediction for multifactorial endpoints by identifying nonlinear structure–activity relationships in high-dimensional chemical and biological data (Pérez Santín et al. 2021). Building on quantitative structure–activity relationships, read-across and multifeatured modeling, modern ML and deep learning frameworks now integrate structural descriptors with biochemical and omics information to increase accuracy and robustness across drugs, industrial chemicals, environmental contaminants and food toxicants. Together, these AI and machine-learning approaches are emerging as central components of next-generation *in silico* toxicology and regulatory new approach methodologies. However, they frequently require large, diligently curated datasets and are sometimes criticized as black-box models. Therefore, rigorous internal cross-validation and external test sets are essential to avoid overfitting and support regulatory acceptance (Jia et al. 2023).

Network toxicology

Within the AI-enabled landscape, network approaches integrate *in silico* target prediction with systems biology to map links between chemicals, protein targets and pathways, effectively organizing multi-source data into mechanistic graphs that machine-learning and graph-analytic tools can further interrogate. Typical workflows first retrieve putative protein targets from resources such as EPA ToxCast, the Comparative Toxicogenomics Database, STITCH and SwissTargetPrediction, and then intersect these targets with disease- or organ-specific gene sets such as liver toxicity genes. Protein-protein interaction networks are then constructed and analyzed using Gene Ontology and KEGG enrichment analyses to highlight crucial nodes and pathways (Yang et al. 2025). For example, a network toxicology study of the phthalate substitute DINCH identified DINCH-associated hepatic genes across multiple databases, built a protein–protein interaction network, and found enrichment in apoptosis and inflammatory pathways. Core hub genes, including TNF, TP53, and PPARG, were identified and subsequently examined in TCGA and GEO datasets to assess their broader biological relevance. These external transcriptomic data provided a supportive plausibility context for TNF-associated hepatic injury pathways but were not considered independent validation of a DINCH-induced hepatotoxic mechanism (Xin et al. 2025). Similarly, network analysis of DEHP and BPA exposures identified approximately 25 putative targets for each compound using SwissTargetPrediction, ToxCast, and related platforms. It revealed hub proteins, including PTGS2, CCNB1, and CYP19A1, involved in steroidogenesis and follicle development (Li et al. 2025). For parabens, a breast

cancer network study identified thirty-five candidate targets and found that ESR1 and ESR2 were central nodes; enrichment analysis suggested estrogenic activity of parabens, and docking confirmed strong binding of paraben molecules to ESR1 and ESR2 (Zhang et al. 2025). Beyond environmental chemicals, network toxicology has also been extended to food safety and botanical-ingredient assessment. For example, network toxicology and molecular docking have been applied to aflatoxin B1, a representative food contaminant, to identify candidate targets and pathways associated with hepatotoxicity (Chu and Zi 2024). Likewise, an integrated network toxicology and feature-based molecular networking strategy has been used to investigate the hepatotoxic constituents and mechanisms of Epimedii Folium, a botanical ingredient used in dietary supplements, highlighting FXR-related bile acid dysregulation and prenylated flavonoids as candidate toxic contributors (Zhao et al. 2023). In summary, these examples show that network toxicology can help generate testable mechanistic hypotheses in food safety assessment, although the predictions still depend on database quality and require experimental confirmation.

Molecular docking simulates how small molecules bind to protein targets, such as hormone receptors or enzymes, to predict potential bioactivity. Docking can identify candidate targets for novel or understudied contaminants. For example, docking has revealed that the paraben methylparaben can bind the estrogen receptors ESR1 and ESR2, as well as the breast-cancer-related protein SERPINE1 (Zhang et al. 2025), which supports its weak estrogenic activity. Similarly, docking of DEHP, a phthalate plasticizer, with predicted endocrine targets, such as CYP19A1 aromatase, helps rationalize its anti-androgenic effects. Docking is often paired with molecular dynamics (MD) simulations to test complex stability. When integrated with network toxicology, these approaches can provide complementary structural information that helps prioritize candidate chemical-protein interactions emerging from broader pathway-level analyses. In the DEHP/BPA-PCOS network toxicology framework, docking energies ranged from −3.0 to −4.4 kcal/mol, indicating weak predicted interactions that are strongly dependent on the scoring function and modeling conditions. In addition, 200-nanosecond molecular dynamics simulations showed relatively stable root-mean-square deviation trajectories, suggesting that the predicted complexes were not immediately unstable under the simulated conditions. These findings were therefore interpreted only as hypothesis-generating support for possible chemical–protein interactions and candidate binding modes, rather than as evidence of strong binding or mechanistic confirmation (Li et al. 2025). Still, their predictive accuracy depends on the quality of the protein structure, the scoring functions used, and the extent to which they capture metabolic and kinetic processes.

