frontiers | Research Topics

Policy and regulation in bioengineering and biotechnology

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Published in
Frontiers in Bioengineering and Biotechnology

ISSN 1664-8714
ISBN 978-2-8325-4098-5
DOI 10.3389/978-2-8325-4098-5

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Policy and regulation in bioengineering and biotechnology

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Citation

Wilcks, A., Quemada, H., eds. (2023). *Policy and regulation in bioengineering and biotechnology*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-4098-5

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frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Editorial
PUBLISHED 27 November 2023
DOI 10.3389/fbioe.2023.1337663

OPEN ACCESS

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RECEIVED 13 November 2023
ACCEPTED 20 November 2023
PUBLISHED 27 November 2023

CITATION
Wilcks A and Quemada H (2023), Editorial: Policy and regulation in bioengineering and biotechnology. *Front. Bioeng. Biotechnol.* 11:1337663. doi: 10.3389/fbioe.2023.1337663

Editorial: Policy and regulation in bioengineering and biotechnology

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KEYWORDS

bioengineering, biotechnology, policy and regulation, new genetic technologies, synthetic biology, genetically modified organism (GM0)

Editorial on the Research Topic
Policy and regulation in bioengineering and biotechnology

Introduction

The field of bioengineering and biotechnology is evolving at an unprecedented pace, making it crucial for policymakers, legislators, and regulatory bodies to ensure safe, sustainable, and efficient advancements. This Research Topic of papers explores the dynamic landscape of policy, legislation, and regulatory guidelines within this domain, highlighting their instrumental roles in shaping the future. The authors of these papers collectively contribute to a more informed and proactive future for bioengineering and biotechnology regulations. By examining, evaluating, and proposing policies, they are paving the way for a more secure and productive global biotech landscape that can meet the challenges of tomorrow. The papers featured in this Research Topic serves a dual purpose: 1) to scrutinize policy-related Research Topic, offering actionable recommendations for legislation in various areas of bioengineering and biotechnology and 2) to underscore the imperative of harmonizing policies and regulations, preferably on a global scale.

Comprising eleven papers, this Research Topic includes six reviews, three original research papers, one perspective paper, and one hypothesis and theory paper submitted by authors from Africa, Europe, Latin America, and the United States.

Advancing risk assessment and management

Several papers propose strategies for enhancing the current risk assessment of bioengineered microbes or plants. For instance, Godbold et al. advocate that the inclusion of annotated sequences of concern (SoC) should be included in the risk assessment together with FunSoCs (Functional sequences of concern) to enhance the evaluation of genetically modified microorganisms, with particular emphasis on dual use research. Mueller aligns with this line of thought, using the origin of SARS-CoV-2 as a starting point to explore biorisk gaps not covered by existing policies. This investigation is

especially pertinent in the context of the rapidly expanding field of synthetic biology. These discussions are crucial, as they pave the way for comprehensive risk assessments that encompass a broad spectrum of potential hazards.

Additionally, Buyel highlights the need for a more nuanced approach when employing plants as molecular farming organisms. He emphasizes that toxic compounds can pose risks even in the absence of replication. Buyel also calls for the assessment of risks associated with the host system, including the presence of toxic secondary metabolites, and the chosen production approach. His comprehensive overview of plant-based production, with a focus on product safety, offers stakeholders actionable recommendations to navigate the complex landscape of bioengineering.

In the realm of agricultural products, two papers scrutinize the existing regulatory framework and propose improvement for regulatory assessment. Kuzma et al. evaluate the regulatory assessment of three food and agricultural biotechnology case studies in the United States. Their evaluation leads to several policy suggestions intended to bolster oversight processes and promote sustainable agrifood products that rely on novel genetic technologies (NGT). Koller and Cieslak delve into the world of unintended genetic changes in plants caused by NGT, shedding lights on the relevance of comprehensive molecular characterization and risk assessment. They underscore the significance of assessing both intended and unintended genetic changes as part of a thorough molecular characterization and risk assessment for NGT plants intended for environmental release or market authorization. Their insights pave the way for more thorough risk evaluations in this burgeoning field.

New applications and regulatory policies

The development of appropriate regulatory policies is paramount when introducing new applications to be released into unmanaged environments. An illustrative example is the release of gene drive-modified mosquitoes designed to control vector-borne diseases, as described by James et al. Their review articulates the importance of considering requirements and data needed before launching new products. This includes an examination of manufacturing and delivery requirements.

The need for harmonized regulations

The diversity of knowledge and regulatory frameworks across countries and regions pose challenges in the field of bioengineering and biotechnology. While the widely differing approaches to regulation have been an obstacle with respect to transgenic organisms, the problem continues when countries deal with gene editing and other new genetic technologies. Several papers in this Research Topic address this Research Topic and offer recommendations to overcome it. Zarate et al. examine agricultural gene editing regulation in nine Latin America and the Caribbean countries. Their findings reveal the positive reception of harmonized regimes throughout the region. The benefits of coordination are evident, demonstrating how streamlined regulations can facilitate the responsible growth of bioengineering and biotechnology.

Masehela and Barros underscore the importance of coordinated policy and regulatory guidelines across the African continent. They highlight the advancements and challenges faced by various African countries in the development and implementation of biosafety policies. They call for an organized and coordinated approach in the region, underpinned by political will and commitment, to facilitate open discussions among scientists, regulators, and policy makers.

Mungeyi et al. provide a detailed overview of Namibian biosafety regulations and the implications for food and feed importers. They advocate for the reduction of administrative burdens, improved dialogue between regulators and the industry, and an increased awareness of regulations for feed and food importers. In line with Masehela and Barros, they propose that Namibia could learn from other countries and regions with established processes, thereby accelerating their own regulatory framework development.

From the European Union (EU) da Silva and Blasimme present a systematic review highlighting the impact of regulatory incentives on the rapid growth of organ chip research. Their analysis showcased how the convergence of research efforts, funding, and regulatory incentives has shaped a robust knowledge ecosystem that places many European research institutions as key international players in the field of organ chip research. This serves as an excellent example of how regional cooperation can advance research and innovation.

Addressing inequities in biotechnology capabilities

Trump et al. investigate how risk culture contributes to disparities in biotechnology capabilities and how this could influence global inequities. They reveal how early adoption of biotechnology and regulatory frameworks can shape the development and acceptance of biotechnological innovations. The concentration of power in a few early adopter nations may hinder global collaboration, impede knowledge sharing, and potentially create a fragmented and competitive global biotech landscape. These findings emphasize the importance of a balanced, collaborative approach to global biotechnology advancement.

Author contributions

AW: Writing–original draft. HQ: Writing–review and editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Review
PUBLISHED 25 April 2023
DOI 10.3389/fbioe.2023.1124100

OPEN ACCESS

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RECEIVED 14 December 2022
ACCEPTED 11 April 2023
PUBLISHED 25 April 2023

CITATION
Godbold GD, Hewitt FC, Kappell AD, Scholz MB, Agar SL, Treangen TJ, Ternus KL, Sandbrink JB and Koblentz GD (2023), Improved understanding of biorisk for research involving microbial modification using annotated sequences of concern.
Front. Bioeng. Biotechnol. 11:1124100.
doi: 10.3389/fbioe.2023.1124100

Improved understanding of biorisk for research involving microbial modification using annotated sequences of concern

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Regulation of research on microbes that cause disease in humans has historically been focused on taxonomic lists of 'bad bugs'. However, given our increased knowledge of these pathogens through inexpensive genome sequencing, 5 decades of research in microbial pathogenesis, and the burgeoning capacity of synthetic biologists, the limitations of this approach are apparent. With heightened scientific and public attention focused on biosafety and biosecurity, and an ongoing review by US authorities of dual-use research oversight, this article proposes the incorporation of sequences of concern (SoCs) into the biorisk management regime governing genetic engineering of pathogens. SoCs enable pathogenesis in all microbes infecting hosts that are 'of concern' to human civilization. Here we review the functions of SoCs (FunSoCs) and discuss how they might bring clarity to potentially problematic research outcomes involving infectious agents. We believe that annotation of SoCs with FunSoCs has the potential to improve the likelihood that dual use research of concern is recognized by both scientists and regulators before it occurs.

KEYWORDS

microbial pathogenesis, DURC, functions of sequences of concern, FunSoCs, biothreat, biorisk, ontology

Introduction

In 2022, the National Institutes of Health (NIH) and Office of Science and Technology Policy (OSTP) began a process to evaluate the effectiveness of dual-use research oversight in the United States and determine whether the current approach sufficiently addresses future potential threats in biological research (Tabak and Jorgenson, 2022). This review encompasses three policies: the March 2012 United States Government Policy for Oversight of Life Sciences Dual Use Research of Concern (United States Government, 2012), the September 2014 United States Government Policy for Institutional Oversight of Life Sciences Dual Use Research of Concern (United States Government, 2014), and the December 2017 Framework for Guiding Funding Decisions about Proposed Research Involving

TABLE 1 Tier 1 pathogens of concern, federal select agent program.

Avian influenza virus (highly pathogenic)
Bacillus anthracis
Botulinum neurotoxin
Burkholderia mallei
Burkholderia pseudomallei
Ebola virus
Foot-and-mouth disease virus
Francisella tularensis
Marburg virus
Reconstructed 1918 Influenza virus
Rinderpest virus
Toxin-producing strains of *Clostridium botulinum*
Variola major virus
Variola minor virus
Yersinia pestis

Enhanced Potential Pandemic Pathogens (P3CO Framework) (Department of Health and Human Services, 2017). The March 2012 and September 2014 dual use research of concern (DURC) policies are complementary and will be considered together since they are both based on a shared list of pathogens and experiments that are subject to oversight. Under the DURC policies, research that is either conducted or funded by a federal agency on fifteen pathogens and toxins (Table 1) that is "reasonably anticipated" to produce one of seven experimental outcomes (Table 2) are subject to review by the funding agency. The list of pathogens is based on those deemed to be Tier 1 high-consequence biological threats by the Federal Select Agent Program (FSAP).

The list-based approach of the DURC policies has been criticized for its static nature and lack of coverage of potentially risky research with pathogens that are not on the Select Agent Tier 1 list. A 2018 study by the National Academies of Science, Engineering, and Medicine highlighted the variety of ways in which biological threats beyond those on this specific list could be generated thanks to our improved understanding of which genotypes generate potentially harmful phenotypes and the diffusion of the expertise, techniques, and technologies needed to apply this knowledge to develop modified genomes with enhanced harmful attributes (National Academies of Science, Engineering, and Medicine, 2018).

The P3CO Framework provides for oversight of research funded by the Department of Health and Human Services (HHS) that is "reasonably anticipated" to enhance the lethality and/or transmissibility of a potential pandemic pathogen (PPP) which is a pathogen capable of "wide and uncontrollable spread" in human populations and able to cause "significant morbidity and/or mortality" in such a population. This type of research is known as "gain of function" since it results in a microbe with enhanced virulence, pathogenicity, transmissibility, or other attribute that poses a higher risk to the host population than the naturally occurring strain. Unlike the DURC policy, the P3CO Framework is not limited to a specified list of pathogens. However, both policies rely on an interpretation of which types of laboratory experiments can be "reasonably anticipated" to have the effects or outcomes covered by both policies. The Government Accountability Office (GAO) has highlighted the lack of a standard for judging what is "reasonably anticipated" as a weakness in the oversight of dual-use research (Government Accountability Office GAO, 2023).

Several of the more controversial "gain of function" experiments were the results of failures by scientists and/or funding agencies to "reasonably anticipate" the outcome of the proposed research. The canonical example is the insertion of the gene coding for the murine interleukin-4 (IL-4) immunomodulator into ectromelia virus (mousepox) by Australian scientists in 2001. This experiment resulted in a strain of the virus that was uniformly lethal to both susceptible and genetically resistant mice and, even more worryingly, killed 60% of mice vaccinated against the virus (Jackson et al., 2001). According to the authors of the study, "this came as a complete surprise and was totally unexpected." However, it has been argued that previous work on IL-4 and poxviruses was such that the "available evidence fully predicted" that a recombinant mousepox IL-4 virus would be more virulent (Müllbacher and Lobigs, 2001), enhancing the harmful consequences of the agent [Table 2], disrupting host immunity [Table 2], and increasing the susceptibility of the host population to the virus [Table 2].

A more recent example also shows that potentially perilous engineering is not always identified in advance. In 2014, EcoHealth Alliance proposed a research project to the NIH to modify coronaviruses, including MERS and bat-related coronaviruses, to evaluate the pandemic risk they posed. NIH determined that these experiments did not fall under the scope of the P3CO Framework because the modifications were "not expected to generate viruses

TABLE 2 Dual-use research of concern (DURC).

a. Enhances the harmful consequences of the agent or toxin
b. Disrupts immunity or the effectiveness of an immunization against the agent or toxin without clinical or agricultural justification
c. Confers to the agent or toxin resistance to clinically or agriculturally useful prophylactic or therapeutic interventions against that agent or toxin or facilitates their ability to evade detection methodologies
d. Increases the stability, transmissibility, or the ability to disseminate the agent or toxin
e. Alters the host range or tropism of the agent or toxin
f. Enhances the susceptibility of a host population to the agent or toxin
g. Generates or reconstitutes an eradicated or extinct agent or toxin

that would be more transmissible or more virulent in humans" despite this being the stated goal of the project. [Letter from NIH Director Francis Collins to Senator Charles Grassley, 28 July2021[1].

The NIH was criticized when it became apparent that the resulting chimeric viruses were, in fact, more virulent in humanized animal models than the original strain[2]. In contrast, the Defense Advanced Research Projects Agency (DARPA) rejected a proposal from EcoHealth Alliance to fund similar research with chimeric coronaviruses using genes associated with the spike protein of SARS-related coronaviruses found in bats[3].

Some countries use taxonomy-based lists for export controls, but only a handful of other countries use such lists to exercise oversight for dual-use research as the United States does. In the United Kingdom, the three largest funders of life sciences research—the Biotechnology and Biological Sciences Research Council, the Medical Research Council, and the Wellcome Trust—review research proposals for dual-use potential using the same list of experiments of concern as in the United States. However, the dual-use review process in the United Kingdom is applicable to all life sciences research, not just that conducted with a list of pathogens as in the United States[4]. Canada requires research institutions to develop plans for managing biorisks, including dual-use issues, and processes for scientists to report to their institution if their research could result in the creation of a "human pathogen with increased virulence, pathogenicity, or communicability, that is resistant to preventative or therapeutic treatments, or produces a toxin with increased toxicity"[5]. Australia does not have an explicit dual-use oversight policy, but research with infectious agents and creation of genetically modified organisms, including as a result of "gain of function" experiments, is subject to monitoring and reporting[6].

Sequences of concern (SoCs) as drivers of infectious diseases

We have been engaged in a multi-year effort to understand the risks of biological sequences and the sorts of threats they pose to humanity. Those that are likely to cause problems if moved to another organism have been called sequences of concern (National Research Council, 2010) which we abbreviate as SoCs. Techniques to transfer sequences from one microbe to another and alter them in ways both minor and major are widely available. We contend that SoCs are not confined to microbes that have historically been feared for their capacity for weaponization, but rather are found in all parasites that have evolved with specific host organisms to cause disease in those organisms. These are commonly called pathogens.

Thousands of published investigations in microbial pathogenesis have provided ample reason to think that the *direct* activity of SoCs on constituent molecules of the host is the primary driver of successful infection and pathogenesis. In the absence of these microbial sequences—which associate with and modify host molecules—infection cannot occur. There are also SoCs that act *indirectly*—by either altering molecules of the parasite or facilitating the operation of direct-acting SoCs (e.g., bacterial secretion system components and chaperones), but these are of secondary importance. Lastly there is the consideration of gene expression. If neither the direct- nor the indirect-acting SoCs of a bacterial or eukaryotic parasite are expressed in sufficient abundance or in a timely, coordinated manner, then the microbe will not be able to successfully exploit the host organism. We also recognize that there are transcriptional, translational, and post-translational influences. There can also be epigenetic effects modulating expression. We are not asserting that SoCs are the only contributors to pathogenic phenotypes, merely that, for microbial parasites, they are the essential contributors to such phenotypes for host organisms with normal immune systems and intact barriers. Without these sequences, the encoding microbes could not cause disease in the healthy, immune-normal hosts with which they co-evolved as pathogens.

In our earlier publication we described how we reviewed thousands of papers to find thousands of virulence factors from bacterial, viral, and eukaryotic parasites that were good candidates for SoCs. We pondered if a sequence, following transfer to another microbe, would be likely to enhance its ability to colonize a susceptible host, increasing the pathological consequences of infection. If the answer was 'probably yes', then we detailed its host-relevant activities and incorporated it in our dataset. For ~100 sequences, the authors *demonstrated* the ability of the transferred sequence to exhibit the same or similar pathogenic function in a different microbe that was previously associated with the expression of that sequence in the original microbe (Godbold et al., 2022).

We developed a controlled vocabulary to describe SoCs called Functions of Sequences of Concern (FunSoCs) (Godbold et al., 2022). We used it for both machine learning and bioinformatic software (Balaji et al., 2022). With FunSoCs, we attempted to capture both the activity and the consequences of these sequences on the host during infection. We identified four types of host *damage* caused by SoCs: (1) cytotoxicity or cell membrane disruption, (2) tissue degradation, (3) organ disabling, and (4) inflammation. We also described types of *innate immune subversion* resulting from SoC activity including: (i) suppression of host immune signaling (with many subtypes), (ii) resisting phagocytosis, (iii) neutralizing host complement, (iv) countering antimicrobial peptide, (v) resisting oxidative killing, (vi) neutralizing host immunoglobulin, (vii) defeating host cytokine, and (viii) inhibiting antigen presentation. Two types of direct SoC activity are characteristic of nearly all infectious organisms: *adherence* and *invasion*. There are two functions of direct-acting SoCs peculiar to intracellular pathogens: *movement within a host cell* and *niche creation*. Finally, some SoCs provide pathogens the ability to *disseminate* within the host organism by subverting host barriers. In addition, we

1 https://www.grassley.senate.gov/imo/media/doc/national_institutes_of_health_to_grassley_-_covid_origins_grant_oversight.pdf.

2 https://theintercept.com/2021/09/09/covid-origins-gain-of-function-research/and https://theintercept.com/2021/10/21/virus-mers-wuhan-experiments/.

3 https://theintercept.com/2021/09/23/coronavirus-research-grant-darpa/.

4 https://cms.wellcome.org/sites/default/files/wtp059491.pdf.

5 https://www.canada.ca/en/public-health/programs/consultation-biosafety-guideline-dual-use-life-science-research/document.html.

6 https://www.nhmrc.gov.au/file/18130/download?token=anGdkE4f.

note which of nine areas of host cell biology (transcription, ubiquitination, etc.) are targeted by SoCs (Godbold et al., 2022).

A valuable adjunct to the consequentialist focus of FunSoCs is the pathogenesis gene ontology (PathGO) developed by researchers at the Johns Hopkins University Applied Physics Laboratory[7]. PathGO consists of ~170 terms which are rooted in biological process and molecular function terms of the Gene Ontology resource (Ashburner et al., 2000; Gene Ontology Consortium et al., 2021). PathGO terms identify the host molecules and pathways that are the targets of SoC activity, and we have employed these to further specify SoCs in our dataset.

In the following sections we address the relative abundance of SoCs in pathogen genomes with reference to SARS-CoV-2 and *Bacillus anthracis*. We discuss whether some SoCs are worse or more dangerous than others with respect to their host-affecting properties and provide some examples of SoCs with multiple functions from bacterial, viral, and eukaryotic pathogens of humans. Next, we emphasize the importance of immune subverting SoCs as these sequences appear critical for producing host susceptibility to microbes. Then we consider the appropriate criteria for determining what microbes from which SoCs should be appropriated. In the final sections we grapple with how annotated SoCs can be used to guide biorisk management decisions. We provide a rubric (Table 6) that exemplifies how they might be applied to the USG dual-use research of concern policy to simplify decision-making processes. We close by drawing out implications of using SoCs to supplement the current taxonomic list-based approach for dual-use research oversight.

How abundant are SoCs in pathogen genomes?

SoCs are more abundant in viral genomes as a fraction of the total genetic material than in other parasites. Of microbes capable of causing disease in humans, viruses are the most genetically compact. Even the largest of these (poxviruses) possess genomes two to three times smaller than that of the smallest bacterial pathogen (*Mycoplasma*). They contain, proportionally, more sequences that confound host immunity than bacterial, fungal, or protozoal parasites. Viruses abound in sequences disrupting innate immune signaling (Godbold et al., 2022). The larger viral pathogens for humans have DNA genomes and can allocate single sequences to one or just a few functions like soaking up host cytokines to blunt the local immune response (Dunlop et al., 2003; Seet et al., 2003; Alvarez-de Miranda et al., 2021). RNA viruses are necessarily more compact with each protein serving many functions. SARS-CoV-2 is an example. Of the ~27 sequences that are translated into proteins (Jungreis et al., 2021), at least 24 are SoCs and 18 of those suppress host cellular immune defenses including Membrane (M), Nsp1, Nsp3, Nsp5, Nsp6, Nsp7, Nsp10, Nsp12, Nsp13, Nsp14, Nsp15, Nucleocapsid (N), Orf3a, Orf6, Orb7b, Orf8, Orf9b, and Spike (S). The hypervariable Orf8 is the only one of these that has so far been demonstrated to be dispensable (Zinzula, 2021). The immune subverting activity for the sequences that suppress host immunity are summarized in Table 3.