Omics integration and mechanistic interpretation

High-throughput omics approaches, including transcriptomics, proteomics and metabolomics, are used to capture system-wide responses to contaminants. *In silico*, these data can be layered onto networks to build adverse outcome pathways (AOPs) or toxicity pathways. For instance, network models may incorporate gene-expression data from resources such as TCGA or GEO to validate i*n silico* predictions (Zhang and Wang 2025). In the DINCH study, predicted DINCH targets were mapped to liver cancer gene sets to assess their relevance to hepatocarcinogenic mechanisms (Xin et al. 2025). Systems toxicology aims to link molecular initiating events to organ-level outcomes through computational models, a concept that underlies the OECD AOP framework. AOPs, however, are not purely computational constructs; rather, they represent a structured integration of evidence from multiple sources and are largely informed by mechanistic experimental data, particularly *in vitro* assays. More broadly, systems toxicology seeks to connect molecular perturbations with higher-level biological outcomes by integrating computational and experimental evidence. While omics-based models are promising for human-relevant mechanistic insights, they remain complex and data-intensive. Integration of multi-omics with computer-based models is an active area of development. Tools such as AOP-Wiki, the Comparative Toxicogenomics Database, and emerging AI methods are being developed to synthesize assorted data streams.

PBPK and risk characterization

PBPK models simulate the absorption, distribution, metabolism, and excretion (ADME) of chemicals in the body using compartments that represent organs and tissues. PBPK models translate external exposure into internal dose metrics, for example, as blood or tissue concentrations and are valuable for risk assessment. For example, a human PBPK model of diethyl phthalate (DEP), which is widely used in personal care products, and its metabolite, monoethyl phthalate (MEP), was calibrated using experimental dermal and inhalation exposure data. The model showed that dermal absorption from personal care products can contribute as much DEP intake as inhalation, particularly when large areas of skin are uncovered (Chen et al. 2024), and it predicted ovarian fluid MEP concentrations of approximately 0.2–0.3 µg/L, which are comparable to levels associated with reduced oocyte yield in IVF patients. Model performance was evaluated by comparing simulated and observed urinary MEP concentrations under hood and no-hood exposure conditions. Food-relevant oral-exposure applications of PBPK/PBTK modeling have also been reported. For dietary acrylamide, PBTK models have been developed for acrylamide and glycidamide in rats and humans and used to support safe intake assessment in humans (Sweeney et al. 2010; Tardiff et al. 2010). For deoxynivalenol, a human PBK model has been adapted to relate dietary exposure to urinary excretion of deoxynivalenol and its glucuronidated metabolites in biomonitoring studies (Notenboom et al. 2023). For ochratoxin A, a PBTK model in rats and humans has been developed to support dose translation and exposure estimation (Su et al. 2024). PBPK models can incorporate age- and life-stage-related variability, coexposures, and interspecies differences, consequently supporting dose–response modeling under physiologically realistic scenarios. However, they require extensive physiological

and chemical-specific data, and uncertainty in key parameters, such as tissue partition coefficients and metabolic rates, can limit their reliability. PBPK models are increasingly accepted in regulatory assessments, as reflected in OECD guidance and EFSA opinions, for deriving internal dose metrics and uncertainty factors (Bhat et al. 2017).

Applications

In practice, integrated computational pipelines (QSAR/read-across, target prediction, network toxicology, docking/MD, and PBPK) are increasingly used to screen personal-care and food-contact chemicals for prioritized hazards. The examples discussed above illustrate how these approaches can be applied across different toxicological domains. In hepatotoxicity research, beyond the DINCH case already described (Xin et al. 2025), structure-based predictions consistently flag phthalates and parabens as potential liver toxins by indicating inhibition of phase I and phase II biotransformation enzymes and activation of oxidative-stress pathways (Liu et al. 2021). These predictions align with experimental observations that phthalate exposure promotes the formation of reactive oxygen species and perturbs Nrf2-dependent detoxification in hepatocytes. For carcinogenicity assessment, network toxicology integrated with docking has been applied to identify cancer-related proteins such as EGFR, JUN, and FN1, as well as transcriptional programs and pathways potentially associated with acrylamide exposure. In this context, PI3K-Akt, HIF-1, and related oncogenic pathways may offer a useful framework for mechanistic interpretation and hypothesis generation regarding acrylamide-associated carcinogenic processes (Duan et al. 2025). Endocrine disruption is still a central focus: high-throughput structure–activity and docking screens quantify binding to estrogen and androgen receptors. At the same time, network analyses reveal consistent disturbances in hormone biosynthesis and signaling. DEHP and BPA disrupt ovarian steroidogenesis and broader hormone-regulation pathways (Li et al. 2025), and parabens modulate estrogen-signaling networks through strong interactions with ESR1 and ESR2 (Zhang et al. 2025).

Physiologically based pharmacokinetic models translate external exposure into internal tissue doses, thereby linking hazard to plausible effects. Human PBPK models for phthalates now incorporate explicit gonadal compartments (Chen et al. 2024), enabling prediction of metabolite concentrations in reproductive organs and permitting evaluation of exposure–fertility relationships under realistic use scenarios (Xin et al. 2025). In conclusion, these applications show that integrated *in silico* pipelines already add value for prioritization and decision support in food and personal-care chemical safety. The following section reviews present constraints and describes future developments needed for reliable translational use and broader regulatory uptake.

Limitations and future perspectives

The main advantage of computational toxicology is the ability to achieve high-throughput and generate mechanistic insight without animal testing. In recent years, a wide range of in silico tools, including QSAR models, target prediction platforms, network toxicology approaches, and molecular docking methods, have been increasingly applied to toxicological assessment and risk evaluation. Many of these tools were originally developed and optimized for drug discovery applications, and their performance may not directly translate to food-related chemicals or dietary exposure scenarios. Their use in food safety assessment should therefore be considered with appropriate caution, particularly with respect to applicability domain, chemical-space coverage, and endpoint relevance. *In silico* tools can rapidly screen large chemical libraries, flagging compounds of concern even when *in vivo* data are limited (Lynch et al. 2024). They also facilitate hypothesis creation on proposed targets and pathways and support weight-of-evidence frameworks. Importantly, many models are built directly on human proteins and human cell data, which may offer better relevance to human risk than traditional animal tests. PBPK models are endorsed in OECD guidance and EFSA opinions for internal dose estimation. Despite these strengths, important shortcomings persist. QSAR and docking results depend heavily on data availability and model validity, and predictions can be unreliable outside the training domain or without experimental confirmation. Network and systems models regularly yield hypotheses with wide confidence intervals and, by themselves, do not prove causality. Machine-learning models can suffer from overfitting or limited interpretability, and their predictions require open reporting, as highlighted by OECD QSAR principles (OECD 2021). In the regulatory context, including EFSA guidance, OECD AOP work, and Integrated Approaches to Testing and Assessment (IATA) frameworks, in silico methods are generally considered NAMs. Within an EFSA-aligned stepwise assessment, these methods are primarily used for problem formulation, grouping, prioritization, and mechanistic support, and their outputs are interpreted within a weight-of-evidence framework alongside toxicological, kinetic, and experimental data. For example, QSAR classifications developed in line with OECD and REACH principles can substitute for experimental testing only if the model is well validated (Pradeep et al. 2017). Similarly, EFSA guidance on chemical grouping advocates using mechanistic and toxicokinetic data, often derived from in silico sources, to support assessment-group formation, while still requiring multiple lines of evidence, including *in vitro* and *in vivo* studies (EFSA Committee et al. 2017). Thus, the translational relevance of any computational method depends on the strength of its underlying data and on its position within an integrated assessment strategy.

The field is swiftly evolving toward integrated platforms and open data. Open databases and tools such as SwissADME and ADMETlab enable researchers to compute ADME and toxicity profiles for new chemicals or foods (Daina et al. 2017; Xiong et al. 2021), and platforms such as PharmMapper and SwissTargetPrediction facilitate target fishing using pharmacophore or similarity-based approaches. Efforts such as the US EPA CompTox Dashboard, the Tox21 bioactivity database and the EU ECHA chemical database provide large repositories of *in vitro* and *in silico* predictions. New

approach methodologies are being organized into integrated testing strategies and AOP-based workflows. Projects such as AOP-Wiki, AOP-helpFinder and OECD adverse outcome pathway guidance encourage linking *in silico* predictions of receptor binding and other molecular events with biological outcomes (Huang et al. 2016). Regulatory interest is growing, and EFSA and OECD are actively studying how to incorporate computational methods into guidance, including grouping chemicals by mode of action, developing read-across frameworks, and standardizing QSAR reporting. For endocrine disruptors, there is an explicit push toward mechanistic evidence of molecular initiating events from QSAR, docking, and omics models (Svingen 2022). Open-source computational toxicology, including machine-learning benchmarks and shared models, is also on the rise (Gadaleta et al. 2024). Looking forward, integrated platforms that combine chemical structure information, target prediction, omics perturbations, and kinetic modeling into so-called virtual tissue models or digital twin approaches are envisioned to form the next generation of risk assessment. Such integrated approaches promise to improve human relevance and efficiency. For instance, combining PBPK-predicted exposure levels with network-modeled pathway perturbations could help quantify risk and uncertainty. Continued development of transparent models and the sharing of data through open databases and collaborations will be key to regulators' trust in and adoption of these tools in safety assessment.

Effects of in vitro digestion on bioaccessibility and bioavailability, mimicking in vivo metabolism and providing greater accuracy for food toxicological protocols

Understanding the health risks associated with dietary compounds requires an accurate assessment of their absorption, metabolism, and potential toxicity. Food safety assessment demands more than measuring the total content of nutrients, contaminants, or bioactive compounds in foods; it requires understanding the transformations and their bioaccessibility and bioavailability. Bioaccessibility refers to the fraction of a compound that is released from its matrix (e.g., food, soil, or pharmaceutical formulation) during gastrointestinal digestion and becomes available for absorption in the intestinal lumen. Operationally, bioaccessibility is frequently measured using *in vitro* digestion models. The outcome is typically expressed as the percentage of the compound solubilized relative to its total content in the original matrix, which represents a physicochemical constraint on exposure and is used as a correction factor for the orally ingested dose. In contrast, bioavailability encompasses the fraction of the compound that is effectively absorbed across the intestinal epithelium and reaches the systemic circulation in an active form. This parameter inherently integrates physiological and metabolic processes, including intestinal permeability, transport mechanisms, and first-pass metabolism in the intestine and liver (Jiménez-Pulido et al. 2024; Minekus et al. 2014; Wojtunik-Kulesza et al. 2020;). Assessing the bioaccessibility of nutrients and bioactive compounds is therefore key in producing effective functional foods and nutraceuticals. It represents an essential step in food toxicology, in which ingested compounds are not necessarily absorbed in their native form. From an operational perspective in risk assessment, bioaccessibility and bioavailability are incorporated at different stages of the exposure-to-dose continuum. Bioaccessibility is applied as a modifying factor of the external (ingested) dose, accounting for the fraction that becomes available for uptake. In contrast, bioavailability is integrated into toxicokinetic modeling frameworks, including physiologically based pharmacokinetic (PBPK) models, to estimate the fraction that ultimately contributes to systemic exposure (Zhou et al. 2023; Jiménez-Pulido et al. 2024).