Our annotations suggest that, in contrast to viruses, the great majority of the encoded sequences of *nonviral* microbes play no role in pathogenesis. These SoCs comprise, at most, a few per cent of the sequences of bacterial, fungal, and protozoal pathogen genomes. Some microbes do not have nearly so many. Out of ~5,800 genes on a single chromosome and two plasmids, *Bacillus anthracis* encodes about a dozen proteins enabling pathogenesis including the 'big three' of protective antigen, edema factor, and lethal factor. The annotated proteins are shown in Table 4.

Are some SoCs 'worse' than others?

We think the following assertions are generally true about the relative danger of SoCs in microbial pathogenesis. First, SoCs that act directly on a host molecule are more concerning than those that act indirectly. Second, SoCs that have a *damaging* effect are more concerning than those that only provide adhesive, invasive, or disseminating capacities. We think that SoCs enabling dissemination of a pathogen that has already colonized a host are more concerning than adhesive or invasive SoCs. Third, SoCs that only provide within-cell motility or the ability to form an intracellular niche are the SoCs of lowest concern of the direct-acting SoCs. Fourth, SoCs that have multiple functions are more concerning than those that have a single function. Some sequences that enable adhesion can also subvert immunity. A subset of SoCs with many functions are detailed in Table 5. *Immune subverting* SoCs are a special case that we address in the next section.

The importance of immune subverting SoCs for host susceptibility

Why are some organisms susceptible to infection by some microbes but not others? Why are immune-compromised persons subject to infection with a broader range of parasites than immune-normal persons? Why do defects in immune detectors and immune effectors of an organism allow microbes that are normally incapable of infection to become competent for infection and pathogenesis? A single amino acid change in an immune effector can mean the difference between life and death during challenge with a virus (Andoniou et al., 2014). The study of human immune deficiencies shows the critical importance of components of innate immunity for defense against the specific, usually narrow, set of parasites against which they defend (Casanova, 2015a; 2015b; Li et al., 2017).

What these phenomena have in common is a host with intact barriers and an immune system that fends off microbes that lack direct-acting sequences evolved to either counter or disrupt key components of the innate immune system of that host (Godbold et al., 2022). These direct-acting sequences, expressed in a combination that varies by parasitic microbe, produce a state of susceptibility in a host, allowing colonization by the parasite (Wickham et al., 2007; Kurupati et al., 2010). Such a set of immune subverting mechanisms is not generic. A parasite with a given set of innate immune subverting

7 https://github.com/jhuapl-bio/pathogenesis-gene-ontology.

TABLE 3 SARS-CoV-2 encoded proteins directly involved in host immune subversion.

SoC	Innate immune subversion	FunSoCs	PathGO
Nsp1	Nsp1 shut down cellular translation, thereby abrogating much of the cellular innate immune defense (Thoms et al., 2020).	Manipulate host translation (Schubert et al., 2020; Thoms et al., 2020; Zhang et al., 2021a; Finkel et al., 2021); Suppress host immune signaling (Lei et al., 2020; Thoms et al., 2020; Shemesh et al., 2021);	PATHGO:0000006 (modulates protein synthesis in another organism) (Thoms et al., 2020); PATHGO:0000370 (mediates mRNA destruction in another organism) (Huang et al., 2011);
Nsp3	The protease Nsp3 cuts ISG15 from proteins to dampen inflammation and antiviral signaling (Klemm et al., 2020). It counteracted host antiviral ADP-ribosylation by poly-ADP-ribose polymerases (Rack et al., 2020; Russo et al., 2021), and also cleaved interferon response factor 3 (IRF3) (Moustaqil et al., 2021).	Manipulate host ubiquitin dynamics (Klemm et al., 2020); Suppress host immune signaling (Klemm et al., 2020; Lei et al., 2020; Rack et al., 2020; Shin et al., 2020; Moustaqil et al., 2021; Correy et al., 2022); Resist other immune effector (Rack et al., 2020; Brosey et al., 2021; Schuller et al., 2021); Degrade tissue (cytopathic effect) (Shin et al., 2020);	PATHGO:0000382 (suppresses interferon signaling in another organism) (Lei et al., 2020; Moustaqil et al., 2021); PATHGO:0000325 (modulates ubiquitin dynamics in another organism) (Klemm et al., 2020); PATHGO:0000330 (mediates de-ISGylation of proteins in another organism) (Klemm et al., 2020); PATHGO:0000365 (mediates de-ADP-ribosylation of proteins in another organism) (Rack et al., 2020);
Nsp5	The Nsp5 protease cut human TAB1, the intracellular pattern recognition receptor NLRP12 (Moustaqil et al., 2021), and human gasdermin (Shi et al., 2022). It promoted the ubiquitination and subsequent destruction of host MAVS. Nsp5 cut the N-terminus of RIG-1 to eliminate its ability to trigger downstream interferon production (Liu et al., 2021). Nsp5 disrupted formation of cellular stress granules and the consequent interaction of RIG-1 and MAVS (Zheng et al., 2022).	Manipulate host ubiquitin dynamics (Liu et al., 2021); Suppress host immune signaling (Liu et al., 2021; Shemesh et al., 2021);	PATHGO:0000325 (modulates ubiquitin dynamics in another organism) (Liu et al., 2021); PATHGO:0000306 (disrupts RIG-I signaling in another organism) (Liu et al., 2021);
Nsp6	Nsp6 associated with TANK-binding kinase 1 to suppress IRF3 phosphorylation and subsequent interferon-beta production (Xia et al., 2020; Vazquez et al., 2021).	Manipulate host membrane dynamics (Díaz, 2020; Mishra et al., 2021); Suppress host immune signaling (Xia et al., 2020; Shemesh et al., 2021);	PATHGO:0000382 (suppresses interferon signaling in another organism) (Xia et al., 2020); PATHGO:0000236 (modulates cell endomembrane dynamics in another organism) (Díaz, 2020);
Nsp12	Nsp12 inhibited IFN promoter activation triggered by overexpression of RIG-I, MDA5, MAVS, and IRF3. This suppression was not dependent upon the polymerase activity of Nsp12 (Wang et al., 2021c).	Suppress host immune signaling (Lei et al., 2020; Wang et al., 2021c);	PATHGO:0000382 (suppresses interferon signaling in another organism) (Wang et al., 2021c);
Nsp13	Nsp13 associated with TANK-binding kinase 1 to suppress IRF3 phosphorylation and subsequent interferon-beta production (Xia et al., 2020; Vazquez et al., 2021). Nsp13 associated with STAT1 to suppress interferon signaling (Feng et al., 2021).	Manipulate host ubiquitin dynamics (Guo et al., 2021); Manipulate host membrane dynamics (Díaz, 2020; Gordon et al., 2020); Suppress host immune signaling (Lei et al., 2020; Xia et al., 2020; Zhang et al., 2021b; Feng et al., 2021);	PATHGO:0000382 (suppresses interferon signaling in another organism) (Xia et al., 2020); PATHGO:0000236 (modulates cell endomembrane dynamics in another organism) (Díaz, 2020); PATHGO:0000325 (modulates ubiquitin dynamics in another organism) (Guo et al., 2021); PATHGO:0000302 (disrupts JAK-STAT signaling in another organism) (Feng et al., 2021);
Nsp14	The NSP14 exonuclease antagonized host cell interferon production and host IRF3 nuclear translocation (Yuen et al., 2020). Nsp14 mediates the cessation of host cell translation. Mutations in the active site of either abolish its ability to inhibit translation. Nsp14 forms a complex with Nsp10 that enhances its ability to inhibit translation and so abolishes the induction of immune evasion genes by interferon (Hsu et al., 2021).	Manipulate host translation (Hsu et al., 2021); Suppress host immune signaling (Lei et al., 2020; Yuen et al., 2020; Hsu et al., 2021);	PATHGO:0000327 (mediates DNA cleavage in another organism) (Yuen et al., 2020); PATHGO:0000382 (suppresses interferon signaling in another organism) (Yuen et al., 2020); PATHGO:0000006 (modulates protein synthesis in another organism) (Hsu et al., 2021);
Nsp15	Nsp15 interfered with IFN-alpha/beta production through its interaction with the host E3 ligase RNF41/Nrdp1 (Gordon et al., 2020).	Manipulate host ubiquitin dynamics (Gordon et al., 2020); Manipulate host membrane dynamics (Díaz, 2020); Suppress host immune signaling (Yuen et al., 2020; Shemesh et al., 2021);	PATHGO:0000236 (modulates cell endomembrane dynamics in another organism) (Díaz, 2020); PATHGO:0000306 (disrupts RIG-I signaling in another organism) (Shemesh et al., 2021); PATHGO:0000325 (modulates ubiquitin dynamics in another organism) (Gordon et al., 2020);
Orf3a	Orf3a upregulated suppressor of cytokine signaling (SOCS1) to inhibit antiviral JAK/STAT signaling (Wang et al., 2021a). It is associated with the host E3 ubiquitin ligase TRIM59 which regulates antiviral immune signaling (Gordon et al., 2020).	Manipulate host transcription (Wang et al., 2021a); Manipulate host ubiquitin dynamics (Gordon et al., 2020); Manipulate host regulated cell death (Ren et al., 2020); Manipulate host membrane dynamics (Chen et al., 2021); Manipulate xenophagy (Chen et al., 2021; Miao et al., 2021; Su et al., 2021; Zhang et al., 2022); Suppress host immune signaling (Gordon et al., 2020; Wang et al., 2021a);	PATHGO:0000335 (induces apoptosis in another organism) (Ren et al., 2020); PATHGO:0000239 (disrupts phagolysosome fusion in another organism) (Zhang et al., 2021d; Miao et al., 2021); PATHGO:0000347 (modulates autophagy or xenophagy in another organism) (Zhang et al., 2021d; Miao et al., 2021); PATHGO:0000302 (disrupts JAK-STAT signaling in another organism) (Wang et al., 2021a); PATHGO:0000326 (modulates transcription in another organism) (Wang et al., 2021a);

(Continued on following page)

TABLE 3 (*Continued*) SARS-CoV-2 encoded proteins directly involved in host immune subversion.

SoC	Innate immune subversion	FunSoCs	PathGO
Orf6	Orf6 associated with importin karyopherin-alpha2 (KPNA2) to inhibit translocation of IRF3 to the nucleus (Xia et al., 2020). The C-terminus of Orf6 directly binds to STAT1 resulting in its exclusion from the host nucleus (Miyamoto et al., 2022).	Manipulate host translation (Gordon et al., 2020; Addetia et al., 2021); Suppress host immune signaling (Lei et al., 2020; Li et al., 2020, 8; Xia et al., 2020; Yuen et al., 2020; Miyamoto et al., 2022);	PATHGO:0000006 (modulates protein synthesis in another organism) (Gordon et al., 2020); PATHGO:0000382 (suppresses interferon signaling in another organism) (Xia et al., 2020);
Orf8	Orf8 mediated immune evasion via downregulation of host MHC-I (Flower et al., 2020; Park, 2020). MHC-I molecules are targeted for lysosomal destruction by autophagy through the host beclin-1-mediated pathway (Zhang et al., 2021c).	Manipulate host membrane dynamics (Díaz, 2020); Suppress host immune signaling (Li et al., 2020, 8); Inhibit host antigen presentation (Flower et al., 2020, 8; Park, 2020, 8; Zhang et al., 2021c; Matsuoka et al., 2022); Induce inflammation (Lin et al., 2021; Zinzula, 2021);	PATHGO:0000308 (disrupts antigen presentation in another organism) (Flower et al., 2020, 8; Park, 2020, 8); PATHGO:0000351 (mediates cytokine sequestration in another organism) (Lin et al., 2021); PATHGO:0000236 (modulates cell endomembrane dynamics in another organism) (Díaz, 2020); PATHGO:0000362 (suppresses anti-inflammatory cytokine activity in another organism) (Lin et al., 2021);
Orf9b	Orf9b localized to the membrane of host mitochondria and suppressed host type I interferon (IFN) responses by targeting host TOM70 (Jiang et al., 2020; Brandherm et al., 2021). Orf9b antagonized the cellular antiviral response by targeting the NFκB essential modulator (NEMO, IKKγ). This association disrupted the polyubiquitination of NEMO and inhibited NFκB signaling (Wu et al., 2021).	Manipulate host ubiquitin dynamics (Wu et al., 2021); Suppress host immune signaling (Kreimendahl and Rassow, 2020; Han et al., 2021; Wu et al., 2021);	PATHGO:0000306 (disrupts RIG-I signaling in another organism) (Kreimendahl and Rassow, 2020); PATHGO:0000325 (modulates ubiquitin dynamics in another organism) (Wu et al., 2021); PATHGO:0000295 (suppresses NFκB signaling in another organism) (Wu et al., 2021); PATHGO:0000300 (suppresses STING signaling in another organism) (Han et al., 2021); PATHGO:0000352 (disrupts TRIM/TRIM-like signaling in another organism) (Han et al., 2021); PATHGO:0000382 (suppresses interferon signaling in another organism) (Han et al., 2021);
M	M localizes to the host ER and Golgi and colocalizes with host TBK1 and TRAF3 but just partially with RIG-I, MDA-5, and MAVS. Membrane prevents the interaction of RIG-I with MAVS, MAVS with TBK1, and TRAF3 with TBK1. IRF3 phosphorylation is inhibited (Zheng et al., 2020). Membrane protein suppresses expression of IFNβ and interferon-stimulated genes by interacting with MDA5, TRAF3, IKKε, and TBK1. Membrane protein induces the degradation of TBK1 by Lys48-linked ubiquitination. Lower levels of TBK1 impair formation of the TRAF3-TANK-TBK1/IKKε complex leading to inhibition of IFN-I (Sui et al., 2021).	Manipulate host ubiquitin dynamics (Sui et al., 2021, 1); Manipulate host regulated cell death (Yang et al., 2022); Manipulate host membrane dynamics (Díaz, 2020); Suppress host immune signaling (Lei et al., 2020; Zheng et al., 2020; Sui et al., 2021, 1); Resist other host immune effector (Zhang et al., 2021b);	PATHGO:0000325 (modulates ubiquitin dynamics in another organism) (Sui et al., 2021, 1); PATHGO:0000236 (modulates cell endomembrane dynamics in another organism) (Díaz, 2020); PATHGO:0000382 (suppresses interferon signaling in another organism) (Lei et al., 2020); PATHGO:0000306 (disrupts RIG-I signaling in another organism) (Fu et al., 2021; Sui et al., 2021, 1); PATHGO:0000314 (modulates TRAF signaling in another organism) (Sui et al., 2021, 1);
N	Nucleocapsid suppressed the interaction between the host TRIM25 proteins and RIG-I (Oh and Shin, 2021). It also interacted with both STAT1 and STAT2 to suppress their nuclear translocation (Mu et al., 2020). Nucleocapsid bound host G3BP1 and thereby contributed to the dispersion of host stress granules where antiviral signaling is facilitated (Biswal et al., 2022; Zheng et al., 2022).	Manipulate host translation (Gordon et al., 2020; Lu et al., 2021); Adherence to another organism (Kumar et al., 2020); Suppress host immune signaling (Li et al., 2020, 8; Mu et al., 2020; Oh and Shin, 2021; Wang et al., 2021b; Zheng et al., 2022); Induce inflammation (Kumar et al., 2020; Magro et al., 2020; Youn et al., 2021);	PATHGO:0000006 (modulates protein synthesis in another organism) (Gordon et al., 2020); PATHGO:0000306 (disrupts host RIG-I signaling) (Mu et al., 2020); PATHGO:0000302 (disrupts JAK-STAT signaling in another organism) (Mu et al., 2020); PATHGO:0000382 (suppresses interferon signaling in another organism) (Mu et al., 2020); PATHGO:0000361 (enhances coagulation in another organism) (Magro et al., 2020; Youn et al., 2021); PATHGO:0000352 (disrupts TRIM/TRIM-like signaling in another organism) (Oh and Shin, 2021); PATHGO:0000072 (mediates binding to cell surface glycoprotein in another organism) (Kumar et al., 2020);
S	The S1 portion of spike directly interacted with STAT1 to interfere with the interaction between JAK1 and STAT1 and suppressed STAT1 phosphorylation (Zhang et al., 2021b).	Manipulate host membrane dynamics (Prelli Bozzo et al., 2021); Adherence to another organism (Cantuti-Castelvetri et al., 2020; Daly et al., 2020; Saputri et al., 2020); Host invasion (Cantuti-Castelvetri et al., 2020; Daly et al., 2020; Walls et al., 2020); Suppress host immune signaling (Zhang et al., 2021b); Induce inflammation (Cao et al., 2020; Barreda et al., 2021; Khan et al., 2021; Shirato and Kizaki, 2021; Youn et al., 2021); Degrade tissue (Buchrieser et al., 2020; Barrett et al., 2021; Rocheleau et al., 2021; Yu et al., 2022);	PATHGO:0000072 (mediates binding to cell surface glycoprotein in another organism) (Saputri et al., 2020); PATHGO:0000368 (mediates host cell invasion by microbe) (Walls et al., 2020); PATHGO:0000003 (modulates ion channel activity in another organism) (Braga et al., 2021, 16); PATHGO:0000358 (mediates release of cell from extracellular matrix in another organism) (Braga et al., 2021, 16); PATHGO:0000162 (disrupts epithelial layer in another organism) (Braga et al., 2021, 16); PATHGO:0000302 (disrupts JAK-STAT signaling in another organism) (Zhang et al., 2021b);

TABLE 4 Sequences of concern of *Bacillus anthracis*.

Sequence of Concern	Function of Sequences of Concern (FunSoCs)	Pathogenesis Gene Ontology (PathGO)
Adenosine synthase A	Suppress host immune signaling (Thammavongsa et al., 2009)	PATHGO:0000220 (suppresses inflammatory cytokine release in another organism) (Thammavongsa et al., 2009)
Anthrolysin O	Adherence to another organism (Mosser and Rest, 2006); Dissemination in host (Bishop et al., 2010); Resist other immune effector (Mosser and Rest, 2006; Heffernan et al., 2007);	PATHGO:0000211 (mediates binding to the cell surface in another organism) (Mosser and Rest, 2006); PATHGO:0000033 (mediates pore formation in another organism) (Mosser and Rest, 2006); PATHGO:0000253 (mediates barrier traversal in another organism) (Bishop et al., 2010);
BclA	Resist host complement (Wang et al., 2016a);	PATHGO:0000341 (mediates binding of complement control protein in another organism) (Wang et al., 2016a);
BslA	Adherence to another organism (Ebrahimi et al., 2009; Kern and Schneewind, 2010; Wang et al., 2016b); Dissemination in host (Ebrahimi et al., 2009);	PATHGO:0000275 (mediates binding to laminin in another organism) (Wang et al., 2016b); PATHGO:0000253 (mediates barrier traversal in another organism) (Ebrahimi et al., 2009); PATHGO:0000211 (mediates binding to the cell surface in another organism) (Ebrahimi et al., 2009);
ClpX	Resist host antimicrobial peptide (McGillivray et al., 2009);	PATHGO:0000104 (disrupts antimicrobial peptide binding in another organism) (McGillivray et al., 2009);
Immune inhibitor A	Dissemination in host (Mukherjee et al., 2011; Tonry et al., 2012);	PATHGO:0000253 (mediates barrier traversal in another organism) (Mukherjee et al., 2011; Tonry et al., 2012);
PI-PLC	Resist other immune effector (Wei et al., 2005; Zenewicz et al., 2005);	PATHGO:0000233 (disrupts toll-like receptor signaling in another organism) (Zenewicz et al., 2005); PATHGO:0000080 (suppresses dendritic cell activation in another organism) (Zenewicz et al., 2005); PATHGO:0000055 (mediates membrane phospholipid cleavage in another organism) (Zenewicz et al., 2005);
Superoxide dismutases (4)	Resist host oxidative killing (Cybulski et al., 2009)	PATHGO:0000230 (mediates free radical detoxification) (Cybulski et al., 2009); PATHGO:0000271 (mediates resistance to oxidative killing in another organism) (Cybulski et al., 2009);
Protective antigen	Adherence to another organism (Vuyisich et al., 2012); Host invasion (Abrami et al., 2005);	PATHGO:0000072 (mediates binding to cell surface glycoprotein in another organism) (Vuyisich et al., 2012); PATHGO:0000369 (mediates cell invasion by macromolecule from another organism) (Abrami et al., 2005); PATHGO:0000033 (mediates pore formation in another organism) (Abrami et al., 2005);
Edema factor	Suppress host immune signaling (Agrawal and Pulendran, 2004; Tournier et al., 2005; van Sorge et al., 2008); Disable organ (Firoved et al., 2005; Guichard et al., 2010; Liu et al., 2013; Hutt et al., 2014)	PATHGO:0000173 (modulates cAMP synthesis within a cell of another organism) (Agrawal and Pulendran, 2004; Friebe et al., 2016); PATHGO:0000220 (suppresses inflammatory cytokine release in another organism) (Tournier et al., 2005; van Sorge et al., 2008); PATHGO:0000080 (suppresses dendritic cell activation in another organism) (Tournier et al., 2005); PATHGO:0000326 (modulates transcription in host cell) (van Sorge et al., 2008);
Lethal factor	Adherence to another organism (Vuyisich et al., 2012); Dissemination in host (Langer et al., 2012); Suppress host immune signaling (Agrawal et al., 2003; Tournier et al., 2005; van Sorge et al., 2008; Friebe et al., 2016; Goldberg et al., 2017); Induce inflammation (Chui et al., 2019); Degrade tissue (Langer et al., 2012); Disable organ (Guichard et al., 2010; Liu et al., 2013; Hutt et al., 2014);	PATHGO:0000211 (mediates binding to the cell surface in another organism) (Vuyisich et al., 2012); PATHGO:0000220 (suppresses inflammatory cytokine release in another organism) (van Sorge et al., 2008; Friebe et al., 2016); PATHGO:0000290 (suppresses MAPK signaling in another organism) (Friebe et al., 2016); PATHGO:0000080 (suppresses dendritic cell activation in another organism) (Agrawal et al., 2003; Tournier et al., 2005); PATHGO:0000349 (enhances inflammasome activation in another organism) (Chui et al., 2019); PATHGO:0000162 (disrupts epithelial layer in another organism) (Langer et al., 2012); PATHGO:0000253 (mediates barrier traversal in another organism) (Langer et al., 2012);

mechanisms is not able to subvert every immune system, but just the limited grouping of species with which it co-evolved as a pathogen. Its encoded molecular armamentarium is specific to counter a relatively narrow set of organism-specific innate signaling pathways and effectors and exploit a specific host biology—including barrier breaching. These encoded sequences are how the pathogen makes a host susceptible. Obviously jumps into new species can happen. In these cases, though, the new species, if it is not immune compromised, is always related to the original species with respect to the innate immune system. A mouse pathogen innate immune subverting mechanisms may (or may not) function on the human ortholog of the mouse innate immune protein. But the sequences encoded by a microbe that make plants susceptible to that particular pathogen by subverting the plant innate immune defenses do not, and cannot, make mammals

TABLE 5 SoCs with multiple functions from bacterial, viral, and eukaryotic pathogens.