Traditional *in vivo* studies have long provided important insights into digestion and absorption, but are costly, time-consuming, and create ethical concerns. Consequently, the development of reliable intermediate methods that reproduce the main physiological conditions of the human digestive tract is increasingly important (Jiménez-Pulido et al. 2024). *In vitro* digestion models, particularly those that simulate gastrointestinal conditions, have proven valuable tools for studying the bioaccessibility and bioavailability of nutrients, contaminants, and bioactive compounds (Cuvas-Limon et al. 2022; Dacoreggio et al. 2024). By mimicking *in vivo* metabolism, these systems bridge the gap between simplified chemical assays and complex animal or human studies, providing more physiologically relevant data for food safety assessment and enabling toxicity evaluation of novel food components in a controlled, human-relevant context (Jiménez-Pulido et al. 2024). As an example, the standardized INFOGEST static model has become the most widely adopted reference protocol. It reproduces oral, gastric, and intestinal conditions to evaluate the stability, transformation, and solubility of food constituents during digestion, thereby generating reproducible, comparable datasets across laboratories (Brodkorb et al. 2019; Zhou et al. 2023).

Recent studies have shown that digestion models can refine toxicological assessments, revealing the formation of degradation products with potential toxicological relevance (Cuvas-Limon et al. 2022; Dacoreggio et al. 2024). These models enable screening of a wide range of compounds and food matrices under varying pH, enzyme activity, and exposure conditions, thereby supporting more precise hazard identification. Therefore, despite progress, limitations remain. Static digestion models, although standardized and robust, cannot fully reproduce the complex physiology of the human gastrointestinal tract, including peristalsis, variable pH gradients, secretion kinetics, and interactions with the gut microbiota (Minekus et al. 2014). To address these limitations, hybrid systems have been developed that combine digestion models with cell-based *in vitro* assays to simulate intestinal absorption and metabolism. Combining the INFOGEST model with Caco-2 monolayers or 3D intestinal organoid cultures supports simultaneous evaluation of permeability, transport, and biotransformation, delivering a more integrated understanding of how food components or contaminants behave post-digestion (Langhans 2018; Jiménez-Pulido et al. 2024). However, the successful integration of *in vitro* digestion models with intestinal cell systems requires careful

experimental planning to ensure reproducibility and physiological relevance across laboratories. One critical aspect is diluting the digested fraction prior to cell exposure, as digestion products may exert nonspecific cytotoxic effects on intestinal cells depending on their concentration. Indeed, studies have demonstrated that exposure to increasing proportions of digested samples containing residual digestive components (particularly bile salts, pancreatic enzymes, and digestion-derived products) reduces Caco-2 cell viability, even at relatively low concentrations, highlighting the need to define non-cytotoxic working ranges. These constituents have been shown to disrupt epithelial integrity, alter transepithelial electrical resistance (TEER), and reduce cell viability, thereby compromising the reliability of transport and metabolism data (Vital et al. 2024). In this case, dilution strategies (e.g., 1:5 to 1:30) are commonly employed to preserve cell integrity while maintaining detectable levels of bioaccessible compounds (Camporesi et al. 2026). Cutting-edge analytical approaches, including metabolomics, proteomics, and isotopic tracing, are now being integrated into these systems to trace digestion-derived metabolites and degradation products (Sheng et al. 2025). These multi-omics strategies enable the identification of transformation processes and exposure biomarkers. Moreover, emerging immune-competent and microbe-inclusive co-culture systems are providing new perspectives on host–microbiome interactions, which modulate xenobiotic metabolism, inflammation, and intestinal barrier integrity. Such multidimensional models exemplify a paradigm shift in food toxicology: moving from simple quantification of compound concentration to a dynamic, mechanistic understanding of exposure and effect (Xavier et al. 2023; Sheng et al. 2025). In this context, accounting for bioaccessibility and bioavailability is critical, since toxicological effects depend not only on the ingested dose but on the fraction of the compound that reaches systemic circulation and interacts with target organs (Hu et al. 2023). The inclusion of these parameters significantly improves the physiological relevance of toxicity assessments, ensuring that safety assessments reflect realistic human exposure scenarios.