SoC, Organism	FunSoCs	PathGO terms
LasB, *Pseudomonas aeruginosa*	Resist host complement (Bastaert et al., 2018); Resist host antimicrobial peptide (Saint-Criq et al., 2018); Resist host oxidative killing (Bastaert et al., 2018); Counter host cytokine (Matheson et al., 2006; Golovkine et al., 2014); Resist other host immune effector (Ijiri et al., 1994); Induce inflammation (Saint-Criq et al., 2018; Sun et al., 2020); Degrade tissue (Leduc et al., 2007; Beaufort et al., 2011; Golovkine et al., 2014); Disable organ (Zhu et al., 2021);	PATHGO:0000271 (mediates resistance to oxidative killing in another organism) (Bastaert et al., 2018); PATHGO:0000353 (modulates reactive oxygen species levels in another organism) (Bastaert et al., 2018); PATHGO:0000100 (mediates resistance to complement system in another organism) (Bastaert et al., 2018); PATHGO:0000104 (disrupts antimicrobial peptide binding in another organism) (Saint-Criq et al., 2018); PATHGO:0000363 (suppresses pro-inflammatory cytokine activity in another organism) (Matheson et al., 2006); PATHGO:0000214 (modifies tight junction or adherens junction in another organism) (Golovkine et al., 2014); PATHGO:0000358 (mediates release of cell from extracellular matrix in another organism) (Leduc et al., 2007);
IbpA, *Histophilus somni*	Manipulate host small GTPase (Zekarias et al., 2010); Manipulate host cytoskeleton dynamics (Zekarias et al., 2010); Adherence to another organism (Zekarias et al., 2010; Corbeil, 2016); Resist host phagocytosis (Pan et al., 2018); Resist host complement (Pan et al., 2018); Counter host immunoglobulin (Corbeil, 2016); Cytotoxicity (Zekarias et al., 2010);	PATHGO:0000355 (mediates deactivation of small GTPase in another organism) (Zekarias et al., 2010); PATHGO:0000216 (mediates filamentous actin depolymerization in another organism) (Zekarias et al., 2010); PATHGO:0000211 (mediates binding to the cell surface in another organism) (Corbeil, 2016); PATHGO:0000232 (suppresses phagocytosis in another organism) (Pan et al., 2018); PATHGO:0000257 (mediates immunoglobulin neutralization in another organism) (Corbeil, 2016); PATHGO:0000100 (mediates resistance to complement system in another organism) (Pan et al., 2018);
IpaB, *Shigella flexneri*	Manipulate host cell cycle (Iwai et al., 2007; Wang et al., 2019, 7); Secretion system component (Blocker et al., 1999; Iwai et al., 2007; Roehrich et al., 2010); Adherence to another organism (Schroeder and Hilbi, 2008); Host invasion (Lafont et al., 2002; Mounier et al., 2009; 2012; Senerovic et al., 2012); Suppress host immune signaling (Hathaway et al., 2002); Induce inflammation (Hilbi et al., 1998; Senerovic et al., 2012); Cytotoxicity (Yang et al., 2015);	PATHGO:0000152 (induces cell cycle arrest in cell of another organism) (Iwai et al., 2007); PATHGO:0000110 (mediates secretion of protein effector) (Blocker et al., 1999; Roehrich et al., 2010); PATHGO:0000234 (mediates binding to integrin in another organism) (Schroeder and Hilbi, 2008); PATHGO:0000368 (mediates host cell invasion by microbe) (Lafont et al., 2002); PATHGO:0000220 (suppresses inflammatory cytokine release in another organism) (Hathaway et al., 2002); PATHGO:0000284 (mediates binding to cholesterol in another organism) (Mounier et al., 2012); PATHGO:0000033 (mediates pore formation in another organism) (Mounier et al., 2009; Senerovic et al., 2012);
TcdA, *Clostridioides difficile*	Manipulate host small GTPase (Aktories et al., 2017); Manipulate host cytoskeleton dynamics (Aktories et al., 2017); Adherence to another organism (Aktories and Just, 2005; Tao et al., 2019); Host invasion (Papatheodorou et al., 2010; Aktories et al., 2017); Induce inflammation (Ng et al., 2010; Cowardin et al., 2016); Degrade tissue (Aktories et al., 2017);	PATHGO:0000285 (mediates carbohydrate-derivative binding in another organism) (Aktories and Just, 2005); PATHGO:0000273 (mediates glycosaminoglycan- or proteoglycan-binding in another organism) (Tao et al., 2019); PATHGO:0000072 (mediates binding to cell surface glycoprotein in another organism) (Tao et al., 2019); PATHGO:0000214 (modifies tight junction or adherens junction in another organism) (Sousa et al., 2005); PATHGO:0000369 (mediates cell invasion by macromolecule from another organism) (Papatheodorou et al., 2010); PATHGO:0000355 (mediates deactivation of small GTPase in another organism) (Aktories et al., 2017); PATHGO:0000214 (modifies tight junction or adherens junction in another organism) (Aktories et al., 2017); PATHGO:0000216 (mediates filamentous actin depolymerization in another organism) (Aktories et al., 2017); PATHGO:0000162 (disrupts epithelium in another organism) (Aktories et al., 2017);
NS1, influenza virus	Manipulate host transcription (Anastasina et al., 2016); Manipulate host translation (Chaimayo et al., 2018); Manipulate host ubiquitin dynamics (Gack et al., 2009); Manipulate host regulated cell death (Bergsbaken et al., 2009); Suppress host immune signaling (Fislová and Kostolanský, 2005; Gack et al., 2009); Resist other host immune effector (Fernandez-Sesma et al., 2006); Suppress antigen presentation (Chien et al., 2004; Bonjardim, 2005);	PATHGO:0000326 (modulates transcription in another organism) (Anastasina et al., 2016); PATHGO:0000006 (modulates protein synthesis in another organism) (Chaimayo et al., 2018); PATHGO:0000325 (modulates ubiquitin dynamics in another organism) (Gack et al., 2009); PATHGO:0000352 (disrupts TRIM/TRIM-like signaling in another organism) (Gack et al., 2009); PATHGO:0000334 (suppresses apoptosis in another organism) (Bergsbaken et al., 2009); PATHGO:0000220 (suppresses inflammatory cytokine release in another organism) (Fislová and Kostolanský, 2005); PATHGO:0000080 (suppresses dendritic cell activation in another organism) (Fernandez-Sesma et al., 2006); PATHGO:0000312 (mediates concealment of foreign nucleic acid in another organism) (Chien et al., 2004; Bonjardim, 2005);
E1A, human adenovirus	Manipulate host transcription (Fonseca et al., 2012; Glenewinkel et al., 2016; King et al., 2018); Manipulate host cell cycle (Ryan, 2010); Manipulate host ubiquitin dynamics (Fonseca et al., 2012); Manipulate host regulated cell death (Miller, 2005); Suppress host immune signaling (Lau et al., 2015); Suppress antigen presentation (Jiao et al., 2010; Berhane et al., 2011)	PATHGO:0000325 (modulates ubiquitin dynamics in another organism) (Fonseca et al., 2012); PATHGO:0000326 (modulates transcription in another organism) (Fonseca et al., 2012; Glenewinkel et al., 2016; King et al., 2018); PATHGO:0000335 (induces apoptosis in another organism) (Miller, 2005); PATHGO:0000300 (disrupts STING signaling in another organism) (Lau et al., 2015); PATHGO:0000308 (disrupts antigen presentation in another organism) (Jiao et al., 2010; Berhane et al., 2011);

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TABLE 5 (*Continued*) SoCs with multiple functions from bacterial, viral, and eukaryotic pathogens.

SoC, Organism	FunSoCs	PathGO terms
NSs, Rift Valley fever virus	Manipulate host transcription (Kainulainen et al., 2014; Terasaki et al., 2016); Manipulate host cell cycle (Baer et al., 2012); Manipulate host ubiquitin dynamics (Kainulainen et al., 2014; 2016); Manipulate host cytoskeleton dynamics (Bamia et al., 2020); Suppress host immune signaling (Le May et al., 2008; Head et al., 2012; Terasaki et al., 2016); Resist other host immune effector (Kainulainen et al., 2016; Terasaki et al., 2016);	PATHGO:0000326 (modulates transcription in another organism) (Kainulainen et al., 2014; Terasaki et al., 2016); PATHGO:0000152 (induces cell cycle arrest in cell of another organism) (Baer et al., 2012); PATHGO:0000325 (modulates ubiquitin dynamics in another organism) (Kainulainen et al., 2014; 2016); PATHGO:0000028 (modulates cytoskeleton in another organism) (Bamia et al., 2020); PATHGO: 0000214 (modifies tight junction or adherens junction in another organism) (Bamia et al., 2020); PATHGO:0000382 (suppresses interferon signaling in another organism) (Le May et al., 2008; Head et al., 2012); PATHGO:0000304 (disrupts PKR activity in another organism) (Kainulainen et al., 2016; Terasaki et al., 2016);
Alp1, *Neosartorya fumigata*	Resists host complement (Behnsen et al., 2010); Counter host immunoglobulin (Behnsen et al., 2010); Degrade tissue (Balenga et al., 2015); Disable organ (Balenga et al., 2015);	PATHGO:0000100 (mediates resistance to complement system in another organism) (Behnsen et al., 2010); PATHGO:0000257 (mediates immunoglobulin neutralization in another organism) (Behnsen et al., 2010); PATHGO:0000226 (disrupts extracellular matrix in another organism) (Balenga et al., 2015);
ROP18/VIR3, *Toxoplasma gondii*	Manipulate host ubiquitin dynamics (Du et al., 2014); Manipulate host programmed cell death (Wu et al., 2016); Suppress host immune signaling (Fentress et al., 2010; Yamamoto et al., 2011; Du et al., 2014; Yang et al., 2017; Xia et al., 2018; Yao et al., 2021)	PATHGO:0000325 (modulates ubiquitin dynamics in another organism) (Du et al., 2014); PATHGO:0000295 (suppresses NFκB signaling in another organism) (Du et al., 2014); PATHGO:0000334 (suppresses apoptosis in another organism) (Wu et al., 2016); PATHGO:0000352 (disrupts TRIM/TRIM-like signaling in another organism) (Yao et al., 2021);

susceptible to that same microbe because of the substantial differences in the innate immune systems of plants and mammals. *Yersinia pestis* infects two different sorts of hosts: insects (fleas) and mammals. The bacterium encodes different sets of sequences to exploit each host and employs temperature-based regulation to switch between them (Vadyvaloo et al., 2010).

We are asserting that the immune subverting mechanisms employed by a specific microbial pathogen are what produce susceptibility in the (typically) narrow range of hosts parasitized by that microbe. Whatever pathogenesis follows from this infection is not just a function of the parasite, but rather an emergent property of the gestalt of the host-parasite interactions shaped by the development of the adaptive immune response (Casadevall and Pirofski, 2003; Pirofski and Casadevall, 2015). For these reasons, we think it is possible that immune subverting mechanisms may be the 'worst' of SoCs since they essentially enable infection and appear (to us) to be the difference between pathogenic and non-pathogenic species. We have documented and annotated a few thousand SoCs from parasites of (mostly) humans, including over 500 that subvert host innate immunity. But this is probably just a tithe of the immune subverting SoCs encoded by human pathogens. While we think that the available evidence points strongly in the direction of these sequences being necessary for infection by making the host susceptible, at present this is merely a hypothesis that requires testing.

From how broad a pool of pathogens should SoCs be drawn? Which hosts?

Every nonviral species in biology serves as a host to its own subset of microbial pathogens. And all those pathogens have sequences that directly exploit the biology of their host. But these are not necessarily SoCs. Why not? Because sequences of concern are only 'concerning' if they are from pathogens capable of infecting humans and other species that humans rely upon for survival. The specific bacteriophage sequences enabling exploitation of strains of *Salmonella* or *Listeria* are not SoCs because humans do not care about the wellbeing of those bacteria. The sequences that allow bacteriophage to exploit these bacteria cannot be used to cause harm to mammalian or crop plant hosts and so would not be considered SoCs. Sequences encoding virulence factors that are designated SoCs should be documented and annotated only from pathogens that afflict humans, our livestock, and our crop plants (Godbold et al., 2022).

As mentioned above, this requires a broadening of microbes beyond those placed on select agent lists. These lists are generally composed of organisms and toxins that have been weaponized or are viewed as being weaponizable. But sequences that effectively interact with human molecules, attach to host cells, invade them, subvert immunity, enable dissemination, and generate pathology are not limited to weaponizable microbes. If microbes that cause disease in immune-compromised people are included, there are at least 1,500 species that probably encode SoCs (Godbold et al., 2022).

That said, we are not sure where the line on infectious microbes should be drawn: all microbes capable of causing disease in any human, however immune-compromised? That would require mining SoCs from opportunistic pathogens. Or should SoCs be taken only from microbes capable of causing disease in immune-normal people? The latter would neglect documenting the many and varied SoCs that have been elegantly investigated in such 'conditional' pathogens as *Pseudomonas aeruginosa*.

We mention SoCs from pathogens of crop plants above, but we acknowledge that is the weakest area of our SoC annotation effort.

TABLE 6 How work on SoCs could correlate with DURC categories.

Function of Sequence of Concern (FunSoC)	Case 1, SoC transferred to other pathogen or Case 2, SoC altered for enhancement of original pathogen	Case 3, SoC transferred to nonpathogen
Damaging	Could enhance the harmful consequences of the agent;	Might enable the nonpathogen to have harmful consequences
Immune Subverting	Enhances the harmful consequences of the agent; Disrupts immunity or the effectiveness of an immunization against the agent; Alters the host range or tropism of the agent; Enhances the susceptibility of a host population to the agent;	Might enable the nonpathogen to have harmful consequences; Might enable the nonpathogen to infect novel hosts; Might enhance the susceptibility of a host population to the agent;
Attachment Protein/Adhesin	Alters the host range or tropism of the agent; Enhances the susceptibility of a host population to the agent;	*Probably none*
Fusion Protein/Invasin	Alters the host range or tropism of the agent; Enhances the susceptibility of a host population to the agent;	*Probably none*
Dissemination	Enhances the harmful consequences of the agent; Increases the transmissibility or the ability to disseminate the agent; Enhances the susceptibility of a host population to the agent;	*Probably none*

This is the case for several reasons. The literature for microbial pathogenesis in plants lags at least 2 decades behind that of mammals. We have documented fewer than 300 SoCs from plant pathogens (viral, bacterial, and fungal). Our terminology for functions of SoCs from plant pathogens needs supplementation/improvement. Why? Because the innate immune system of plants, while at least as complex as that of mammals, is substantially different. The goals and 'principles' of immune defense are the same, but the individual cases and host molecules effecting the defensive effort are distinct. We are not as familiar with them. We plan on improving our understanding and our annotation effort for plant pathogen SoCs.

As the NSABB reviews the conduct of dual-use research oversight, it should consider how to incorporate our growing knowledge of SoCs into the biorisk management regime to ensure that life sciences research is conducted safely, securely, and responsibly. In the following section, we suggest several ways in which SoCs could be used to guide biorisk management decisions.

How might annotated SoCs guide biorisk management decisions?

Since neither the DURC policies nor P3CO Framework provide guidance for scientists to judge whether proposed research is "reasonably anticipated" to result in a modified microbe with enhanced pathogenic properties, we propose leveraging our annotated SoCs as one indicator which could trigger greater scrutiny. We think our conception of SoCs provide clearer guidance than what currently exists. If the sequence being manipulated in the investigation is a direct-acting sequence of concern, and it is being expressed in an organism capable of causing disease in humans, then that work may require a higher level of oversight. This will depend on the likelihood that the resulting manipulated microbe poses a greater risk of infection or transmission than the unmodified microbe. So how might that risk be better adjudicated using SoCs annotated with FunSoCs and PathGO terms?

Application to the USG DURC policy

Among the experiments of concern listed in USG DURC policy (Table 2), our conception of SoCs and their functions (FunSoCs) can help illuminate potential risks. Research that involves any one of three research activities: (i) transfer of a SoC to a different pathogen, (ii) alteration of a SoC such that the existing abilities of the original pathogen might be enhanced, or (iii) transfer of an SoC to a nonpathogenic microbe would trigger oversight. Our reflections on how FunSoCs might be used to better understand DURC follow and are summarized in Table 6.

Damaging SoCs: We briefly detailed four categories of damaging SoCs in our previous work (Godbold et al., 2022) and recapitulated them above. Inserting damaging SoCs into a microbe could violate Table 2 as it would be expected to enhance the harmful consequence of the agent. Such a result might also follow the alteration of a damaging SoC in its native microbe.

Immune subverting SoCs: As we discuss above, SoCs that subvert innate immunity may be more consequential than damaging SoCs. Results of experiments involving addition of these sequences to other microbes as well as modifications that might enhance their immune subverting abilities are also the most difficult to anticipate prior to the experiment. Such could "disrupt immunity against the agent" [Table 2] or "enhance the susceptibility of a host population to the agent" [Table 2]. It could also "increase the harmful consequence of the agent" [Table 2]. Alterations in some poxviral immune-evading sequences can change the host tropism of the virus [Table 2] (Bratke et al., 2013; Rahman and McFadden, 2017; 2020). Of course, these modifications depend on the experimental system and could very well be allowed after review. The study of immune subverting mechanisms of microbes in experimental infections of host organisms has produced numerous and important breakthroughs in our understanding of immunity.

Adhesins and **Invasins**: Adhesive properties are particularly abundant in biology. Adhesins are the molecules which primarily condition what cell types and what taxa are targeted by an infectious agent. As a result, their transplantation into a new

organism might enable a change in host tropism [Table 2] or enhance the susceptibility of a new host population [Table 2]. Likewise, alterations of adhesins with the intention of altering host cell tropism should trigger a review. For viruses and for many other infectious agents that have an intracellular life cycle, the principal attachment protein (adhesin) is also responsible for viral fusion and subsequent cellular invasion. But there are dozens of bacterial invasins, not also adhesins, which manipulate extracellular matrix molecules or the cytoskeleton thereby leading to invasion. It is conceivable that altering these within a pathogen could lead to changes in host tropism [Table 2] or enhance the susceptibility of a new host population [Table 2]. Expressing adhesins/invasins from pathogens in nonpathogenic species will not generally violate DURC rules as they do not, by themselves, make a nonpathogenic microbe pathogenic (Schubert et al., 2004; Uchiyama et al., 2006; Pisano et al., 2012; Schmidgen et al., 2014).

Dissemination factors: There are disparate modes of action for dissemination factors, but the effect is that the infectious agent can spread within the host organism beyond what would be possible in the absence of the dissemination factor. This often occurs through the temporary subversion of host barriers. Addition of a foreign dissemination factor to an existing pathogen could lead to consequences that could increase the harmful effects of the agent [Table 2], increase the ability of the agent to disseminate in the host [Table 2], or even enhance the susceptibility of a host population to the agent [Table 2]. Modifications to a dissemination factor could conceivably affect each of these as well. Expression of a dissemination factor in a nonpathogen would be unlikely to make it pathogenic.

Of course, additions or alterations of SoCs to study the mechanism(s) would be less risky if performed in a microbe that was either not competent to replicate or otherwise incapable of causing human infection. If a SoC-based biosecurity regime were adopted, development of safer systems to study SoC function should be a focus of funding agencies.

Implications for biosafety, biosecurity, and dual-use research oversight

As the global biorisk landscape evolves, it is necessary to update biorisk management policies and practices. As the NIH and OSTP reviews US dual-use research oversight policy, we think our approach to categorizing the functions of SoCs based on the published literature and using these as an aid for considering outcomes of organismal manipulation is a valuable addition and will strengthen existing policy. The rubric provided in Table 6, which maps the functions of SoCs onto different classes of experiments to suggest which DURC categories might be involved, could be helpful for considering the consequences of microbial modifications.

The United States has not provided any guidance for how to judge when the standard of "reasonably anticipated," as used by the DURC policies and P3CO Framework, is met. This lack of detail and ambiguous terminology can be confusing for both researchers submitting proposals as well as scientists and funding agency officials involved in the peer review process. Therefore, NIH and OSTP should consider recommending that inserting or modifying SoCs with certain functions could be "reasonably anticipated" to lead to an enhanced phenotype covered by either set of policies. While this rule of thumb would not be the only determinant of whether an experiment was covered by DURC, it would increase the likelihood that potentially concerning research is subject to review under the appropriate policy. This approach will be particularly useful if NIH and OSTP adopts the recommendations from the National Science Advisory Board for Biosecurity (NSABB) to expand the scope and coverage of the P3CO and DURC policies. For example, NSABB proposed reducing the threshold for oversight of experiments with potential pandemic pathogens from those that are reasonably anticipated to generate a highly virulent or transmissible pathogen to those likely to generate a moderately virulent or transmissible pathogen. NSABB also recommended that the scope of the DURC policy be expanded from Tier 1 Select Agents to all human, animal, and plant pathogens (National Science Advisory Board for Biosecurity, 2023). These recommendations, when taken together, will subject a much broader swathe of pathogen research subject to oversight, necessitating the development of tools that can aid researchers and review entities in determining if proposed experiments could be "reasonably anticipated" to generate enhanced pathogens that require the implementation of risk mitigation measures prior to or following the research.

A similar lacuna in guidance for researchers on how to identify potential dual-use research exists in other countries that exercise some degree of oversight of dual-use research such as Australia, Canada, and the United Kingdom. These countries might also benefit from adopting functional criteria (like FunSoCs) into their education and awareness-raising activities to help scientists identify potential dual-use research. In addition, funding agencies in these countries could use FunSoCs as part of their screening process for grant proposals to determine if the research poses any dual-use risks that require mitigation.

For microbes that are increasingly synthetic, having their constituent sequences drawn from an expanding set of organisms, a screening approach based on taxonomy is likely to be of decreasing utility. In such cases a standard list of 'bad sequences' should be helpful in determining what microbes are likely to be concerning. An accurate computational assessment of the infectiousness of a synthetic microbe is not currently possible nor is it likely to be in the next decade. We think our work and that of others can provide pointers for how such as assessment might be attempted (Gemler et al., 2022; Godbold et al., 2022). Our criteria for functions of sequences of concern were described in our earlier publication and are available to the scientific community. Here we offer them as a useful framework for assessing risk in the context of dual-use research of concern.

SoCs cannot replace taxonomic lists of 'bad bugs', particularly in the case of viruses pathogenic for humans, which must remain part of any policy framework. But the addition of SoCs categorized by functions necessary for pathogenesis provides a useful supplement to such lists. The transfer of such sequences and their modification in ways that can be reasonably anticipated to enhance their damaging, disseminating, adhesive, invasive, or immune subverting effects should be noted in

research proposals. Such a list of SoCs might allow the de-regulation of thousands of sequences from bacterial and eukaryotic pathogens that are presently deemed controlled. As suggested in **Table 4** for *Bacillus anthracis*, over 99.5% of its 5,800 sequences play no distinct role in pathogenesis. Documenting and regulating the sequences that enable pathogenesis in nonviral organisms make it easier for researchers to investigate, *without oversight*, the biology of the remaining (and overwhelming) majority.

The revised guidance on DNA synthesis screening issued by the Department of Health and Human Services is undertaking a shift from a pathogen-based to a sequence-based approach[8]. Under the previous guidance, DNA synthesis providers were only required to screen orders against the genomes of a list of regulated pathogens. Under the revised guidance, "sequences that contribute to toxicity or pathogenicity" are considered sequences of concern that are covered by the guidance even if these are not encoded by a regulated biological agent. The NIH and OSTP could explore the desirability and feasibility of applying the broadly defined "sequences of concern" by the new HHS guidance to DURC oversight.

How can we be sure that the sequences enabling pathogenesis for these disease-causing microbes have been sufficiently investigated to find them all? This is something we cannot know, though there has been a great deal of work on most of the microbes found on select agent lists. Investing in research on the less well-investigated pathogens would help ensure that the most important pathogenic sequences are characterized. In addition, knowledge of the commonalities of sequences enabling pathogenesis that are a consequence of categorizing them might drive development of pathogen-agnostic therapeutics that may be able to neutralize widely shared mechanisms of pathogenesis.

One strategy to mitigate the risk of research involving SoCs is for funding authorities to encourage researchers to develop more, and more suitable, nonpathogenic microbial chassis to support the safe discovery of SoC functions. Once approved, these chassis could be used with decreased oversight. The use of non-replicating pseudoviruses can also be encouraged as a safer alternative to the insertion or modification of SoCs in pathogenic, replicating viruses.

A standardized and official list of SoCs with a set of approved annotations should be devised by governments whose scientists are involved in microbial pathogenesis research. How this list should be selected, maintained, and used is something that will need to be resolved. The process should involve consultation among experts in infectious diseases and policy as well as relevant biodefense professionals. The first question for such a group involves deciding which host taxa needing protection should be selected. Humans are the primary concern, but animals and plants that dominate a country's agriculture should probably also be considered. Once the hosts are established, the pathogens that afflict these hosts can be determined. Then SoCs will be documented from this list of pathogens.

The availability of such a list and the type of information it should provide is also something to be decided. Should it be an open list of sequence names? A list of sequence names with accession numbers? The names, accession numbers, and a tabular list of problematic functions (i.e., damaging, immune subverting, adhesive, etc.)? Should terse but specific descriptions of pathogenic activity such as FunSoCs and PathGO be associated with each SoC? Or a more detailed description of how it interacts with host molecules? Should citations/references of the primary or secondary literature be required to justify the functional determinations for each sequence?

Who should have access to these lists? Should it be publicly available to the scientific community at large? Should only institutional review entities responsible for implementing DURC oversight of research conducted at their institution have access to this information? Should different groups have access to lists of differing comprehensiveness? The utility of such a tool for enhancing DURC oversight needs to be balanced with the information hazards presented by an accessible compilation of sequences that enable pathogenesis. Those making these decisions will be threading the needle to best serve the interests of public safety, open research, and international security.

Conclusion

Thoughtful researchers who work with pathogenic microbes are usually aware of the hazards involved in introducing changes into sequences involved in pathogenesis. We think SoCs annotated with FunSoCs will bring further clarity to help ascertain when more care should be taken in experiments, especially in fully replication-competent organisms. We believe that SoCs can be a useful component of the regulatory regime that governs sequences acceptable for insertion and alteration in pathogenic agents. We have delineated bioengineering situations that could be 'reasonably anticipated' to improve the disease-causing capacity of pathogens. We believe that these guidelines have the potential to reduce the risk of accidentally generating an 'improved' pathogen while promoting awareness of the phenotype effects of potentially concerning genotypic changes. We think considering SoCs by function improves the probability that potentially concerning research is subject to the appropriate level of oversight to ensure that such research is conducted safely, securely, and responsibly.

Author contributions

GG conceived, drafted and revised the article as well as collected, analyzed, and interpreted the data. GK conceived, drafted and revised the article. AK and MS collected and analyzed data, as well as critiqued and revised the article. FH, SA, TT, KT, and JS critiqued and revised the article. FH, TT, and KT acquired funding support for the research. TT and KT equally exercised oversight and supervision for the research.

Funding

GG, FH, AK, MS, KT, and TT were partially supported by the Fun GCAT program from the Office of the Director of National Intelligence (ODNI), Intelligence Advanced Research Projects Activity (IARPA), via the Army Research Office (ARO) under federal award no. W911NF-17-2-0089. GG, KT, and TT were also partially supported by the Centers for Disease Control (CDC) contract 75D30121C11180.

8 https://aspr.hhs.gov/legal/syndna/Pages/default.aspx.

Acknowledgments

The views and conclusion contained herein are those of the authors and should not be interpreted as necessarily representing the official policies or endorsements, either expressed or implied, of the ODNI, IARPA, ARO, CDC, or the U.S. Government.

Conflict of interest.

Authors GD, FH, AK, MS, SA, and KT were employed by Signature Science LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Abrami, L., Reig, N., and van der Goot, F. G. (2005). Anthrax toxin: The long and winding road that leads to the kill. *Trends Microbiol.* 13, 72–78. doi:10.1016/j.tim.2004.12.004

Addetia, A., Lieberman, N. A. P., Phung, Q., Hsiang, T.-Y., Xie, H., Roychoudhury, P., et al. (2021). SARS-CoV-2 ORF6 disrupts bidirectional nucleocytoplasmic transport through interactions with Rae1 and Nup98. *mBio* 12, e00065–21. doi:10.1128/mBio.00065-21

Agrawal, A., Lingappa, J., Leppla, S. H., Agrawal, S., Jabbar, A., Quinn, C., et al. (2003). Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin. *Nature* 424, 329–334. doi:10.1038/nature01794

Agrawal, A., and Pulendran, B. (2004). Anthrax lethal toxin: A weapon of multisystem destruction. *Cell Mol. Life Sci.* 61, 2859–2865. doi:10.1007/s00018-004-4251-4

Aktories, K., and Just, I. (2005). Clostridial Rho-inhibiting protein toxins. *Curr. Top. Microbiol. Immunol.* 291, 113–145. doi:10.1007/3-540-27511-8_7

Aktories, K., Schwan, C., and Jank, T. (2017). *Clostridium difficile* toxin biology. *Annu. Rev. Microbiol.* 71, 281–307. doi:10.1146/annurev-micro-090816-093458

Alvarez-de Miranda, F. J., Alonso-Sánchez, I., Alcamí, A., and Hernaez, B. (2021). TNF decoy receptors encoded by poxviruses. *Pathogens* 10, 1065. doi:10.3390/pathogens10081065

Anastasina, M., Le May, N., Bugai, A., Fu, Y., Söderholm, S., Gaelings, L., et al. (2016). Influenza virus NS1 protein binds cellular DNA to block transcription of antiviral genes. *Biochim. Biophys. Acta* 1859, 1440–1448. doi:10.1016/j.bbagrm.2016.09.005

Andoniou, C. E., Sutton, V. R., Wikstrom, M. E., Fleming, P., Thia, K. Y. T., Matthews, A. Y., et al. (2014). A natural genetic variant of granzyme B confers lethality to a common viral infection. *PLoS Pathog.* 10, e1004526. doi:10.1371/journal.ppat.1004526

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. (2000). Gene ontology: Tool for the unification of biology. *Nat. Genet.* 25, 25–29. doi:10.1038/75556

Baer, A., Austin, D., Narayanan, A., Popova, T., Kainulainen, M., Bailey, C., et al. (2012). Induction of DNA damage signaling upon Rift Valley fever virus infection results in cell cycle arrest and increased viral replication. *J. Biol. Chem.* 287, 7399–7410. doi:10.1074/jbc.M111.296608

Balaji, A., Kille, B., Kappell, A. D., Godbold, G. D., Diep, M., Elworth, R. A. L., et al. (2022). SeqScreen: Accurate and sensitive functional screening of pathogenic sequences via ensemble learning. *Genome Biol.* 23, 133. doi:10.1186/s13059-022-02695-x

Balenga, N. A., Klichinsky, M., Xie, Z., Chan, E. C., Zhao, M., Jude, J., et al. (2015). A fungal protease allergen provokes airway hyper-responsiveness in asthma. *Nat. Commun.* 6, 6763. doi:10.1038/ncomms7763

Bamia, A., Marcato, V., Boissière, M., Mansuroglu, Z., Tamietti, C., Romani, M., et al. (2020). The NSs protein encoded by the virulent strain of rift valley fever virus targets the expression of Abl2 and the actin cytoskeleton of the host, affecting cell mobility, cell shape, and cell-cell adhesion. *J. Virol.* 95, e01768–20. doi:10.1128/JVI.01768-20

Barreda, D., Santiago, C., Rodríguez, J. R., Rodríguez, J. F., Casasnovas, J. M., Mérida, I., et al. (2021). SARS-CoV-2 spike protein and its receptor binding domain promote a proinflammatory activation profile on human dendritic cells. *Cells* 10, 3279. doi:10.3390/cells10123279

Barrett, C. T., Neal, H. E., Edmonds, K., Moncman, C. L., Thompson, R., Branttie, J. M., et al. (2021). Effect of clinical isolate or cleavage site mutations in the SARS-CoV-2 spike protein on protein stability, cleavage, and cell-cell fusion. *J. Biol. Chem.* 297, 100902. doi:10.1016/j.jbc.2021.100902

Bastaert, F., Kheir, S., Saint-Criq, V., Villeret, B., Dang, P. M.-C., El-Benna, J., et al. (2018). *Pseudomonas aeruginosa* LasB subverts alveolar macrophage activity by interfering with bacterial killing through downregulation of innate immune defense, reactive oxygen species generation, and complement activation. *Front. Immunol.* 9, 1675. doi:10.3389/fimmu.2018.01675

Beaufort, N., Corvazier, E., Hervieu, A., Choqueux, C., Dussiot, M., Louedec, L., et al. (2011). The thermolysin-like metalloproteinase and virulence factor LasB from pathogenic *Pseudomonas aeruginosa* induces anoikis of human vascular cells. *Cell Microbiol.* 13, 1149–1167. doi:10.1111/j.1462-5822.2011.01606.x

Behnsen, J., Lessing, F., Schindler, S., Wartenberg, D., Jacobsen, I. D., Thoen, M., et al. (2010). Secreted Aspergillus fumigatus protease Alp1 degrades human complement proteins C3, C4, and C5. *Infect. Immun.* 78, 3585–3594. doi:10.1128/IAI.01353-09

Bergsbaken, T., Fink, S. L., and Cookson, B. T. (2009). Pyroptosis: Host cell death and inflammation. *Nat. Rev. Microbiol.* 7, 99–109. doi:10.1038/nrmicro2070

Berhane, S., Aresté, C., Ablack, J. N., Ryan, G. B., Blackbourn, D. J., Mymryk, J. S., et al. (2011). Adenovirus E1A interacts directly with, and regulates the level of expression of, the immunoproteasome component MECL1. *Virology* 421, 149–158. doi:10.1016/j.virol.2011.09.025

Bishop, B. L., Lodolce, J. P., Kolodziej, L. E., Boone, D. L., and Tang, W. J. (2010). The role of anthrolysin O in gut epithelial barrier disruption during Bacillus anthracis infection. *Biochem. Biophys. Res. Commun.* 394, 254–259. doi:10.1016/j.bbrc.2010.02.091

Biswal, M., Lu, J., and Song, J. (2022). SARS-CoV-2 nucleocapsid protein targets a conserved surface groove of the NTF2-like domain of G3BP1. *J. Mol. Biol.* 434, 167516. doi:10.1016/j.jmb.2022.167516

Blocker, A., Gounon, P., Larquet, E., Niebuhr, K., Cabiaux, V., Parsot, C., et al. (1999). The tripartite type III secreton of Shigella flexneri inserts IpaB and IpaC into host membranes. *J. Cell Biol.* 147, 683–693. doi:10.1083/jcb.147.3.683

Bonjardim, C. A. (2005). Interferons (IFNs) are key cytokines in both innate and adaptive antiviral immune responses-and viruses counteract IFN action. *Microbes Infect.* 7, 569–578. doi:10.1016/j.micinf.2005.02.001

Braga, L., Ali, H., Secco, I., Chiavacci, E., Neves, G., Goldhill, D., et al. (2021). Drugs that inhibit TMEM16 proteins block SARS-CoV-2 spike-induced syncytia. *Nature* 594, 88–93. doi:10.1038/s41586-021-03491-6

Brandherm, L., Kobaš, A. M., Klöhn, M., Brüggemann, Y., Pfaender, S., Rassow, J., et al. (2021). Phosphorylation of SARS-CoV-2 Orf9b regulates its targeting to two binding sites in TOM70 and recruitment of Hsp90. *Int. J. Mol. Sci.* 22, 9233. doi:10.3390/ijms22179233

Bratke, K. A., McLysaght, A., and Rothenburg, S. (2013). A survey of host range genes in poxvirus genomes. *Infect. Genet. Evol.* 14, 406–425. doi:10.1016/j.meegid.2012.12.002

Brosey, C. A., Houl, J. H., Katsonis, P., Balapiti-Modarage, L. P. F., Bommagani, S., Arvai, A., et al. (2021). Targeting SARS-CoV-2 Nsp3 macrodomain structure with insights from human poly(ADP-ribose) glycohydrolase (PARG) structures with inhibitors. *Prog. Biophys. Mol. Biol.* 163, 171–186. doi:10.1016/j.pbiomolbio.2021.02.002

Buchrieser, J., Dufloo, J., Hubert, M., Monel, B., Planas, D., Rajah, M. M., et al. (2020). Syncytia formation by SARS-CoV-2-infected cells. *EMBO J.* 39, e106267. doi:10.15252/embj.2020106267

Cantuti-Castelvetri, L., Ojha, R., Pedro, L. D., Djannatian, M., Franz, J., Kuivanen, S., et al. (2020). Neuropilin-1 facilitates SARS-CoV-2 cell entry and infectivity. *Science* 370, 856–860. doi:10.1126/science.abd2985

Cao, X., Tian, Y., Nguyen, V., Zhang, Y., Gao, C., Yin, R., et al. (2020). Spike protein of SARS-CoV-2 activates macrophages and contributes to induction of acute lung inflammations in mice. *bioRxiv* 2020, 414706. doi:10.1101/2020.12.07.414706

Casadevall, A., and Pirofski, L. (2003). The damage-response framework of microbial pathogenesis. *Nat. Rev. Microbiol.* 1, 17–24. doi:10.1038/nrmicro732

Casanova, J.-L. (2015a). Human genetic basis of interindividual variability in the course of infection. *Proc. Natl. Acad. Sci. U.S.A.* 112, E7118–E7127. doi:10.1073/pnas.1521644112

Casanova, J.-L. (2015b). Severe infectious diseases of childhood as monogenic inborn errors of immunity. *Proc. Natl. Acad. Sci. U.S.A.* 112, E7128–E7137. doi:10.1073/pnas.1521651112

Chaimayo, C., Dunagan, M., Hayashi, T., Santoso, N., and Takimoto, T. (2018). Specificity and functional interplay between influenza virus PA-X and NS1 shutoff activity. *PLoS Pathog.* 14, e1007465. doi:10.1371/journal.ppat.1007465

Chen, D., Zheng, Q., Sun, L., Ji, M., Li, Y., Deng, H., et al. (2021). ORF3a of SARS-CoV-2 promotes lysosomal exocytosis-mediated viral egress. *Dev. Cell* 56, 3250–3263.e5. doi:10.1016/j.devcel.2021.10.006

Chien, C., Xu, Y., Xiao, R., Aramini, J. M., Sahasrabudhe, P. V., Krug, R. M., et al. (2004). Biophysical characterization of the complex between double-stranded RNA and the N-terminal domain of the NS1 protein from influenza A virus: Evidence for a novel RNA-binding mode. *Biochemistry* 43, 1950–1962. doi:10.1021/bi030176o

Chui, A. J., Okondo, M. C., Rao, S. D., Gai, K., Griswold, A. R., Johnson, D. C., et al. (2019). N-terminal degradation activates the NLRP1B inflammasome. *Science* 364, 82–85. doi:10.1126/science.aau1208

Corbeil, L. B. (2016). Histophilus somni surface proteins. *Curr. Top. Microbiol. Immunol.* 396, 89–107. doi:10.1007/82_2015_5011

Correy, G. J., Kneller, D. W., Phillips, G., Pant, S., Russi, S., Cohen, A. E., et al. (2022). The mechanisms of catalysis and ligand binding for the SARS-CoV-2 NSP3 macrodomain from neutron and X-ray diffraction at room temperature. *bioRxiv* 2022, 479477. doi:10.1101/2022.02.07.479477

Cowardin, C. A., Jackman, B. M., Noor, Z., Burgess, S. L., Feig, A. L., and Petri, W. A. (2016). Glucosylation drives the innate inflammatory response to *Clostridium difficile* toxin A. *Infect. Immun.* 84, 2317–2323. doi:10.1128/IAI.00327-16

Cybulski, R. J., Sanz, P., Alem, F., Stibitz, S., Bull, R. L., and O'Brien, A. D. (2009). Four superoxide dismutases contribute to Bacillus anthracis virulence and provide spores with redundant protection from oxidative stress. *Infect. Immun.* 77, 274–285. doi:10.1128/IAI.00515-08

Daly, J. L., Simonetti, B., Klein, K., Chen, K.-E., Williamson, M. K., Antón-Plágaro, C., et al. (2020). Neuropilin-1 is a host factor for SARS-CoV-2 infection. *Science* 370, 861–865. doi:10.1126/science.abd3072

Department of Health and Human Services (2017). Framework for guiding funding decisions about proposed research involving enhanced potential pandemic pathogens. Available at: https://www.phe.gov/s3/dualuse/Documents/P3CO.pdf (Accessed June 7, 2022).