From a toxicological standpoint, *in vitro* digestion provides important insights into how complex food matrices modulate the release, transformation, and subsequent bioavailability of potentially harmful compounds (Hu et al. 2023). Digestion can either attenuate or potentiate toxicity by altering chemical solubility, stability, or reactivity. For example, gastrointestinal conditions can convert polyphenols or Maillard reaction products into metabolites with distinct biological activity or generate reactive intermediates from lipid oxidation or processing contaminants (Wojtunik-Kulesza et al. 2020). The outputs generated by *in vitro* digestion models can be directly used to inform physiologically based pharmacokinetic (PBPK/PBK) modeling, thereby bridging the gap between external exposure and internal dose. The chemical speciation and transformation products identified in the digested fraction can be incorporated as distinct input species in PBPK models, allowing the simulation of multiple compounds with different absorption kinetics, distribution patterns, and metabolic pathways. Specifically, the bioaccessible fraction obtained after digestion provides an experimentally derived estimate of the fraction available for absorption (Fa). Furthermore, digestion-derived matrices can be coupled with intestinal cell models to generate quantitative data on permeability and transport, which inform key PBPK parameters such as the absorption rate constant (ka) and intestinal permeability coefficients, thereby improving predictions of systemic bioavailability (Chen et al. 2024). This is particularly relevant for compounds that undergo extensive transformation during digestion, as metabolites rather than the parent compound may drive their toxicity. In this context, integrating digestion outputs into PBPK modeling frameworks enables more mechanistic, physiologically relevant estimates of internal dose, supporting risk assessment approaches that move beyond conservative default assumptions toward data-driven exposure scenarios. In conclusion, these transformations highlight the need to integrate *in vitro* digestion into early stages of hazard identification and risk assessment frameworks, using dynamic gut models and multi-organ microphysiological systems, which constitute the next frontier in food toxicology.

Trends in food toxicological safety assessments and final remarks

To operationalize the concepts reviewed above, we propose a decision-oriented roadmap for the safety evaluation of emerging foods and functional ingredients (Table 4). The framework begins with rigorous chemical characterization and exposure definition, then applies tiered *in vitro* testing using relevant native and/or digestion-adjusted materials. Compounds showing positive, equivocal, or exposure-relevant bioactivity trigger higher-complexity assays, including advanced 3D or microphysiological systems, HTS, and omics-based profiling, to resolve mechanisms and identify pathway-level perturbations. These bioactivity data are subsequently translated into human-relevant internal exposure metrics using qIVIVE, which is supported by PBPK/PBK modeling. Finally, analytical, biological, mechanistic, kinetic, and exposure data are integrated under a weight-of-evidence framework to support regulatory outputs such as authorization under proposed conditions of use, authorization with restrictions, requests for additional targeted data, or non-authorization. Importantly, the computational toxicology workflow functions as a cross-cutting decision-support layer across this roadmap. Early QSAR, read-across, ADME prediction, and exposure modeling define hazard hypotheses and prioritize testing. Target prediction, network toxicology, docking, molecular dynamics, and omics integration then support mechanistic interpretation of *in vitro* and HTS findings. Finally, qIVIVE and PBPK/PBK models translate bioactivity concentrations into human-equivalent internal doses, allowing *in silico* evidence to contribute quantitatively to WoE integration, uncertainty analysis, and regulatory decision-making.

The scientific field of food toxicology is rapidly being redefined by the heightened demand for applications of metabolomic/chemical technologies, ultrastructural investigations, and computational toxicology, which are promoting an innovative paradigm for product/ingredient/compound

Table 4. Decision-oriented roadmap aligning *in silico* toxicology with chemical characterization, tiered *in vitro* testing, HTS/omics triggers, qIVIVE/PBPK translation, weight-of-evidence integration, and regulatory decision outputs.

Stage	Main purpose	Key elements/methods	Decision-oriented trigger (advance/refine/stop)	Decision-relevant output
1. Chemical characterisation and exposure framing	Define and anchor what is being assessed and under which use-relevant exposure scenario(s) before any biological testing.	Source/origin and manufacturing process; compositional profile and batch-to-batch variability; impurities/contaminants; stability; physicochemical properties; chromatographic/spectrometric characterisation; definition of test article; intended use; use level; target population; and life-cycle stage; initial exposure estimates (dietary, consumer, occupational, environmental) using read-across, QSAR, or screening models; preliminary bioavailability considerations to inform dose ranges for *in vitro* testing. ***In silico* contribution:** chemical structure curation into canonical SMILES/InChI; identification of structural alerts; QSAR and read-across screening; preliminary ADME and bioavailability prediction; exposure modelling; prioritisation of constituents of concern in complex mixtures or UVCBs.	**Advance** if identity, composition, and exposure scenarios are sufficiently defined to support biologically relevant testing. **Refine** if key uncertainties remain (e.g. unidentified constituents, unstable test article, poorly bounded exposure). **Stop/defer** biological testing until analytical characterisation and/or exposure definition is adequate to avoid uninterpretable results.	Fit-for-purpose, well-defined test article; clearly bounded, use-relevant exposure scenario(s); initial exposure metrics to contextualise in vitro concentrations; explicit hazard hypotheses and assumptions forming the basis for downstream tiered testing and decision-making.
2. Tier 1 in vitro screening	Rapidly identify potential hazard signals using simple, human-relevant in vitro systems, prioritising protection against false negatives at exposure-relevant concentrations	Relevant 2D cell systems and digestion-adjusted test materials where applicable; assays for cell viability and metabolic activity; membrane integrity (e.g. LDH release); oxidative stress/ROS; mitochondrial and metabolic perturbation; barrier integrity; core genotoxicity battery; basic allergenicity-related or immune-activation screening where appropriate; concentration–response alignment with plausible human exposure ranges. ***In silico* contribution:** QSAR/read-across alerts guide endpoint selection, concentration ranges, metabolic activation needs, and cell model selection; predicted ADME and exposure estimates help align *in vitro* concentrations with plausible human exposure.	**Stop/de-prioritise** if biologically relevant effects are observed **Advance** to Tier 2 and/or HTS/omics if clear, reproducible bioactivity overlaps exposure estimates **Refine** if responses are marginal, non-monotonic, or confounded by cytotoxicity, assay interference, or technical artefacts	Early hazard flagging is sufficient to: (i) conclude low concern at relevant exposure (with uncertainty), (ii) justify escalation to higher-tier mechanistic testing, or (iii) identify specific endpoints/pathways requiring targeted follow-up; transparent documentation of uncertainty and confidence in the screening outcome
3. Tier 2 in vitro refinement	Improve human relevance and resolve tissue- or pathway-specific mechanistic questions raised by Tier 1 screening, reducing uncertainty about adversity and relevance to in vivo biology	*in vitro* Models selected based on Tier-1 signals and exposure route, including Caco-2 permeability/absorption models; co-cultures; 3D spheroids, organoids, or organ-on-chip systems; repeated or low-concentration designs; erythrocyte oxidative stress/antioxidant models where relevant; targeted assays for intestinal, hepatic, immune, endocrine, or other identified target systems; integration of concentration–response data with exposure context. ***In silico* contribution:** predicted target organs, molecular targets, metabolic liabilities, and pathway alerts guide selection of refined models; read-across and target prediction support prioritisation of tissue-specific assays.	**Advance** to HTS/omics and/or qIVIVE/PBPK translation if Tier-2 results confirm biologically plausible, exposure-relevant perturbations with tissue or pathway specificity **Refine** assay selection, concentration design, or exposure assumptions if results are inconsistent, context-dependent, or exposure-decoupled **Stop/de-prioritise** escalation if refined models show no adverse-relevant effects within plausible human exposure ranges	Refined concentration–response relationships; identification of relevant target organs/systems and key modes of action; strengthened or reduced confidence in mechanistic plausibility; determination of whether remaining uncertainty warrants higher-throughput mechanistic interrogation or supports concluding low concern at relevant exposure