Díaz, J. (2020). SARS-CoV-2 molecular network structure. *Front. Physiol.* 11, 870. doi:10.3389/fphys.2020.00870

Du, J., An, R., Chen, L., Shen, Y., Chen, Y., Cheng, L., et al. (2014). Toxoplasma gondii virulence factor ROP18 inhibits the host NF-κB pathway by promoting p65 degradation. *J. Biol. Chem.* 289, 12578–12592. doi:10.1074/jbc.M113.544718

Dunlop, L. R., Oehlberg, K. A., Reid, J. J., Avci, D., and Rosengard, A. M. (2003). Variola virus immune evasion proteins. *Microbes Infect.* 5, 1049–1056. doi:10.1016/s1286-4579(03)00194-1

Ebrahimi, C. M., Kern, J. W., Sheen, T. R., Ebrahimi-Fardooee, M. A., van Sorge, N. M., Schneewind, O., et al. (2009). Penetration of the blood-brain barrier by Bacillus anthracis requires the pXO1-encoded BslA protein. *J. Bacteriol.* 191, 7165–7173. doi:10.1128/JB.00903-09

Feng, K., Min, Y.-Q., Sun, X., Deng, F., Li, P., Wang, H., et al. (2021). Interactome profiling reveals interaction of SARS-CoV-2 NSP13 with host factor STAT1 to suppress interferon signaling. *J. Mol. Cell Biol.* 13, 760–762. doi:10.1093/jmcb/mjab068

Fentress, S. J., Behnke, M. S., Dunay, I. R., Mashayekhi, M., Rommereim, L. M., Fox, B. A., et al. (2010). Phosphorylation of immunity-related GTPases by a Toxoplasma gondii-secreted kinase promotes macrophage survival and virulence. *Cell Host Microbe* 8, 484–495. doi:10.1016/j.chom.2010.11.005

Fernandez-Sesma, A., Marukian, S., Ebersole, B. J., Kaminski, D., Park, M.-S., Yuen, T., et al. (2006). Influenza virus evades innate and adaptive immunity via the NS1 protein. *J. Virol.* 80, 6295–6304. doi:10.1128/JVI.02381-05

Finkel, Y., Gluck, A., Nachshon, A., Winkler, R., Fisher, T., Rozman, B., et al. (2021). SARS-CoV-2 uses a multipronged strategy to impede host protein synthesis. *Nature* 594, 240–245. doi:10.1038/s41586-021-03610-3

Firoved, A. M., Miller, G. F., Moayeri, M., Kakkar, R., Shen, Y., Wiggins, J. F., et al. (2005). Bacillus anthracis edema toxin causes extensive tissue lesions and rapid lethality in mice. *Am. J. Pathol.* 167, 1309–1320. doi:10.1016/S0002-9440(10)61218-7

Fislová, T., and Kostolanský, F. (2005). The factors of virulence of influenza a virus. *Acta Virol.* 49, 147–157.

Flower, T. G., Buffalo, C. Z., Hooy, R. M., Allaire, M., Ren, X., and Hurley, J. H. (2020). Structure of SARS-CoV-2 ORF8, a rapidly evolving coronavirus protein implicated in immune evasion. *bioRxiv* 2020, 270637. doi:10.1101/2020.08.27.270637

Fonseca, G. J., Thillainadesan, G., Yousef, A. F., Ablack, J. N., Mossman, K. L., Torchia, J., et al. (2012). Adenovirus evasion of interferon-mediated innate immunity by direct antagonism of a cellular histone posttranslational modification. *Cell Host Microbe* 11, 597–606. doi:10.1016/j.chom.2012.05.005

Friebe, S., van der Goot, F. G., and Bürgi, J. (2016). The ins and outs of anthrax toxin. *Toxins (Basel)* 8, E69. doi:10.3390/toxins8030069

Fu, Y.-Z., Wang, S.-Y., Zheng, Z.-Q., Huang, Yi, Li, W.-W., Xu, Z.-S., et al. (2021). SARS-CoV-2 membrane glycoprotein M antagonizes the MAVS-mediated innate antiviral response. *Cell Mol. Immunol.* 18, 613–620. doi:10.1038/s41423-020-00571-x

Gack, M. U., Albrecht, R. A., Urano, T., Inn, K.-S., Huang, I.-C., Carnero, E., et al. (2009). Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. *Cell Host Microbe* 5, 439–449. doi:10.1016/j.chom.2009.04.006

Gemler, B. T., Mukherjee, C., Howland, C. A., Huk, D., Shank, Z., Harbo, L. J., et al. (2022). Function-based classification of hazardous biological sequences: Demonstration of a new paradigm for biohazard assessments. *Front. Bioeng. Biotechnol.* 10, 979497. doi:10.3389/fbioe.2022.979497

Gene Ontology Consortium Carbon, S., Douglass, E., Good, B. M., Unni, D. R., Harris, N. L., Mungall, C. J., et al. (2021). The gene ontology resource: Enriching a GOld mine. *Nucleic Acids Res.* 49, D325–D334. doi:10.1093/nar/gkaa1113

Glenewinkel, F., Cohen, M. J., King, C. R., Kaspar, S., Bamberg-Lemper, S., Mymryk, J. S., et al. (2016). The adaptor protein DCAF7 mediates the interaction of the adenovirus E1A oncoprotein with the protein kinases DYRK1A and HIPK2. *Sci. Rep.* 6, 28241. doi:10.1038/srep28241

Godbold, G. D., Kappell, A. D., LeSassier, D. S., Treangen, T. J., and Ternus, K. L. (2022). Categorizing sequences of concern by function to better assess mechanisms of microbial pathogenesis. *Infect. Immun.* 90, e0033421. doi:10.1128/IAI.00334-21

Goldberg, A. B., Cho, E., Miller, C. J., Lou, H. J., and Turk, B. E. (2017). Identification of a substrate-selective exosite within the metalloproteinase anthrax lethal factor. *J. Biol. Chem.* 292, 814–825. doi:10.1074/jbc.M116.761734

Golovkine, G., Faudry, E., Bouillot, S., Voulhoux, R., Attrée, I., and Huber, P. (2014). VE-cadherin cleavage by LasB protease from *Pseudomonas aeruginosa* facilitates type III secretion system toxicity in endothelial cells. *PLoS Pathog.* 10, e1003939. doi:10.1371/journal.ppat.1003939

Gordon, D. E., Jang, G. M., Bouhaddou, M., Xu, J., Obernier, K., White, K. M., et al. (2020). A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature* 583, 459–468. doi:10.1038/s41586-020-2286-9

Government Accountability Office (GAO) (2023). Public Health preparedness: HHS could improve oversight of research involving enhanced potential pandemic pathogens. Available at: https://www.gao.gov/assets/gao-23-105455.pdf (Accessed February 1, 2023).

Guichard, A., McGillivray, S. M., Cruz-Moreno, B., van Sorge, N. M., Nizet, V., and Bier, E. (2010). Anthrax toxins cooperatively inhibit endocytic recycling by the Rab11/Sec15 exocyst. *Nature* 467, 854–858. doi:10.1038/nature09446

Guo, G., Gao, M., Gao, X., Zhu, B., Huang, J., Luo, K., et al. (2021). SARS-CoV-2 non-structural protein 13 (nsp13) hijacks host deubiquitinase USP13 and counteracts host antiviral immune response. *Signal Transduct. Target Ther.* 6, 119. doi:10.1038/s41392-021-00509-3

Han, L., Zhuang, M.-W., Deng, J., Zheng, Y., Zhang, J., Nan, M.-L., et al. (2021). SARS-CoV-2 ORF9b antagonizes type I and III interferons by targeting multiple components of the RIG-I/MDA-5-MAVS, TLR3-TRIF, and cGAS-STING signaling pathways. *J. Med. Virol.* 93, 5376–5389. doi:10.1002/jmv.27050

Hathaway, L. J., Griffin, G. E., Sansonetti, P. J., and Edgeworth, J. D. (2002). Human monocytes kill Shigella flexneri but then die by apoptosis associated with suppression of proinflammatory cytokine production. *Infect. Immun.* 70, 3833–3842. doi:10.1128/IAI.70.7.3833-3842.2002

Head, J. A., Kalveram, B., and Ikegami, T. (2012). Functional analysis of Rift Valley fever virus NSs encoding a partial truncation. *PLoS One* 7, e45730. doi:10.1371/journal.pone.0045730

Heffernan, B. J., Thomason, B., Herring-Palmer, A., and Hanna, P. (2007). Bacillus anthracis anthrolysin O and three phospholipases C are functionally redundant in a murine model of inhalation anthrax. *FEMS Microbiol. Lett.* 271, 98–105. doi:10.1111/j.1574-6968.2007.00713.x

Hilbi, H., Moss, J. E., Hersh, D., Chen, Y., Arondel, J., Banerjee, S., et al. (1998). Shigella-induced apoptosis is dependent on caspase-1 which binds to IpaB. *J. Biol. Chem.* 273, 32895–32900. doi:10.1074/jbc.273.49.32895

Hsu, J. C.-C., Laurent-Rolle, M., Pawlak, J. B., Wilen, C. B., and Cresswell, P. (2021). Translational shutdown and evasion of the innate immune response by SARS-CoV-2 NSP14 protein. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2101161118. doi:10.1073/pnas.2101161118

Huang, C., Lokugamage, K. G., Rozovics, J. M., Narayanan, K., Semler, B. L., and Makino, S. (2011). SARS coronavirus nsp1 protein induces template-dependent endonucleolytic cleavage of mRNAs: Viral mRNAs are resistant to nsp1-induced RNA cleavage. *PLoS Pathog.* 7, e1002433. doi:10.1371/journal.ppat.1002433

Hutt, J. A., Lovchik, J. A., Drysdale, M., Sherwood, R. L., Brasel, T., Lipscomb, M. F., et al. (2014). Lethal factor, but not edema factor, is required to cause fatal anthrax in cynomolgus macaques after pulmonary spore challenge. *Am. J. Pathol.* 184, 3205–3216. doi:10.1016/j.ajpath.2014.08.008

Ijiri, Y., Matsumoto, K., Kamata, R., Nishino, N., Okamura, R., Kambara, T., et al. (1994). Suppression of polymorphonuclear leucocyte chemotaxis by *Pseudomonas aeruginosa* elastase *in vitro*: A study of the mechanisms and the correlation with ring abscess in pseudomonal keratitis. *Int. J. Exp. Pathol.* 75, 441–451.

Iwai, H., Kim, M., Yoshikawa, Y., Ashida, H., Ogawa, M., Fujita, Y., et al. (2007). A bacterial effector targets Mad2L2, an APC inhibitor, to modulate host cell cycling. *Cell* 130, 611–623. doi:10.1016/j.cell.2007.06.043

Jackson, R. J., Ramsay, A. J., Christensen, C. D., Beaton, S., Hall, D. F., and Ramshaw, I. A. (2001). Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox. *J. Virol.* 75, 1205–1210. doi:10.1128/JVI.75.3.1205-1210.2001

Jiang, H.-W., Zhang, H.-N., Meng, Q.-F., Xie, J., Li, Y., Chen, H., et al. (2020). SARS-CoV-2 Orf9b suppresses type I interferon responses by targeting TOM70. *Cell Mol. Immunol.* 17, 998–1000. doi:10.1038/s41423-020-0514-8

Jiao, J., Guan, H., Lippa, A. M., and Ricciardi, R. P. (2010). The N terminus of adenovirus type 12 E1A inhibits major histocompatibility complex class I expression by preventing phosphorylation of NF-κB p65 ser ^{276} through direct binding. *J. Virol.* 84, 7668–7674. doi:10.1128/JVI.02317-09

Jungreis, I., Sealfon, R., and Kellis, M. (2021). SARS-CoV-2 gene content and COVID-19 mutation impact by comparing 44 Sarbecovirus genomes. *Nat. Commun.* 12, 2642. doi:10.1038/s41467-021-22905-7

Kainulainen, M., Habjan, M., Hubel, P., Busch, L., Lau, S., Colinge, J., et al. (2014). Virulence factor NSs of rift valley fever virus recruits the F-box protein FBXO3 to degrade subunit p62 of general transcription factor TFIIH. *J. Virol.* 88, 3464–3473. doi:10.1128/JVI.02914-13

Kainulainen, M., Lau, S., Samuel, C. E., Hornung, V., and Weber, F. (2016). NSs virulence factor of rift valley fever virus engages the F-box proteins FBXW11 and β-TRCP1 to degrade the antiviral protein kinase PKR. *J. Virol.* 90, 6140–6147. doi:10.1128/JVI.00016-16

Kern, J., and Schneewind, O. (2010). BslA, the S-layer adhesin of B. anthracis, is a virulence factor for anthrax pathogenesis. *Mol. Microbiol.* 75, 324–332. doi:10.1111/j.1365-2958.2009.06958.x

Khan, S., Shafiei, M. S., Longoria, C., Schoggins, J., Savani, R. C., and Zaki, H. (2021). SARS-CoV-2 spike protein induces inflammation via TLR2-dependent activation of the NF-κB pathway. *bioRxiv*, 10, e68563. doi:10.7554/eLife.68563

King, C. R., Zhang, A., Tessier, T. M., Gameiro, S. F., and Mymryk, J. S. (2018). Hacking the cell: Network intrusion and exploitation by adenovirus E1A. *mBio* 9, e00390–18. doi:10.1128/mBio.00390-18

Klemm, T., Ebert, G., Calleja, D. J., Allison, C. C., Richardson, L. W., Bernardini, J. P., et al. (2020). Mechanism and inhibition of the papain-like protease, PLpro, of SARS-CoV-2. *EMBO J.* 39, e106275. doi:10.15252/embj.2020106275

Kreimendahl, S., and Rassow, J. (2020). The mitochondrial outer membrane protein tom70-mediator in protein traffic, membrane contact sites and innate immunity. *Int. J. Mol. Sci.* 21, E7262. doi:10.3390/ijms21197262

Kumar, N. A., Kunnakkadan, U., Thomas, S., and Johnson, J. B. (2020). In the crosshairs: RNA viruses OR complement? *Front. Immunol.* 11, 573583. doi:10.3389/fimmu.2020.573583

Kurupati, P., Turner, C. E., Tziona, I., Lawrenson, R. A., Alam, F. M., Nohadani, M., et al. (2010). Chemokine-cleaving Streptococcus pyogenes protease SpyCEP is necessary and sufficient for bacterial dissemination within soft tissues and the respiratory tract. *Mol. Microbiol.* 76, 1387–1397. doi:10.1111/j.1365-2958.2010.07065.x

Lafont, F., Tran Van Nhieu, G., Hanada, K., Sansonetti, P., and van der Goot, F. G. (2002). Initial steps of Shigella infection depend on the cholesterol/sphingolipid raft-mediated CD44-IpaB interaction. *EMBO J.* 21, 4449–4457. doi:10.1093/emboj/cdf457

Langer, M., Duggan, E. S., Booth, J. L., Patel, V. I., Zander, R. A., Silasi-Mansat, R., et al. (2012). Bacillus anthracis lethal toxin reduces human alveolar epithelial barrier function. *Infect. Immun.* 80, 4374–4387. doi:10.1128/IAI.01011-12

Lau, L., Gray, E. E., Brunette, R. L., and Stetson, D. B. (2015). DNA tumor virus oncogenes antagonize the cGAS-STING DNA-sensing pathway. *Science* 350, 568–571. doi:10.1126/science.aab3291

Le May, N., Mansuroglu, Z., Léger, P., Josse, T., Blot, G., Billecocq, A., et al. (2008). A SAP30 complex inhibits IFN-beta expression in Rift Valley fever virus infected cells. *PLoS Pathog.* 4, e13. doi:10.1371/journal.ppat.0040013

Leduc, D., Beaufort, N., de Bentzmann, S., Rousselle, J.-C., Namane, A., Chignard, M., et al. (2007). The *Pseudomonas aeruginosa* LasB metalloproteinase regulates the human urokinase-type plasminogen activator receptor through domain-specific endoproteolysis. *Infect. Immun.* 75, 3848–3858. doi:10.1128/IAI.00015-07

Lei, X., Dong, X., Ma, R., Wang, W., Xiao, X., Tian, Z., et al. (2020). Activation and evasion of type I interferon responses by SARS-CoV-2. *Nat. Commun.* 11, 3810. doi:10.1038/s41467-020-17665-9

Li, J.-Y., Liao, C.-H., Wang, Q., Tan, Y.-J., Luo, R., Qiu, Y., et al. (2020). The ORF6, ORF8 and nucleocapsid proteins of SARS-CoV-2 inhibit type I interferon signaling pathway. *Virus Res.* 286, 198074. doi:10.1016/j.virusres.2020.198074

Li, J., Vinh, D. C., Casanova, J.-L., and Puel, A. (2017). Inborn errors of immunity underlying fungal diseases in otherwise healthy individuals. *Curr. Opin. Microbiol.* 40, 46–57. doi:10.1016/j.mib.2017.10.016

Lin, X., Fu, B., Yin, S., Li, Z., Liu, H., Zhang, H., et al. (2021). ORF8 contributes to cytokine storm during SARS-CoV-2 infection by activating IL-17 pathway. *iScience* 24, 102293. doi:10.1016/j.isci.2021.102293

Liu, S., Zhang, Y., Moayeri, M., Liu, J., Crown, D., Fattah, R. J., et al. (2013). Key tissue targets responsible for anthrax-toxin-induced lethality. *Nature* 501, 63–68. doi:10.1038/nature12510

Liu, Y., Qin, C., Rao, Y., Ngo, C., Feng, J. J., Zhao, J., et al. (2021). SARS-CoV-2 Nsp5 demonstrates two distinct mechanisms targeting RIG-I and MAVS to evade the innate immune response. *mBio* 12, e0233521. doi:10.1128/mBio.02335-21

Lu, S., Ye, Q., Singh, D., Cao, Y., Diedrich, J. K., Yates, J. R., et al. (2021). The SARS-CoV-2 nucleocapsid phosphoprotein forms mutually exclusive condensates with RNA and the membrane-associated M protein. *Nat. Commun.* 12, 502. doi:10.1038/s41467-020-20768-y

Magro, C., Mulvey, J. J., Berlin, D., Nuovo, G., Salvatore, S., Harp, J., et al. (2020). Complement associated microvascular injury and thrombosis in the pathogenesis of severe COVID-19 infection: A report of five cases. *Transl. Res.* 220, 1–13. doi:10.1016/j.trsl.2020.04.007

Matheson, N. R., Potempa, J., and Travis, J. (2006). Interaction of a novel form of *Pseudomonas aeruginosa* alkaline protease (aeruginolysin) with interleukin-6 and interleukin-8. *Biol. Chem.* 387, 911–915. doi:10.1515/BC.2006.115

Matsuoka, K., Imahashi, N., Ohno, M., Ode, H., Nakata, Y., Kubota, M., et al. (2022). SARS-CoV-2 accessory protein ORF8 is secreted extracellularly as a glycoprotein homodimer. *J. Biol. Chem.* 298, 101724. doi:10.1016/j.jbc.2022.101724

McGillivray, S. M., Ebrahimi, C. M., Fisher, N., Sabet, M., Zhang, D. X., Chen, Y., et al. (2009). ClpX contributes to innate defense peptide resistance and virulence phenotypes of <i>Bacillus anthracis</i>. *J. Innate Immun.* 1, 494–506. doi:10.1159/000225955

Miao, G., Zhao, H., Li, Y., Ji, M., Chen, Y., Shi, Y., et al. (2021). ORF3a of the COVID-19 virus SARS-CoV-2 blocks HOPS complex-mediated assembly of the SNARE complex required for autolysosome formation. *Dev. Cell* 56, 427–442.e5. doi:10.1016/j.devcel.2020.12.010

Miller, C. S. (2005). Pleiotropic mechanisms of virus survival and persistence. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 100, S27–S36. doi:10.1016/j.tripleo.2005.03.017

Mishra, D., Suri, G. S., Kaur, G., and Tiwari, M. (2021). Comparative insight into the genomic landscape of SARS-CoV-2 and identification of mutations associated with the origin of infection and diversity. *J. Med. Virol.* 93, 2406–2419. doi:10.1002/jmv.26744

Miyamoto, Y., Itoh, Y., Suzuki, T., Tanaka, T., Sakai, Y., Koido, M., et al. (2022). SARS-CoV-2 ORF6 disrupts nucleocytoplasmic trafficking to advance viral replication. *Commun. Biol.* 5, 483. doi:10.1038/s42003-022-03427-4

Mosser, E. M., and Rest, R. F. (2006). The Bacillus anthracis cholesterol-dependent cytolysin, Anthrolysin O, kills human neutrophils, monocytes and macrophages. *BMC Microbiol.* 6, 56. doi:10.1186/1471-2180-6-56

Mounier, J., Boncompain, G., Senerovic, L., Lagache, T., Chrétien, F., Perez, F., et al. (2012). Shigella effector IpaB-induced cholesterol relocation disrupts the Golgi complex and recycling network to inhibit host cell secretion. *Cell Host Microbe* 12, 381–389. doi:10.1016/j.chom.2012.07.010

Mounier, J., Popoff, M. R., Enninga, J., Frame, M. C., Sansonetti, P. J., and Van Nhieu, G. T. (2009). The IpaC carboxyterminal effector domain mediates Src-dependent actin polymerization during Shigella invasion of epithelial cells. *PLoS Pathog.* 5, e1000271. doi:10.1371/journal.ppat.1000271

Moustaqil, M., Ollivier, E., Chiu, H.-P., Van Tol, S., Rudolffi-Soto, P., Stevens, C., et al. (2021). SARS-CoV-2 proteases PLpro and 3CLpro cleave IRF3 and critical modulators of inflammatory pathways (NLRP12 and TAB1): Implications for disease presentation across species. *Emerg. Microbes Infect.* 10, 178–195. doi:10.1080/22221751.2020.1870414

Mu, J., Fang, Y., Yang, Q., Shu, T., Wang, A., Huang, M., et al. (2020). SARS-CoV-2 N protein antagonizes type I interferon signaling by suppressing phosphorylation and nuclear translocation of STAT1 and STAT2. *Cell Discov.* 6, 65. doi:10.1038/s41421-020-00208-3

Mukherjee, D. V., Tonry, J. H., Kim, K. S., Ramarao, N., Popova, T. G., Bailey, C., et al. (2011). Bacillus anthracis protease InhA increases blood-brain barrier permeability and contributes to cerebral hemorrhages. *PLoS ONE* 6, e17921. doi:10.1371/journal.pone.0017921

Müllbacher, A., and Lobigs, M. (2001). Creation of killer poxvirus could have been predicted. *J. Virol.* 75, 8353–8355. doi:10.1128/jvi.75.18.8353-8355.2001

National Academies of Science, Engineering, and Medicine (2018). Biodefense in the age of synthetic biology. Available at: https://nap.nationalacademies.org/catalog/24890/biodefense-in-the-age-of-synthetic-biology#:~:text=Making%20informed%20decisions%20about%20how,capabilities%20that%20could%20be%20misused.&text=Biodefense%20in%20the%20Age%20of%20Synthetic%20Biology%20explores,potential%20misuses%20of%20synthetic%20biology (Accessed June 7, 2022).

National Research Council (2010). *Sequence-based classification of select agents*. Washington, DC: A Brighter Line. doi:10.17226/12970

National Science Advisory Board for Biosecurity (2023). Proposed biosecurity oversight framework for the future of science. Available at: https://osp.od.nih.gov/wp-content/uploads/2023/01/DRAFT-NSABB-WG-Report.pdf (Accessed February 1, 2023).

Ng, J., Hirota, S. A., Gross, O., Li, Y., Ulke-Lemee, A., Potentier, M. S., et al. (2010). *Clostridium difficile* toxin-induced inflammation and intestinal injury are mediated by the inflammasome. *Gastroenterology* 139, 542–552. doi:10.1053/j.gastro.2010.04.005

Oh, S. J., and Shin, O. S. (2021). SARS-CoV-2 nucleocapsid protein targets RIG-I-like receptor pathways to inhibit the induction of interferon response. *Cells* 10, 530. doi:10.3390/cells10030530

Pan, Y., Tagawa, Y., Champion, A., Sandal, I., and Inzana, T. J. (2018). Histophilus somni survives in bovine macrophages by interfering with phagosome-lysosome fusion but requires IbpA for optimal serum resistance. *Infect. Immun.* 86, e00365–18. doi:10.1128/IAI.00365-18

Papatheodorou, P., Zamboglou, C., Genisyuerek, S., Guttenberg, G., and Aktories, K. (2010). Clostridial glucosylating toxins enter cells via clathrin-mediated endocytosis. *PLoS One* 5, e10673. doi:10.1371/journal.pone.0010673

Park, M. D. (2020). Immune evasion via SARS-CoV-2 ORF8 protein? *Nat. Rev. Immunol.* 20, 408. doi:10.1038/s41577-020-0360-z

Pirofski, L., and Casadevall, A. (2015). What is infectiveness and how is it involved in infection and immunity? *BMC Immunol.* 16, 13. doi:10.1186/s12865-015-0076-1

Pisano, F., Kochut, A., Uliczka, F., Geyer, R., Stolz, T., Thiermann, T., et al. (2012). *In vivo*-induced InvA-like autotransporters Ifp and InvC of Yersinia pseudotuberculosis promote interactions with intestinal epithelial cells and contribute to virulence. *Infect. Immun.* 80, 1050–1064. doi:10.1128/IAI.05715-11

Prelli Bozzo, C., Nchioua, R., Volcic, M., Koepke, L., Krüger, J., Schütz, D., et al. (2021). IFITM proteins promote SARS-CoV-2 infection and are targets for virus inhibition *in vitro*. *Nat. Commun.* 12, 4584. doi:10.1038/s41467-021-24817-y

Rack, J. G. M., Zorzini, V., Zhu, Z., Schuller, M., Ahel, D., and Ahel, I. (2020). Viral macrodomains: A structural and evolutionary assessment of the pharmacological potential. *Open Biol.* 10, 200237. doi:10.1098/rsob.200237

Rahman, M. M., and McFadden, G. (2017). Myxoma virus dsRNA binding protein M029 inhibits the type I IFN-induced antiviral state in a highly species-specific fashion. *Viruses* 9, E27. doi:10.3390/v9020027

Rahman, M. M., and McFadden, G. (2020). Myxoma virus-encoded host range protein M029: A multifunctional antagonist targeting multiple host antiviral and innate immune pathways. *Vaccines (Basel)* 8, E244. doi:10.3390/vaccines8020244

Ren, Y., Shu, T., Wu, D., Mu, J., Wang, C., Huang, M., et al. (2020). The ORF3a protein of SARS-CoV-2 induces apoptosis in cells. *Cell Mol. Immunol.* 17, 881–883. doi:10.1038/s41423-020-0485-9

Rocheleau, L., Laroche, G., Fu, K., Stewart, C. M., Mohamud, A. O., Côté, M., et al. (2021). Identification of a high-frequency intrahost SARS-CoV-2 spike variant with enhanced cytopathic and fusogenic effects. *mBio* 12, e0078821. doi:10.1128/mBio.00788-21

Roehrich, A. D., Martinez-Argudo, I., Johnson, S., Blocker, A. J., and Veenendaal, A. K. J. (2010). The extreme C terminus of Shigella flexneri IpaB is required for regulation of type III secretion, needle tip composition, and binding. *Infect. Immun.* 78, 1682–1691. doi:10.1128/IAI.00645-09

Russo, L. C., Tomasin, R., Matos, I. A., Manucci, A. C., Sowa, S. T., Dale, K., et al. (2021). The SARS-CoV-2 Nsp3 macrodomain reverses PARP9/DTX3L-dependent ADP-ribosylation induced by interferon signaling. *J. Biol. Chem.* 297, 101041. doi:10.1016/j.jbc.2021.101041

Ryan, K. M. (2010). Cancer: Viruses' backup plan. *Nature* 466, 1054–1055. doi:10.1038/4661054a

Saint-Criq, V., Villeret, B., Bastaert, F., Kheir, S., Hatton, A., Cazes, A., et al. (2018). *Pseudomonas aeruginosa* LasB protease impairs innate immunity in mice and humans by targeting a lung epithelial cystic fibrosis transmembrane regulator-IL-6-antimicrobial-repair pathway. *Thorax* 73, 49–61. doi:10.1136/thoraxjnl-2017-210298

Saputri, D. S., Li, S., van Eerden, F. J., Rozewicki, J., Xu, Z., Ismanto, H. S., et al. (2020). Flexible, functional, and familiar: Characteristics of SARS-CoV-2 spike protein evolution. *Front. Microbiol.* 11, 2112. doi:10.3389/fmicb.2020.02112

Schmidgen, T., Kaiser, P. O., Ballhorn, W., Franz, B., Göttig, S., Linke, D., et al. (2014). Heterologous expression of Bartonella adhesin A in *Escherichia coli* by exchange of trimeric autotransporter adhesin domains results in enhanced adhesion properties and a pathogenic phenotype. *J. Bacteriol.* 196, 2155–2165. doi:10.1128/JB.01461-13

Schroeder, G. N., and Hilbi, H. (2008). Molecular pathogenesis of Shigella spp.: Controlling host cell signaling, invasion, and death by type III secretion. *Clin. Microbiol. Rev.* 21, 134–156. doi:10.1128/CMR.00032-07

Schubert, A., Zakikhany, K., Pietrocola, G., Meinke, A., Speziale, P., Eikmanns, B. J., et al. (2004). The fibrinogen receptor FbsA promotes adherence of Streptococcus agalactiae to human epithelial cells. *Infect. Immun.* 72, 6197–6205. doi:10.1128/IAI.72.11.6197-6205.2004

Schubert, K., Karousis, E. D., Jomaa, A., Scaiola, A., Echeverria, B., Gurzeler, L.-A., et al. (2020). SARS-CoV-2 Nsp1 binds the ribosomal mRNA channel to inhibit translation. *Nat. Struct. Mol. Biol.* 27, 959–966. doi:10.1038/s41594-020-0511-8

Schuller, M., Correy, G. J., Gahbauer, S., Fearon, D., Wu, T., Díaz, R. E., et al. (2021). Fragment binding to the Nsp3 macrodomain of SARS-CoV-2 identified through crystallographic screening and computational docking. *Sci. Adv.* 7, eabf8711. doi:10.1126/sciadv.abf8711

Seet, B. T., Johnston, J. B., Brunetti, C. R., Barrett, J. W., Everett, H., Cameron, C., et al. (2003). Poxviruses and immune evasion. *Annu. Rev. Immunol.* 21, 377–423. doi:10.1146/annurev.immunol.21.120601.141049

Senerovic, L., Tsunoda, S. P., Goosmann, C., Brinkmann, V., Zychlinsky, A., Meissner, F., et al. (2012). Spontaneous formation of IpaB ion channels in host cell membranes reveals how Shigella induces pyroptosis in macrophages. *Cell Death Dis.* 3, e384. doi:10.1038/cddis.2012.124

Shemesh, M., Aktepe, T. E., Deerain, J. M., McAuley, J. L., Audsley, M. D., David, C. T., et al. (2021). SARS-CoV-2 suppresses IFNβ production mediated by NSP1, 5, 6, 15, ORF6 and ORF7b but does not suppress the effects of added interferon. *PLoS Pathog.* 17, e1009800. doi:10.1371/journal.ppat.1009800

Shi, F., Lv, Q., Wang, T., Xu, J., Xu, W., Shi, Y., et al. (2022). Coronaviruses Nsp5 antagonizes porcine gasdermin D-mediated pyroptosis by cleaving pore-forming p30 fragment. *mBio* 13, e0273921. doi:10.1128/mbio.02739-21

Shin, D., Mukherjee, R., Grewe, D., Bojkova, D., Baek, K., Bhattacharya, A., et al. (2020). Papain-like protease regulates SARS-CoV-2 viral spread and innate immunity. *Nature* 587, 657–662. doi:10.1038/s41586-020-2601-5

Shirato, K., and Kizaki, T. (2021). SARS-CoV-2 spike protein S1 subunit induces pro-inflammatory responses via toll-like receptor 4 signaling in murine and human macrophages. *Heliyon* 7, e06187. doi:10.1016/j.heliyon.2021.e06187

Sousa, S., Lecuit, M., and Cossart, P. (2005). Microbial strategies to target, cross or disrupt epithelia. *Curr. Opin. Cell Biol.* 17, 489–498. doi:10.1016/j.ceb.2005.08.013

Su, W.-Q., Yu, X.-J., and Zhou, C.-M. (2021). SARS-CoV-2 ORF3a induces incomplete autophagy via the unfolded protein response. *Viruses* 13, 2467. doi:10.3390/v13122467

Sui, L., Zhao, Y., Wang, W., Wu, P., Wang, Z., Yu, Y., et al. (2021). SARS-CoV-2 membrane protein inhibits type I interferon production through ubiquitin-mediated degradation of TBK1. *Front. Immunol.* 12, 662989. doi:10.3389/fimmu.2021.662989

Sun, J., LaRock, D. L., Skowronski, E. A., Kimmey, J. M., Olson, J., Jiang, Z., et al. (2020). The *Pseudomonas aeruginosa* protease LasB directly activates IL-1β. *EBioMedicine* 60, 102984. doi:10.1016/j.ebiom.2020.102984

Tabak, L. A., and Jorgenson, L. A. (2022). 2022 charge to the NSABB. Available at: https://osp.od.nih.gov/wp-content/uploads/Tabak_and_Jorgenson-2022_Charge_to_the_NSABB.pdf [Accessed May 11, 2022].

Tao, L., Tian, S., Zhang, J., Liu, Z., Robinson-McCarthy, L., Miyashita, S.-I., et al. (2019). Sulfated glycosaminoglycans and low-density lipoprotein receptor contribute to *Clostridium difficile* toxin A entry into cells. *Nat. Microbiol.* 4, 1760–1769. doi:10.1038/s41564-019-0464-z

Terasaki, K., Ramirez, S. I., and Makino, S. (2016). Mechanistic insight into the host transcription inhibition function of rift valley fever virus NSs and its importance in virulence. *PLoS Negl. Trop. Dis.* 10, e0005047. doi:10.1371/journal.pntd.0005047

Thammavongsa, V., Kern, J. W., Missiakas, D. M., and Schneewind, O. (2009). *Staphylococcus aureus* synthesizes adenosine to escape host immune responses. *J. Exp. Med.* 206, 2417–2427. doi:10.1084/jem.20090097

Thoms, M., Buschauer, R., Ameismeier, M., Koepke, L., Denk, T., Hirschenberger, M., et al. (2020). Structural basis for translational shutdown and immune evasion by the Nsp1 protein of SARS-CoV-2. *Science* 369, 1249–1255. doi:10.1126/science.abc8665

Tonry, J. H., McNichol, B. A., Ramarao, N., Chertow, D. S., Kim, K. S., Stibitz, S., et al. (2012). Bacillus anthracis protease InhA regulates BslA-mediated adhesion in human endothelial cells. *Cell. Microbiol.* 14, 1219–1230. doi:10.1111/j.1462-5822.2012.01791.x

Tournier, J.-N., Quesnel-Hellmann, A., Mathieu, J., Montecucco, C., Tang, W.-J., Mock, M., et al. (2005). Anthrax edema toxin cooperates with lethal toxin to impair cytokine secretion during infection of dendritic cells. *J. Immunol.* 174, 4934–4941. doi:10.4049/jimmunol.174.8.4934

Uchiyama, T., Kawano, H., and Kusuhara, Y. (2006). The major outer membrane protein rOmpB of spotted fever group rickettsiae functions in the rickettsial adherence to and invasion of Vero cells. *Microbes Infect.* 8, 801–809. doi:10.1016/j.micinf.2005.10.003

United States Government (2014). United States government policy for institutional oversight of life sciences dual use research of concern. Available at: https://www.phe.gov/s3/dualuse/Documents/durc-policy.pdf [Accessed June 7, 2022].

United States Government (2012). United States government policy for oversight of life sciences dual use research of concern. Available at: https://www.phe.gov/s3/dualuse/Documents/us-policy-durc-032812.pdf [Accessed June 7, 2022].

Vadyvaloo, V., Jarrett, C., Sturdevant, D. E., Sebbane, F., and Hinnebusch, B. J. (2010). Transit through the flea vector induces a pretransmission innate immunity resistance phenotype in *Yersinia pestis*. *PLoS Pathog.* 6, e1000783. doi:10.1371/journal.ppat.1000783

van Sorge, N. M., Ebrahimi, C. M., McGillivray, S. M., Quach, D., Sabet, M., Guiney, D. G., et al. (2008). Anthrax toxins inhibit neutrophil signaling pathways in brain endothelium and contribute to the pathogenesis of meningitis. *PLoS One* 3, e2964. doi:10.1371/journal.pone.0002964

Vazquez, C., Swanson, S. E., Negatu, S. G., Dittmar, M., Miller, J., Ramage, H. R., et al. (2021). SARS-CoV-2 viral proteins NSP1 and NSP13 inhibit interferon activation through distinct mechanisms. *PLoS One* 16, e0253089. doi:10.1371/journal.pone.0253089

Vuyisich, M., Sanders, C. K., and Graves, S. W. (2012). Binding and cell intoxication studies of anthrax lethal toxin. *Mol. Biol. Rep.* 39, 5897–5903. doi:10.1007/s11033-011-1401-2

Walls, A. C., Park, Y.-J., Tortorici, M. A., Wall, A., McGuire, A. T., and Veesler, D. (2020). Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 181, 281–292.e6. doi:10.1016/j.cell.2020.02.058

Wang, R., Yang, X., Chang, M., Xue, Z., Wang, W., Bai, L., et al. (2021a). ORF3a protein of severe acute respiratory syndrome coronavirus 2 inhibits interferon-activated janus kinase/signal transducer and activator of transcription signaling via elevating suppressor of cytokine signaling 1. *Front. Microbiol.* 12, 752597. doi:10.3389/fmicb.2021.752597

Wang, S., Dai, T., Qin, Z., Pan, T., Chu, F., Lou, L., et al. (2021b). Targeting liquid-liquid phase separation of SARS-CoV-2 nucleocapsid protein promotes innate antiviral immunity by elevating MAVS activity. *Nat. Cell Biol.* 23, 718–732. doi:10.1038/s41556-021-00710-0

Wang, W., Zhou, Z., Xiao, X., Tian, Z., Dong, X., Wang, C., et al. (2021c). SARS-CoV-2 nsp12 attenuates type I interferon production by inhibiting IRF3 nuclear translocation. *Cell Mol. Immunol.* 18, 945–953. doi:10.1038/s41423-020-00619-y

Wang, X., Pernicone, N., Pertz, L., Hua, D., Zhang, T., Listovsky, T., et al. (2019). REV7 has a dynamic adaptor region to accommodate small GTPase RAN/Shigella IpaB ligands, and its activity is regulated by the RanGTP/GDP switch. *J. Biol. Chem.* 294, 15733–15742. doi:10.1074/jbc.RA119.010123

Wang, Y., Jenkins, S. A., Gu, C., Shree, A., Martinez-Moczygemba, M., Herold, J., et al. (2016a). Bacillus anthracis spore surface protein BclA mediates complement factor H binding to spores and promotes spore persistence. *PLoS Pathog.* 12, e1005678. doi:10.1371/journal.ppat.1005678

Wang, Y., Wei, Y., Yuan, S., Tao, H., Dong, J., Zhang, Z., et al. (2016b). Bacillus anthracis S-layer protein BslA binds to extracellular matrix by interacting with laminin. *BMC Microbiol.* 16, 183. doi:10.1186/s12866-016-0802-8

Wei, Z., Schnupf, P., Poussin, M. A., Zenewicz, L. A., Shen, H., and Goldfine, H. (2005). Characterization of Listeria monocytogenes expressing anthrolysin O and phosphatidylinositol-specific phospholipase C from Bacillus anthracis. *Infect. Immun.* 73, 6639–6646. doi:10.1128/IAI.73.10.6639-6646.2005

Wickham, M. E., Brown, N. F., Boyle, E. C., Coombes, B. K., and Finlay, B. B. (2007). Virulence is positively selected by transmission success between mammalian hosts. *Curr. Biol.* 17, 783–788. doi:10.1016/j.cub.2007.03.067

Wu, J., Shi, Y., Pan, X., Wu, S., Hou, R., Zhang, Y., et al. (2021). SARS-CoV-2 ORF9b inhibits RIG-I-MAVS antiviral signaling by interrupting K63-linked ubiquitination of NEMO. *Cell Rep.* 34, 108761. doi:10.1016/j.celrep.2021.108761

Wu, L., Wang, X., Li, Y., Liu, Y., Su, D., Fu, T., et al. (2016). Toxoplasma gondii ROP18: Potential to manipulate host cell mitochondrial apoptosis. *Parasitol. Res.* 115, 2415–2422. doi:10.1007/s00436-016-4993-6

Xia, H., Cao, Z., Xie, X., Zhang, X., Chen, J. Y.-C., Wang, H., et al. (2020). Evasion of type I interferon by SARS-CoV-2. *Cell Rep.* 33, 108234. doi:10.1016/j.celrep.2020.108234

Xia, J., Kong, L., Zhou, L.-J., Wu, S.-Z., Yao, L.-J., He, C., et al. (2018). Genome-wide bimolecular fluorescence complementation-based proteomic analysis of toxoplasma gondii ROP18's human interactome shows its key role in regulation of cell immunity and apoptosis. *Front. Immunol.* 9, 61. doi:10.3389/fimmu.2018.00061

Yamamoto, M., Ma, J. S., Mueller, C., Kamiyama, N., Saiga, H., Kubo, E., et al. (2011). ATF6β is a host cellular target of the Toxoplasma gondii virulence factor ROP18. *J. Exp. Med.* 208, 1533–1546. doi:10.1084/jem.20101660

Yang, S.-C., Hung, C.-F., Aljuffali, I. A., and Fang, J.-Y. (2015). The roles of the virulence factor IpaB in Shigella spp. in the escape from immune cells and invasion of epithelial cells. *Microbiol. Res.* 181, 43–51. doi:10.1016/j.micres.2015.08.006

Yang, Y., Wu, Y., Meng, X., Wang, Z., Younis, M., Liu, Y., et al. (2022). SARS-CoV-2 membrane protein causes the mitochondrial apoptosis and pulmonary edema via targeting BOK. *Cell Death Differ.* 29, 1395–1408. doi:10.1038/s41418-022-00928-x

Yang, Z., Hou, Y., Hao, T., Rho, H.-S., Wan, J., Luan, Y., et al. (2017). A human proteome array approach to identifying key host proteins targeted by toxoplasma kinase ROP18. *Mol. Cell Proteomics* 16, 469–484. doi:10.1074/mcp.M116.063602

Yao, L., Xu, L., Zhou, L., Wu, S., Zou, W., Chen, M., et al. (2021). Toxoplasma gondii type-I ROP18 targeting human E3 ligase TRIM21 for immune escape. *Front. Cell Dev. Biol.* 9, 685913. doi:10.3389/fcell.2021.685913