(*Continued*)

Table 4. Continued.

Stage	Main purpose	Key elements/methods	Decision-oriented trigger (advance/refine/stop)	Decision-relevant output
4. High-throughput screening (HTS)/omics trigger module	Identify molecular and cellular pathways of perturbation and define mechanistic anchors linking in vitro bioactivity to potential adverse outcomes	HTS; high-content imaging; transcriptomics; proteomics; metabolomics; toxicogenomics; AOP mapping and network-based analysis; in vitro–in vivo concordance considerations; mixture-focused profiling where relevant; benchmark concentration modelling across multiple endpoints. ***In silico* contribution:** AI/ML target prediction; network toxicology; protein–protein interaction mapping; Gene Ontology/KEGG enrichment; docking and molecular dynamics to evaluate chemical–target interactions; integration of omics datasets with predicted pathways and AOPs; mixture-focused computational profiling where relevant.	**Advance** to qIVIVE/PBPK translation if consistent, exposure-relevant pathway perturbations are observed with plausible linkage to adversity and sufficient concordance across platforms **Refine** if signals are complex, discordant across assays, mixture-driven, or sensitive to experimental context, requiring focused follow-up or targeted Tier-2 refinement **Stop/limit escalation** if pathway-level responses lack coherence, biological plausibility, or overlap with plausible human exposure ranges	Mechanistic fingerprint defining molecular initiating and key events; prioritised pathways and modes of action; omics-derived points of departure or benchmark concentrations suitable for qIVIVE/PBPK modelling; strengthened mechanistic confidence to support downstream dose translation and regulatory weight-of-evidence (WoE) evaluation
5. Quantitative in vitro–to–in vivo extrapolation (qIVIVE)/ PBPK translation	Translate in vitro bioactivity into human-relevant internal exposure metrics to determine whether observed effects are plausible under intended use conditions	Qivive incorporating free-concentration corrections; reverse dosimetry; ADME considerations (absorption, distribution, metabolism, elimination); bioavailability and biokinetic adjustments; physiologically based pharmacokinetic modelling to compare bioactive concentrations with predicted human internal doses across relevant exposure scenarios; uncertainty and variability analysis. ***In silico* contribution:** PBPK/PBK model construction; parameter estimation using physicochemical and ADME predictions; simulation of internal blood/tissue concentrations; comparison of predicted human internal exposure with *in vitro* points of departure.	**Advance** to the integrated WoE conclusion if the margins between bioactive concentrations and predicted human internal exposures are sufficiently protective **Refine/restrict** assessment if bioactive concentrations overlap or approach plausible human internal exposures, requiring refined exposure estimates, model parameterisation, or risk-management considerations **Stop escalation** if human internal exposures are clearly orders of magnitude below bioactivity thresholds, supporting low concern	Human-equivalent point(s) of departure suitable for margin-of-exposure assessment; quantitative exposure margins under intended use scenarios; transparent characterisation of uncertainty; decisive input for WoE integration and downstream regulatory decision-making
6. WoE integration	Integrate all evidence streams into a transparent, structured regulatory narrative that supports a defensible safety conclusion	Structured WoE approach (e.g. EFSA-style) considering reliability, relevance, biological plausibility, consistency, and uncertainty; integration of chemical characterisation, exposure assessment, tiered in vitro data, HTS/ omics, qIVIVE/PBPK outputs, and any available in silico, in chemico, or human data; explicit documentation of assumptions, data gaps, and uncertainty sources. ***In silico* contribution:** QSAR/read-across confidence; applicability-domain assessment; target/pathway predictions; docking/MD support; network/AOP evidence; PBPK simulations; uncertainty from computational models explicitly documented and weighted.	**Advance** to regulatory decision outputs if evidence is coherent, internally consistent, and sufficient to support a clear safety conclusion with defined uncertainty **Refine** if inconsistencies, critical data gaps, or unresolved uncertainties remain, triggering targeted follow-up testing or exposure refinement **Stop/defer** regulatory conclusion if evidence is insufficient to support a defensible decision without additional data	Integrated safety conclusion stating level of concern (or lack thereof), confidence and uncertainty bounds, key drivers of the assessment, and rationale for any remaining data needs; explicit basis for downstream regulatory decision-making
7. Regulatory decision outputs	Translate the integrated WoE conclusion into enforceable regulatory decisions under defined conditions of use	Risk-management interpretation of the complete dataset, including hazard characterisation, exposure assessment, kinetic considerations, margins of exposure, and uncertainty; alignment with applicable regulatory criteria, guidance, and protection goals. ***In silico* contribution:** supports risk management by identifying exposure scenarios, sensitive pathways, data gaps, and conditions under which safety margins are adequate or inadequate.	**Authorise as proposed** if margins are adequate and uncertainty is low **Authorise with conditions** if concerns can be managed through specifications, use restrictions, labelling, monitoring, or mitigation measures **Request additional data** if key uncertainties materially affect the decision and are feasibly resolvable **Do not authorise/require reformulation** if exposure-relevant concern remains despite refinement	Explicit regulatory outcome, including authorisation status; conditions of use or risk-management measures (if applicable); requirements for post-market monitoring or additional data generation; and a transparent rationale linking the final decision to the underlying WoE and uncertainty assessment

Note: The roadmap outlines a flexible, exposure-led decision framework adaptable across regulatory contexts.

discovery throughout multiple areas of food processing technology. Food toxicological safety assessments are shifting from traditional animal-based testing to NAMs, including high-throughput screening and computational toxicology, which focus on pathways of toxicity and molecular mechanisms. NAMs will be the main tools for NGRA, using non-animal methodologies such as *in vitro* and in silico approaches. They are increasingly used for risk identification and assessment, although regulatory adoption is still in progress. This transition strives to improve efficiency, accuracy, and relevance to human health while handling the complexity inherent in modern food systems (Yang et al. 2023).

In parallel, the integration of advanced analytical and digital technologies additionally supports this paradigm shift. Mass spectrometry, biosensors, spectroscopic and spectral imaging techniques, and surface-enhanced Raman scattering (SERS) enable the rapid, sensitive, and multiplexed detection of food toxins and contaminants at ultra-low levels, including marine biotoxins, mycotoxins, and plant-derived toxins. It also examines the application of non-target approaches, such as metabolomics and proteomics, to discover novel and emerging food toxins, thereby increasing our understanding of potential hazards in the food supply chain (Ahuja et al. 2023). Beyond chemical detection, omics-based technologies, such as genomics, transcriptomics, proteomics, and metabolomics, are reshaping the analytical foundations of food toxicology. These datasets allow the mapping of AOPs, clarifying molecular initiating events and key intermediate steps that precede adverse phenotypes (Zhang and Wang 2025). These omics-based strategies interact with high-level analytical and digital technologies, creating opportunities for more predictive, data-driven food toxicology. In this context, toxicology is undergoing a digital transformation driven by AI, machine learning (ML), and emerging digital tools, including mobile applications, sensors, blockchain, and wearable devices (Singh et al. 2023). These advances allow real-time data integration, enhanced traceability, and predictive analytics in food safety. *In silico* toxicology further accelerates this shift through AI-supported frameworks, including QSAR models, read-across methods, PBPK simulations, and network toxicology approaches, which translate *in vitro* data into human-relevant outcomes and map molecular interactions underlying adverse effects. In addition, wearable technologies (e.g., biosensors and continuous monitoring devices) can generate individual-level physiological and exposure data such as biomarkers, dietary patterns, or environmental exposures, which may be integrated with AI-driven models to support more dynamic and personalized risk assessment. The strategies presented here address various challenges and tasks in food toxicology by integrating experimental and computational tools within a coherent, decision-oriented workflow to improve predictive performance, overcome limitations, and pave the way for more accurate and efficient toxicity assessment in the future, while providing an overview of the requirements for such models to be used within the EFSA regulatory context. Together, these methods define the current trend toward mechanism-based, data-driven food toxicology and more transparent risk assessment aligned with regulatory science.

Regulatory structures orchestrate this ongoing transformation in food toxicology through legislation by international agencies, such as EFSA, FDA, and OECD, which have begun integrating NAMs into risk-assessment pipelines. This regulatory momentum aligns with trends in food toxicology and converges with key EU policy initiatives that guide the future of chemical food safety. The End-of-Waste Directive illustrates this shift, promoting circular-economy practices that, while sustainable, introduce risks from chemical contaminants and call for advanced analytical methods for risk assessment. Together with the European Green Deal and its primary initiatives, such as the Farm to Fork Strategy, the Biodiversity Strategy, and the REFIT program, these actions are redefining EU food systems to secure fairness, safety, and sustainability (D'Amore et al. 2025).