Youn, Y.-J., Lee, Y.-B., Kim, S.-H., Jin, H. K., Bae, J.-S., and Hong, C.-W. (2021). Nucleocapsid and spike proteins of SARS-CoV-2 drive neutrophil extracellular trap formation. *Immune Netw.* 21, e16. doi:10.4110/in.2021.21.e16

Yu, S., Zheng, X., Zhou, B., Li, J., Chen, M., Deng, R., et al. (2022). SARS-CoV-2 spike engagement of ACE2 primes S2' site cleavage and fusion initiation. *Proc. Natl. Acad. Sci. U. S. A.* 119, e2111199119. doi:10.1073/pnas.2111199119

Yuen, C.-K., Lam, J.-Y., Wong, W.-M., Mak, L.-F., Wang, X., Chu, H., et al. (2020). SARS-CoV-2 nsp13, nsp14, nsp15 and orf6 function as potent interferon antagonists. *Emerg. Microbes Infect.* 9, 1418–1428. doi:10.1080/22221751.2020.1780953

Zekarias, B., Mattoo, S., Worby, C., Lehmann, J., Rosenbusch, R. F., and Corbeil, L. B. (2010). Histophilus somni IbpA DR2/Fic in virulence and immunoprotection at the natural host alveolar epithelial barrier. *Infect. Immun.* 78, 1850–1858. doi:10.1128/IAI.01277-09

Zenewicz, L. A., Wei, Z., Goldfine, H., and Shen, H. (2005). Phosphatidylinositol-specific phospholipase C of Bacillus anthracis down-modulates the immune response. *J. Immunol.* 174, 8011–8016. doi:10.4049/jimmunol.174.12.8011

Zhang, K., Miorin, L., Makio, T., Dehghan, I., Gao, S., Xie, Y., et al. (2021a). Nsp1 protein of SARS-CoV-2 disrupts the mRNA export machinery to inhibit host gene expression. *Sci. Adv.* 7, eabe7386. doi:10.1126/sciadv.abe7386

Zhang, Q., Chen, Z., Huang, C., Sun, J., Xue, M., Feng, T., et al. (2021b). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) membrane (M) and spike (S) proteins antagonize host type I interferon response. *Front. Cell Infect. Microbiol.* 11, 766922. doi:10.3389/fcimb.2021.766922

Zhang, X., Yang, Z., Pan, T., Long, X., Sun, Q., Wang, P.-H., et al. (2022). SARS-CoV-2 ORF3a induces RETREG1/FAM134B-dependent reticulophagy and triggers sequential ER stress and inflammatory responses during SARS-CoV-2 infection. *Autophagy* 18, 2576–2592. doi:10.1080/15548627.2022.2039992

Zhang, Y., Chen, Y., Li, Y., Huang, F., Luo, B., Yuan, Y., et al. (2021c). The ORF8 protein of SARS-CoV-2 mediates immune evasion through down-regulating MHC-I. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2024202118. doi:10.1073/pnas.2024202118

Zhang, Y., Sun, H., Pei, R., Mao, B., Zhao, Z., Li, H., et al. (2021d). The SARS-CoV-2 protein ORF3a inhibits fusion of autophagosomes with lysosomes. *Cell Discov.* 7, 31. doi:10.1038/s41421-021-00268-z

Zheng, Y., Deng, J., Han, L., Zhuang, M.-W., Xu, Y., Zhang, J., et al. (2022). SARS-CoV-2 NSP5 and N protein counteract the RIG-I signaling pathway by suppressing the formation of stress granules. *Signal Transduct. Target Ther.* 7, 22. doi:10.1038/s41392-022-00878-3

Zheng, Y., Zhuang, M.-W., Han, L., Zhang, J., Nan, M.-L., Zhan, P., et al. (2020). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) membrane (M) protein inhibits type I and III interferon production by targeting RIG-I/MDA-5 signaling. *Signal Transduct. Target Ther.* 5, 299. doi:10.1038/s41392-020-00438-7

Zhu, Y., Ge, X., Xie, D., Wang, S., Chen, F., and Pan, S. (2021). Clinical strains of *Pseudomonas aeruginosa* secrete LasB elastase to induce hemorrhagic diffuse alveolar damage in mice. *J. Inflamm. Res.* 14, 3767–3780. doi:10.2147/JIR.S322960

Zinzula, L. (2021). Lost in deletion: The enigmatic ORF8 protein of SARS-CoV-2. *Biochem. Biophys. Res. Commun.* 538, 116–124. doi:10.1016/j.bbrc.2020.10.045

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Perspective
PUBLISHED 07 June 2023
DOI 10.3389/fbioe.2023.1211789

OPEN ACCESS

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RECEIVED 25 April 2023
ACCEPTED 26 May 2023
PUBLISHED 07 June 2023

CITATION
Masehela TS and Barros E (2023), The African continent should consider a harmonized consultative and collaborative effort towards coordinated policy and regulatory guidelines across the fields of biotechnology.
Front. Bioeng. Biotechnol. 11:1211789.
doi: 10.3389/fbioe.2023.1211789

The African continent should consider a harmonized consultative and collaborative effort towards coordinated policy and regulatory guidelines across the fields of biotechnology

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The advances in the field of biotechnology (and bioengineering) over the past decades has allowed the precise development of new products across the agricultural, environmental, and pharmaceutical sectors. This has led to the need to evaluate the relevance and applicability of existing policies and frameworks that regulate the current transgenic technologies. On the African continent, there are delays in the development and implementation of biosafety policies and regulations. Most African countries formulate their policies, regulations, and frameworks by following The Convention on Biological Diversity's (CBD) guidelines. Although the CBD documents are continually evolving, this happens at a slower pace. It is becoming increasingly important for countries to deal swiftly with the advances in biotechnology in a manner that *balances* the regulatory complexities, while safeguarding the *net gains* for human health, the environment, and the economy. For the African countries, some of these *net gains* are similar, while concerns and perceived risks associated with the adoption and use of the technology are also common. Furthermore, the challenges relating to capacity, knowledge, and skills to address some of the regulatory complexities. In this article we explore the advancement of some African countries in the development and implementation of various biosafety policies and detail the challenges and constraints faced by those countries that are lagging behind. We conclude by outlining identified opportunities for neighbouring and regional countries to assist one another and work in a more organised and coordinated approach towards developing, implementing, and strengthening their respective biosafety policies, regulations, and frameworks.

KEYWORDS

Africa, biotechnology, biosafety, regulatory guidelines, policy, convention on biological diversity (CBD), genome editing, new breeding technologies (NBTs)

Introduction

The field of biotechnology has overtime been recognised to be rapid in terms of new improvements and advancements towards supporting innovation across the different fields of research and development (Barragán-Ocaña, 2020; Ma, 2021). The significant potential for their applications cuts across many fields and disciplines, with the major ones being

agriculture and health (medicine). In these two fields, biotechnology has presented to the human population several useful products by using enzymes, microbes, proteins, and various metabolic machinery of plants and animals (Masson et al., 2001; Khan, 2014; Pham, 2018).

The biggest impact of biotechnology has been in the field of agriculture mainly because of the need for more sustainable food production to feed the ever-increasing world population (Giller et al., 2021). Working with agricultural farmers, scientists have developed biotechnology tools to complement conventional crop improvement methodologies to produce genetically modified crops (GMOs). These crops are better adapted to grow in different environments, to be more resistant to agricultural biotic and abiotic stresses, to be better protected against pests and to have improved nutritional quality (Tran et al., 2010; Abdallah et al., 2014; Kamthan et al., 2016). The latest plant-breeding technology tool that has the potential to revolutionize agriculture is the development of genome edited crops. If the African continent is to benefit from these biotechnology developments there is an urgent need for discussion, debate, and harmonization of guidelines across the continent.

The adoption, application, and use, of biotechnology has not always been positive, as it has been marked with various concerns and controversies (Bauer, 2002). The debates on this subject comes mainly from the public and goes as far back as the early introductions of Genetically Modified (GM) products (Hielscher et al., 2016). In their early years, Genetically Modified crops, and foods, were to a large degree met with different perceptions and a strong level of mistrust–especially those based on personal or religious beliefs (Phillips, 2008). In most instances, the discussions and perceptions remain highly emotional, and focused on the potential economic, environmental, human health and social risks (Carr & Levidow, 2000; Goyal & Gurtoo, 2011; Lucht, 2015). Although, the trend on concerns varies across the continents, common issues are centred around ethical standards of practice, the morality and unpredictable results that come with different gene manipulations and experiments (Deane-Drummond et al., 2001). In some instances, questions are raised around the impacts on small-scale farmers and communities when it comes to seed rights and the socio-economic implications (direct/indirect), issuing of patents, and the equitable sharing of some of the proceeds from the biological resources and genetic material derived from regions/countries (Masehela et al., 2021). Furthermore, arguments remain that the GM technology depicts and promotes a particular narrative around a solution towards the global food crisis focusing on crops and traits (Stone & Glover, 2011; Stone & Glover, 2017). At the same time, others argue that a lot of the debates and criticism of the technology discredits various benefits already achieved with its application and use (Klümper & Qaim, 2014; Smyth, 2020).

The dawn of GMOs on the African continent has forever been marked by the hesitancy to accept, emanating from unfavourable policies and a wide array of public opinions (Gbadegesin et al., 2022). Besides the general lack of knowledge base, education and awareness of the technology and its application to the public (Gastrow et al., 2018), undecisive political attitude to GMOs has also been noted to have added more confusion and indirectly increased mistrust within the technology space (de Cheveigné et al., 2002). It is for this reason that there has been calls for care-based approach to ethics and politics so that social, economic, and ethical considerations are strategically incorporated into biotechnology governance and regulatory assessments (Wickson et al., 2017). For the African continent, this is important given that public trust is critical for the technology's success and its benefits to be realised. However, this does not mean that the longstanding concerns, implications, and questions around safety should be forgotten (Trump et al., 2022). We know now that the world has begun embracing New Breeding Technologies (NBTs), spearheaded by the likes of CRISPR-Cas9 and other gene editing techniques (de Graeff et al., 2019). Already, we are seeing several concerns and oppositions to these technologies across the world (Helliwell et al., 2017), and since the African continent has not fully advanced from its GMOs challenges and drawbacks, it might be difficult to advance to the new politics and governance of these new technologies.

Countries and governments across the globe have set up regulatory agencies (bodies and committees) that will have oversight and make decisions regarding the validity of the research, development and the safety in the application of the technology and its derived products (McLean et al., 2012; Komen et al., 2020; Turnbull et al., 2021). However, the level in which the various regulations, biosafety frameworks and policy instruments are designed, implemented, enforced, and monitored differs depending on the country/government needs (Cantley, 2007). The focus areas are to a large extend guided, shaped and controlled, by country priorities, political influences and leadership, and the economic elements. For those countries that are signatories and party to the Cartegena Protocol on Biosafety to the Convention on Biological Diversity (CBD), the treaty was and remains instrumental in providing guidance and governance on the movements of living modified organisms (LMOs) resulting from modern biotechnology (Glass, 2000). Subsequent, several supplementary protocols and agreements have been put in place, recognising that with the rapid advances in the field of biotechnology; there is a need to protect biological diversity from the potential risks posed by living modified organisms (Shibata, 2014). At the same time, these key protocols have had their own shortcomings as they have not fully kept up with the fast developments within the biotechnology space and this is evident with the lack of clear definitions and guidance in fields such as Synthetic Biology (Hokanson, 2019; Groenewald, 2021). Although this can be viewed as a drawback, it should not undermine the substantial work done over the years through the various committees, expert and working groups [e.g., Ad Hoc Technical Expert Group (AHTEG)] and online forums of the CBD.

One of the major challenges for countries/parties has been that of taking on the guidance documents, training manuals and other supplementary materials for further development in line with their country needs (Pertry et al., 2014). Often, this failure is attributed to the lack of political will, lack of financial resources, relevant expertise, knowledge and experience in the respective policy and framework areas (Kameri-Mbote, 2002; Falkner & Gupta, 2004). This is particularly true for the African continent and remains a great challenge for most countries–in turn, lack of progression when it comes to exploring the potential applications of biotechnology and its associated bioengineering tools (Makinde et al., 2009). In this article, we explore: 1) the relevance and applicability of agricultural biotechnology to the African continent; 2) review and outline

African countries that have made good strides in developing relevant biosafety protocols towards regulating the use of the technology; 3) explore some of the drawbacks of progress or reluctance in formulating and implementing biosafety protocols; and 4) propose or put forward an approach that could benefit the continent towards achieving various components of their frameworks, policy and biosafety protocols for guidance when considering the adoption and use of biotechnology–and bioengineering tools/options.

The context and relevance of biotechnology for the African continent

Biotechnology has a strong significance for the African continent in terms of contribution towards solving and/or offering options in mitigating a multitude of problems in both the agriculture and health sectors. Several studies do recognise the massive potential that biotechnology has to offer to the continent when it comes to improving agricultural production (Juma, 2015), improving economic growth, contributing to food and nutrition security (Binswanger-Mkhize et al., 2010; Kedir & Kararach, 2019), strengthening scientific capacity and advancement, providing alternative solutions to waste management, and improving health as well pharmaceutical options in the medicinal field (Bediako, 2022). The 2009 publication by the New Partnerships for Africa's Development (NEPAD), outlines challenges facing the African continent on biotechnology and biosafety (Makinde et al., 2009). Among others, the report highlighted the financial challenges, the lack/loss of trained technical expertise; slow development of the biotechnology sector; inadequate Intellectual Property Rights infrastructure; lack of political will and government leadership. Today, these shortcomings remain prevalent and are evident in the lack of progress in biotechnology policy advancement and/or development of national biosafety frameworks (NBFs) across the continent.

Without these laws, regulations, guidelines, or policies related to biotechnology, it remains difficult to carry out or conduct any biotechnology related activities in the respective countries. Paarlberg (2009) indicates that one of the major constrains exploring new technologies in agriculture for Africa, stems from the lack of formulation–subsequently, implementation of relevant policies and regulations that would be geared towards agricultural advancement through science. In fact, they specifically cite the disapprovals on modern agricultural biotechnology because of inadequate policy frameworks to support its update. Similarly, Egwang (2001) and Bediako (2022), demonstrates that biotechnology has the potential to transform the health and the economies of most African countries, and that for this to be realised, African governments must create enabling environments through positive policies and the availability of resources.

African countries continue to face challenges when it comes to food production and medicinal needs (Pinstrup-Andersen & Watson, 2011). Countries find it difficult to provided adequate healthcare (and products/medicines), while farmers find it difficult to control and manage agricultural pests. At the same time, multilevel approaches are needed to overcome these challenges that are further exacerbated by increasing environmental, economic, and social challenges. Moreover, biotechnology has moved far beyond the *basic principles* of GMOs, offering some of the most powerful technological tools as options for mitigating most challenges and constraints in both agriculture and medical fields. Wambugu (1999), Machuka (2001), Nitin et al. (2022), Mfutso-Bengo & Muula (2007) and Sammut (2021) outline some of the potential benefits that can be realised for the African continent in agriculture and medicine, respectively.

Brief overview of biosafety policies, regulations and/or frameworks for African countries

The regulatory landscape of genetically modified products in Africa is still very diverse and harmonization of its regulatory processes has not yet been archived. There are many obstacles facing the commercial release of GM crops and they include biosafety factors, public and farmer acceptance as well as, political will and support (Akinbo et al., 2021). The 55 member states of the African union have developed specific regulatory agencies to approve seed regulation and variety regulation of crops produced by conventional methodologies under the Seed Act in addition to a National Biosafety Authority (NBA) that regulates crops developed using biotechnological approaches, like GMOs. Under the Seed Act regulation many African countries require approval by the National Performance Trial Committee (NPTC) and the National Variety Release Committee (NVRC) for the release and commercialization of conventionally derived seeds. Regarding the environmental release and commercialization of GMOs, African countries are at different levels of adoption of GM crops and only a few have approved the commercial release of crops for farmer adoption. To consider a joint and co-ordinated regulatory guideline for the continent one needs to understand where they are at, what regulations are in place and where the regulatory process could be fast tracked. An outline of the process used by Kenya, Nigeria, Eswatini, Ethiopia, Ghana, Malawi, Mozambique, Sudan and South Africa is summarized in Table 1 (Akinbo et al., 2021). These countries have commercialized GM crops (e.g., Bt cotton) but have their own specific Seed Laws and Regulations, and follow different steps some of which maybe more laborious resulting in a fast or slow approval of GM crops.

With the rapid advances in biotechnology, it is crucial for African countries to work together and try to harmonize their science-based regulatory guidelines to be ready for the release and approval of products developed using CRISPR/Cas9-mediated genome editing. CRISPR-Cas9-based genome editing has become the most prevalent genetic engineering approach to develop improved crop varieties in addition to conventional technologies due to its simplicity, precision, and accuracy (Arora & Narula, 2017; Montecillo et al., 2020). Genome editing technologies enable the targeted manipulation of plant genomes and therefore it speeds up the breeding processes enabling breeders to address urgent goals with greater precision (Ceasar et al., 2016; Rao & Wang, 2021). Although globally there is not yet a definite consensus on how to regulate genome editing products, some countries have opted to regulate genome-edited crops based on

TABLE 1 Regulatory processes adopted by different African countries (adopted and modified from Akinbo et al., 2021).

	Biosafety regulatory framework	Seed acts and implementing regulations
Kenya		
Laws and Regulations	Biosafety Act 2009 and implementing regulations to cover contained use, environmental release, import, export, and transit	Seed and Plant Varieties Act (Seed Act; Cap.326 (Gok, 2012) and the Seeds and Plant Varieties Regulations (NPT Regulations)
Agencies/Department	National biosafety Authority is the Competent Authority	KEPHIS, Ministry of Agriculture
Committees	Scientific Advisory Committee	National Performance Trial Committee National Variety Release Committee
Nigeria		
Laws and Regulations	National Biosafety Management Agency Act 2015 revised in 2019 to National Biosafety Management Agency Act 2019	National Agricultural Seeds Act, N5 Laws of Nigeria, 2004 revised to give National Seed Act (NSC) Act 2019
Agencies/Department	National biosafety Management Agency (NBMA) is the National Biosafety Authority	National Agricultural Seeds Council (NASC), an agency of the Federal Ministry of Agriculture and Rural Development
Committees/ PARTNERSHIPS	The Nigeria Agricultural Seed Council; National Agricultural Quarantine Service; Nigeria Customs Service; National Agency for Food and Drug Administration and Control; Federal Ministry of Agriculture (Department of Veterinary and Pest Control); Standard Organization of Nigeria; Federal Competition and Consumer Protection Commission	National Crop Varieties and Livestock Breeds Registration and Release Committee
Eswatini		
Laws and Regulations	Biosafety Act of 2012 (under review)	Plant Control Act, 1981 (under review); Seeds and Plant Varieties Act of 2000 and Plant Varieties Regulations
Agencies/Department	Eswatini Environmental Authority	Seed Quality Control Services, under the Ministry of Agriculture
Committees	National Biosafety Advisory Committee	National Variety Release Committee
Ethiopia		
Laws and Regulations	Biosafety Proclamations (Proclamation No. 655/2009 and the Amendment into Proclamation No. 896/2015	Seed Proclamation (Proclamation No. 782/2013) revised to give Proclamation No. 206/2000 in 2000
Agencies/Department	Environment, Forest, and Climate Change Commission	National Seed Quality Control and Certification Division under MoARD
Committees	National Biosafety Advisory Committee	National Crop Improvement Committee
Ghana		
Laws and Regulations	Biosafety Act 831, 2011 and Implementing Regulations	Plants and Fertilizer act of 2010 (803)
Agencies/Department	National Biosafety Authority	National Crop Improvement Committee
Committees	Board consisting of experts in biotechnology and related biological sciences, including biosafety	Plant Protection and Regulatory Services Directorate
Malawi		
Laws and Regulations	Biosafety Act was passed in 2002 and implemented in 2007 and National Biotechnology and Biosafety Policy was enacted in 2008	Seed Act of 2005 and recently published seed Regulations 2018
Agencies/Department	National Biosafety Regulatory Committee (NBRC) is the Competent Authority	The Seed Services Unit of DARS (Department of Agricultural Research Services)
Committees	National Biosafety Regulatory Committee, which includes Reviewers, Inspectors and Biosafety Registrar	Agricultural Technology Clearing Committee (ATCC)
Mozambique		
Laws and Regulations	Decree no. 6/2007 (regulation) with an amendment in 2014 to allow for the commercialization of GMOs to give Decree 71/2014 of 28 November 2014	12/2013 Seed Regulation Decree
Agencies/Department	Minister of Science and Technology, Higher and Technical Vocational Education, is competent authority on matters pertaining to GMO approvals	National Seed Committee (NaSC) in Ministry of Agriculture and the Variety Registration and Release Committee
Committees	The Grupo Inter-Institucional Sobre Bio-Segurança, (GIBS) serve as advisory committee to the Minister of Science and Technology, Higher and Technical Vocational Education	Department of Seeds in the Ministry of Agriculture

(Continued on following page)

TABLE 1 (*Continued*) Regulatory processes adopted by different African countries (adopted and modified from Akinbo et al., 2021).