Current legislative trends in food toxicology, as outlined by the European Food Safety Authority (Miccoli et al. 2022; Lin et al. 2023; Recoules et al. 2025), point toward a deep regulatory modernization centered on mechanistic, non-animal, and data-informed approaches. Regulatory structures are progressively integrating NAMs, including *in vitro* and in silico systems, physiologically based kinetic (PBK) models, and network toxicology, to improve the mechanistic understanding of toxic effects. The development of Adverse Outcome Pathways and the use of exposome concepts reflect a shift toward holistic, pathway-based assessments that consider mixtures, co-exposures, and human variability. These advances require harmonized guidance for data integration, validation, and reporting to ensure regulatory acceptance. Altogether, these schemes define the NGRA paradigm, promoting more predictive, ethical, and transparent food safety governance across the EU. As innovation in food systems expands to encompass functional ingredients, nanomaterials, and biotechnologically derived compounds, the ability to predict toxicity using molecular- and system-level insights becomes central to regulatory science and risk governance.

In this framework, safety is no longer inferred from descriptive endpoints but predicted through mechanistic understanding, quantitative modeling, and data-based inference. From a practical standpoint, some of these approaches can already be incorporated into safety assessments, such as *in silico* models (e.g., QSAR), high-throughput data (e.g., HTS) integrated with weight-of-evidence strategies, and toxicokinetic models (PBPK/PBK) for internal dose estimation. In contrast, the routine application of omics-based approaches, microphysiological systems, and artificial intelligence-based predictive models still faces limitations related to validation, standardization, and regulatory acceptance. Currently, bodies such as the EFSA tend to accept this approach when accompanied by a clearly defined context of use, methodological transparency, evidence of reproducibility, and adequate characterization of uncertainties. However, gaps remain, especially in harmonizing protocols, performance criteria, and reporting formats, which continue to limit the consistent integration of these approaches into regulatory processes. As cooperative partnerships among academia, industry, and regulatory agencies expand, food

toxicology will evolve into a systems-based, computationally enhanced discipline that can accurately predict risks, foster responsible innovation, and ensure that the safety of food systems is evaluated with the same sophistication as their design. Thus, although significant progress is already underway, the consolidation of a fully mechanistic and data-driven food toxicology will depend on coordinated efforts to standardize and validate it, ensuring its practical applicability in decision-making.

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Abbreviations

AAPH	2,2′-Azobis(2-methylpropionamidine) dihydrochloride
ADME	Absorption, Distribution, Metabolism, and Excretion
AI	Artificial Intelligence
AOPs	Adverse Outcome Pathways
AR	Amplex Red
BPA	Bisphenol A
CAT	Catalase
CI	Combination Index
DCFH-DA	Dichlorodihydrofluorescein Diacetate
DEHP	Di(2-Ethylhexyl) Phthalate
DEP	Diethyl Phthalate
DHE	Dihydroethidium
DINCH	Di(isononyl) Cyclohexane-1,2-Dicarboxylate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EPAA	European Partnership for Alternative Approaches to Animal Testing
EU	European Union
FDA	U.S. Food and Drug Administration
GI	Growth inhibition concentration
GPx	Glutathione Peroxidase
GSH	Glutathione
HAT	Hydrogen Atom Transfer
HERYCA-P	High-Throughput Synchronous Erythrocyte Cellular Antioxidant Activity
HIV	Human Immunodeficiency Virus
HTS	High-Throughput Screening
IC	Inhibitory concentration
InChI	IUPAC International Chemical Identifier
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Lethal concentration
LDH	Lactate Dehydrogenase
MCODE	(Molecular Complex Detection)
MD	Molecular Dynamics
MDA	Malondialdehyde
MEP	Mono-Ethyl Phthalate
ML	Machine Learning
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NAMs	New Approach Methodologies
NBT	Nitroblue Tetrazolium
NCI	U.S. National Cancer Institute
NF	Novel Foods
NGRA	Next Generation Risk Assessments
NRC	National Research Council
OECD	OrganisationOrganization for Economic Co-operation and Development
OS	Oxidative Stress
PBK	Physiologically Based Kinetic
PBPK	Physiologically Based Pharmacokinetic (modellingmodeling/model)
PCA	Principal Component Analysis
PPI	(Protein-Protein Interaction)
PUFA	Polyunsaturated Fatty Acids
qIVIVE	(quantitative in vitro to in vivo extrapolation)
QSAR	Quantitative Structure–Activity Relationship
RBCs	Red Blood Cells
REACH	Registration, Evaluation, AuthorisationAuthorization and Restriction of Chemicals
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SDGs	Sustainable Development Goals
SERS	Surface-Enhanced Raman Scattering

SET	Single Electron Transfer
SI	Selectivity Index
SOD	Superoxide Dismutase
UN	United Nations
UVCBs	Substances of unknown or variable composition, complex reaction products, or biological materials
UV-Vis	Ultraviolet-Visible Radiation
WoE	Weight-of-Evidence

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