	Biosafety regulatory framework	Seed acts and implementing regulations
Sudan		
Laws and Regulations	Biological Safety Act 2020	New Seed Law in 2009
Agencies/Department	Sudan National Biosafety Council (SNBC)	National Seed Council
Committees	—	—
South Africa		
Laws and Regulations	Genetically Modified Organisms Act 1977 (Act No.15 of 1997) revised in 2006 to Genetically Modified Organisms Act No. 23 of 2006	Plant Breeder's Rights Act 1976 (Act No. 15 of 1976)
Agencies/Department	Formerly Minister for Agriculture, Forestry and Fisheries and now Minister of Agriculture, Land Reform and Rural Development	Formerly Minister for Agriculture, Forestry and Fisheries and now Minister of Agriculture, Land Reform and Rural Development
Committees	Advisory Committee (AC) and Executive Council (EC)	—

the presence/absence of foreign DNA integration. So, genome-edited crops that do not have any foreign gene and the edited gene is not harmful to other plants and its safety attributes are comparable to its conventionally bred crops, does not require regulatory evaluation. Likewise, genome-edited foods whose safety attributes are comparable to those produced by conventionally bred crops, do not require regulatory evaluation.

Here, we are not suggesting or advocating that the African continent take a limited oversight on gene edited products, but rather explore paths towards homogeneity within the regulatory space of these new technology-based products, in line with their country specific needs and economical advancements. We also note that the scope of the technology and its applications will continue to advance, and the flexibility to accommodate these future developments will be of great importance. Therefore, bringing into the spotlight the need for effective risk management, responsible governance, and a robust approach to regulatory coherence.

To date, Nigeria was the first African country to develop biosafety guidelines through the National Biosafety Management Agency (NBMA 2020) to regulate genome editing products followed by Kenya. Both countries have adopted a case-by-case biosafety regulations for genome-edited products. As a result, when the genetic manipulation process requires the use of recombinant DNA sequences or the genome-edited product has a novel combination of genetic material, the product will be regulated as a GMO. But if the genetic changes do not include foreign DNA and thus introduces genetic changes that are comparable to conventional breeding outcomes, the product will be treated as a non-GMO and are therefore exempt from GMO regulations. South Africa has adopted the approach that gene-edited products should be treated as GMOs and as such to be regulated as GMOs (DALRRD Public Notice, 2021). Since the CRISPR/Cas9 technology was discovered, many African countries have been using it in the improvement of the major staple food crops (Tripathi et al., 2022). Currently, Burkina Faso, Egypt, Ethiopia, Ghana, Kenya, South Africa, and Uganda are the only African countries with active projects that involve the use of gene editing techniques (Gakpo, 2021; Karembu, 2021; Sprink et al., 2022).

Current efforts on policies and biosafety regulations development on the African continent

Over the years, there has been various suggestions on how African countries can better approach processes of product development, deployment, and commercialization of biotech products (Makinde et al., 2009; Glover et al., 2018; Akinbo et al., 2021). Most common in these suggestions, is the regulatory process by legislative means that needs to be agile, proactive towards advancing tools and mechanisms of biotechnology, and overall harmonisation of the various steps within the evaluation and decision-making processes. The development of biosafety legislation across African countries, has not seen much improvement or progress since 2016. However, the efforts of NEPAD in establishing the African Biosafety Network of Expertise (ABNE) Programme in 2009, has contributed immensely to assisting African countries to develop functional biosafety systems, followed by the implementation of the Cartagena Protocol on Biosafety. At regional level, both Economic Community of West African States (ECOWAS) and Common Market for Eastern and Southern Africa (COMESA) have made commendable efforts towards development and harmonization of biosafety regulations for their members (Akinbo et al., 2021). The envisaged action plans on biotechnology and biosafety are mainly geared towards increased investment and promoting economic trade opportunities in the region. The AUDA-NEPAD (African Union Development Agency–New Partnership for Africa Development), transformed in July 2018, has also initiated the establishment of the Integrated Vector Management (IVM) Programme to strengthen or build regulatory capacities to enable scientists to explore genetic engineering for potential novel vector control tools on the continent (Savadogo, 2022). According to NEPAD, one of the key IVM Programme objectives includes bringing together biosafety regulators and health-related regulators to ensure safe development and potential deployment of Genetically Based Vector Control innovative tools.

Proposed coordinated approach for regions and the continent

The delay in the acceptance of GM crops in the African continent indicate that the introduction of similar or more advanced technologies, their envisaged benefits, their safety reservations/challenges and the associated safety guidelines should be addressed in a more transparent and coordinated manner to avoid a similar reaction towards NBT crops, that have already been adopted in some parts of the global north. So, policymakers should be given science-based information that would enable decision making in terms of biosafety, based on each country's sovereign policies aiming at achieving the safe approval of GM crops and NBT/genome edited crops in the region, that would be environmentally and human safe and enable them to benefit from the advances in biotechnology (Akinbo et al., 2021). In the sections below, we identify areas where regions and the continent can work together, in a well-coordinated manner through a consultative approach towards advancing their biosafety regulations and biotechnology regulatory frameworks and policies.

Identifying common needs and addressing them through dedicated networks

Across the four recognised African regions, the challenges and needs in terms of the economic advancement, addressing poverty, hunger, health and education are the same if not similar. The needs are in line with the African Union's goals and priorities of Agenda 2063, whereby goal 3, 5 and 7, are specific to healthy and well-nourished citizens, modern agriculture for increased productivity and production, as well as environmentally sustainable and climate resilient economies and communities, respectfully (African Union Agenda, 2063, 2015). Furthermore, the Agenda 2063 links the various goals to the various Sustainable Development Goals (SDGs), an indication that the continent is geared towards realising a better and more sustainable future for all.

In this article, we have already demonstrated how biotechnology can help improve some of the current conditions for the African continent in the agriculture sector. Already, these regions address some of the political and economic challenges and conflicts they face through their joint regional committees, and the same should be done when it comes to other areas that are not necessarily political. Already, the AU-NEPAD Africa's Science and Technology Consolidated Plan of Action (CPA) was adopted in 2005, reaffirming the continent's collective action for using technological innovations (Makinde et al., 2009). The CPA work has been coordinated through the different centres, namely, 1) North African Biosciences Network (NABNet); 2) West African Biosciences Network (WABNet); 3) Southern African Network for Biosciences (SANBio) and 4) Biosciences eastern and central Africa Network (BecNet). Each of these centres (nodes) has its own focus area of work depending on the region's needs aligned with various technological development and advancements. However, not much is known about these networks and what work they do or what their annual targets are in terms of their plans, focus work area and scope. Therefore, the goals of these networks need to be well communicated and coordinated across the regions so that those willing to get involved know how to do so. Also, there needs to be strong partnerships with various stakeholders and multidisciplinary teams to ensure efficiency and that all projects are implemented in a coherent manner.

Being proactive through a horizon scanning initiative

Horizon scanning has been an effective tool to help adequately prepare for any future activities or for the anticipation of new challenges. If performed consistently, it can assist towards identifying the areas of needs, gaps, and there could be plans formulated towards addressing any of these. Also, horizon scanning is an effective tool for bringing different skills set and knowledge (expertise) in different subject areas together, to not only unpack common challenges, but to also find viable and sustainable solutions. Within the regions, initiatives such as the African Scientists Directory, administered by the Academy of Science of South Africa (Mark, 2020), can be used to bring different experts across the fields of biosafety and biotechnology together to work through any challenges or to plan ahead for Africa's needs and challenges. Through such initiatives, capacity building can also be fast tracked by encouraging knowledge sharing and exchange of programs with the various institutions of higher education. However, it is important that participation in all of these forums and initiatives include all countries to make sure that no one is left behind.

Addressing concerns on risks in the adoption and use of biotechnology

As already indicated, the African continent like many countries in the world is still grappling with the major areas of concern around the adoption and use of biotechnology. The major areas of concern remain, but not limited to the unintended harmful effects, environmental and food safety as well as ethical consideration. The social attitudes (and cultural aspects) also play a big role as they contribute to the public trust in the various processes governing the regulation and approval of GMOs on the continent. As a result, there remains strong doubts and to some degree prevalent acts of rebellion on any new form of biotechnology. Several studies have shown how the public is less aware and/or educated on the use and application of the technology across the continent (Zerbe, 2008; Clark et al., 2014; Gastrow et al., 2018). In some instances, it is also the general lack of understanding when it comes to the nature of genetic modification, its related techniques, and subsequent products (Marris, 2001; Aerni, 2013). It is also of note that even when such educational initiatives are put in place, there remains a greater degree of no interest, lack of participation or outright ignorance (Ahteensuu. 2012). Therefore, it remains an individual's choice on how to receive and use the information at their disposal in the communication and debates related to the technology.

Other contributing factors relates to how the lack of transparency from governments is perceived by the public also

contributes towards the erosion of trust on the newly deployed technologies. For example, the recent decision by the Kenyan government to lift a 10-year ban on GMOs brought about intense public opinion and debates (Oloo, 2022; The East African, 2022). Furthermore, it sparked *fears* that the country will be exposed to the control of seeds by multinational corporations, while biodiversity will continue to be at risk from GM crop cultivation. Also, the regulatory capacity was brought into question, with most activist groups and Non-Governmental Organizations (NGOs) believing that the country lacks the right approach to make the *correct decisions* on GMOs (Langat, 2022). Here, we witness once again the lack in proactiveness by regulatory authorities to take the public into their confidence in the decision taken on GMOs and addressing concerns on perceived risks. At the same time, we must acknowledge that it can also be difficult or close to impossible to try and convince the public to accept the decision on GMOs. However, it comes back to education and awareness, and the efforts to communicate transparently and in time, while allowing for a public participation process to take place. When such matters are debated vigorously in one country, it is bound to trickle to neighbouring countries and the region, making it difficult to manage any new ventures with the fear of the same (similar) setbacks. It is therefore important that the education and awareness on perceived risks associated with biotechnology be driven at regional level, with the help of experts in the field and the networks already established in the regions to deal with research and development of biotechnology.

The need to prioritize

The African continent faces many challenges, yet the resources required to address many of the challenges are never adequate, especially in those countries that need them the most. This has over the years contributed to the growing gap between country advancements in many areas. While some countries continue to do well in the markets and other elements of trade and development, other countries continue to lag behind. Although the urgency to address certain challenges will vary from country to country, there are those that are common within the agriculture, environment and health sectors that affect countries similarly if not equally. Also, the impacts thereafter often means that countries end up assisting each other or relying on one another for certain services and/or aid. Therefore, through the use of tools such as the horizon scanning process, countries and regions can begin to narrow down on what needs to be done or achieved first, followed by a phased in plan and strategies of common interest and how to achieve them. The knowledge and expertise through the expert's consultation would be critical for identifying the skills sets and resources needed to achieve the identified goals or priority areas. Central to this process, would be to identify the lead institutions or networks–per region, to champion the process. Here, various oversight, monitoring and reporting mechanisms would need to be in place for all reporting purposes and to account for any activities within the programs.

Formulating a guided process on "process versus product" regulatory approach

The emerging and advancing biotechnology tools and methods have led to the regulatory authorities having to rethink the long-adopted approach of process-based regulations, previously developed for the GMO technology. In recent times, countries such as Argentina, Australia, Brazil, China, Japan, the United States, Nigeria, have taken the product-based approach (Lloyd et al., 2022). In both instances, the case-by-case basis evaluation in line with the CBD guidelines remains applicable. The debate is still out there in terms of the pros' versus cons' on the two regulatory approaches, but with the view that when it comes to CRISPR/Cas9-mediated (based) genome editing, there needs to be less regulatory burden as this hampers innovation; and this technology *only modifies* existing genetic material of the desired plant/animal (Lassoued et al., 2021). Therefore, the argument is that the same or similar regulations for GMOs, *should not* be subjected to genome edited products. For majority of the African countries (if not all), these new technologies are tried and licensed to foreign multinational companies and countries also remain importers of the "final product(s)", derived through the new technologies.

As indicated, only seven (7) countries on the continent currently make use of the gene editing technology in various areas of research and development (Gakpo, 2021; Karembu, 2021; Sprink et al., 2022). Therefore, countries might remain net importers of GE derived products, making it difficult for them to apply the process-based risk analysis and regulations. Also, with the reality of the situation of porous borders between countries on the African continent where there is movement of people (including farmers), legally or illegally, may result in the exchange of seeds and food products where they are not approved or regulated formally. On the African continent, communities and small holder farmers have relied on informal seed systems for decades (Almekinders et al., 1994; Jones et al., 2001). This has served as a reliable and most important seed source of traditional food crops (Hlatshwayo et al., 2021). Furthermore, seed exchanges are central to the some of the traditional norms, are central to food sovereignty and strengthen social as well as cultural value systems among communities (van Niekerk & Wynberg, 2017). In addition, informal seed exchanges are not always restricted to or between farmers, as the practice can extend across villages or different regions (Pratap & Gupta, 2020).

Although the exchange of GM seeds or those developed using the technology is not established on the continent, it has been recorded that farmers do save GM derived seeds in South Africa (Masehela & Gouse, 2021). This makes it critical for countries to develop, finalise and implement their regulatory frameworks, and the process versus product regulatory approach will no doubt be central to deliberations involving the adoption and use of new technologies. As a result, countries and regions will need to engage in a more joint and coordinated manner to formulate their respective approaches in this regard, knowing very well that the option not to regulate, does not mean you will not have to deal with the product being present in the country.

The political will, commitment, and action

While the field of biotechnology suffers from its own politics, the politics of governance–per country also needs to be decisive and favourable for research and development to thrive. It has been shown that government policies and positive political commitment to the biotechnology industry can have influence on how various investments are channelled for funding (Zarrilli, 2007). Africa also suffers from the formulation of many frameworks, action plans and the establishment of "working groups or committees". Often, these groups come up with great regional approach and policy documents, which are signed off and endorsed by countries and regions, but hardly get implemented or reviewed for the effectiveness in terms of implementation. In some instances, no feedback is ever shared or given in terms of any progress or achievements. As a result, this adds to the frustrations in every attempt to fully implement biosafety regimes across the continent. Furthermore, managing public expectations becomes difficult as the overall public confidence and acceptance of biotechnology is pinned against the much-desired transparency and political goodwill.

Currently, there is a strong regional approach towards issues of trade (import/export) across the continent through the Inter Africa Trade discussions and policy developments, under the African Continental Free Trade Area (AfCFTA). These discussions also cover, to a large extent, country specific and regional orientated needs, challenges, and priorities. It is at this level that the biotechnology developments and advancements also need to take place, if they are to be taken seriously through any political agenda of the continent. Ultimately, harmonizing regulations and standards for biotechnology products, facilitating trade and economies is necessary for the advancement and adoption of new technologies in Africa.

Concluding remarks

We are not the first authors to identify challenges in the acceptance and adoption of GMOs in the African continent. Also, pointing out that this currently impacts on how the new and emerging technologies are being view in the public domain. While the development and implementation of various biosafety regulations and policies remain a challenge for many African countries, a few have made good strides and have also started utilizing new technologies such as genome editing. This is because they realise the potential to harness the products that will benefit the countries towards addressing several challenges relating to, among others, economic growth and trade, the impacts associated with climate change, hunger and nutrition, crop diseases and pests, as well as health and pharmaceutical needs. All these developments cannot be successful if there is limited involvement of African scientists, regulators and policymakers in the development and harmonization of regulations and policies that favours the adoption and use of new and emerging technologies. It is for these reasons that we put forward a few consultative and collaborative based approaches that the countries, regions and continent must consider if they are to fully give the technology and its various developmental stages a chance on the African continent. Central to this proposal is the political will, commitment, and action. Ultimately, the scientists, regulators and policymakers need to come together and openly discuss how they view the impact of these technologies, address any reservations that potentially may cause delays in the implementation of regulatory frameworks and policies.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

TM and EB were both responsible for the conception and design of the article. TM wrote the outline approach to the article, with inputs and guidance from EB. Both TM and EB had dedicated sections to write for the manuscript. TM carried out the final revisions and edits, while EB carried out the final proof reading for the submitted version. All authors contributed to the article and approved the submitted version.

Conflict of interest

Author EB is the director of EB Biosciences and Consulting (Pty) Ltd. EB is a consultant in the field of biotechnology and in the risk assessment space, and serves in the Advisory Committee (AC) for Genetically Modified Organisms in the Department of Agriculture, Land Reform and Rural Development (DALRRD). EB has not produced any commercial products or patents.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Abdallah, N. A., Moses, V., and Prakash, C. S. (2014). The impact of possible climate changes on developing countries: The needs for plants tolerant to abiotic stresses. *GM crops food* 5 (2), 77–80. doi:10.4161/gmcr.32208

Aerni, P. (2013). Resistance to agricultural biotechnology: The importance of distinguishing between weak and strong public attitudes. *Biotechnol. J.* 8 (10), 1129–1132. doi:10.1002/biot.201300188

African Union Agenda 2063 (2015). *The Africa we want*. African Union Commission. 978-92-95104-23-5.

Ahteensuu, M. (2012). Assumptions of the deficit model type of thinking: Ignorance, attitudes, and science communication in the debate on genetic engineering in agriculture. *J. Agric. Environ. ethics* 25, 295–313. doi:10.1007/s10806-011-9311-9

Akinbo, O., Obukosia, S., Ouedraogo, J., Sinebo, W., Savadogo, M., Timpo, S., et al. (2021). Commercial release of genetically modified crops in Africa: Interface between biosafety regulatory systems and varietal release systems. *Front. Plant Sci.* 12, 605937. doi:10.3389/fpls.2021.605937

Almekinders, C. J., Louwaars, N. P., and De Bruijn, G. H. (1994). Local seed systems and their importance for an improved seed supply in developing countries. *Euphytica* 78, 207–216. doi:10.1007/bf00027519

Arora, L., and Narula, A. (2017). Gene editing and crop improvement using CRISPR-Cas9 system. *Front. Plant Sci.* 8, 1932. doi:10.3389/fpls.2017.01932

Barragán-Ocaña, A., Silva-Borjas, P., Olmos-Peña, S., and Polanco-Olguín, M. (2020). Biotechnology and bioprocesses: Their contribution to sustainability. *Processes* 8 (4), 436. doi:10.3390/pr8040436

Bauer, M. W. (2002). Controversial medical and agri-food biotechnology: A cultivation analysis. *Public Underst. Sci.* 11 (2), 93–111. doi:10.1088/0963-6625/11/2/301

Bediako, Y. (2022). African biotech holds the key to transforming not just the health of African people, but our economies as well. Online at: https://speakingofmedicine.plos.org/2022/11/29/african-biotech-holds-the-key-to-transforming-not-just-the-health-of-african-people-but-our-economies-as-well/.

Binswanger-Mkhize, H. P., McCalla, A. F., and Patel, P. (2010). Structural transformation and African agriculture. *Glob. J. Emerg. Mark. Econ.* 2 (2), 113–152. doi:10.1177/097491011000200203

Cantley, M. (2007). *An overview of regulatory tools and frameworks for modern biotechnology: A focus on agro-food*.

Carr, S., and Levidow, L. (2000). Exploring the links between science, risk, uncertainty, and ethics in regulatory controversies about genetically modified crops. *J. Agric. Environ. ethics* 12, 29–39. doi:10.1023/a:1009595924500

Ceasar, S. A., Rajan, V., Prykhozhij, S. V., Berman, J. N., and Ignacimuthu, S. (2016). Insert, remove or replace: A highly advanced genome editing system using CRISPR/cas9. *Biochimica Biophysica Acta (BBA)-Molecular Cell Res.* 1863 (9), 2333–2344. doi:10.1016/j.bbamcr.2016.06.009

Clark, N., Mugabe, J., and Smith, J. (2014). *Governing agricultural biotechnology in Africa building public confidence and capacity for policy-making.*

DALRRD Public Notice (2021). Notice SA's regulatory approach for NBT's 2021. Online at: http://www.old.dalrrd.gov.za/Branches/Agricultural-Production-Health-Food-Safety/Genetic-Resources/Biosafety/Notifications.

de Cheveigné, S., Daniel, B., and Galloux, J. C. (2002). *Les biotechnologies en débat: Pour une démocratie scientifique*. Balland, 253.

de Graeff, N., Jongsma, K. R., Johnston, J., Hartley, S., and Bredenoord, A. L. (2019). The ethics of genome editing in non-human animals: A systematic review of reasons reported in the academic literature. *Philosophical Trans. R. Soc. B* 374 (1772), 20180106. doi:10.1098/rstb.2018.0106

Deane-Drummond, C., Grove-White, R., and Szerszynski, B. (2001). Genetically modified theology: The religious dimensions of public concerns about agricultural biotechnology. *Stud. Christian Ethics* 14 (2), 23–41. doi:10.1177/095394680101400203

Egwang, T. G. (2001). Biotechnology issues in Africa. *Electron. J. Biotechnol.* 4 (3), 23–24. doi:10.2225/vol4-issue3-fulltext-12

Falkner, R., and Gupta, A. (2004). *Implementing the biosafety protocol: Key challenges*. Royal Institute of International Affairs.

Gakpo, J. O. (2021). Ghana scientist turns to gene editing to improve sweet potato crop. Online at: https://allianceforscience.org/blog/2021/02/ghana-scientist-turns-to-gene-editing-to-improve-sweet-potato-crop/.

Gastrow, M., Roberts, B., Reddy, V., and Ismail, S. (2018). Public perceptions of biotechnology in South Africa. *South Afr. J. Sci.* 114 (1-2), 9. doi:10.17159/sajs.2018/20170276

Gbadegesin, L. A., Ayeni, E. A., Tettey, C. K., Uyanga, V. A., Aluko, O. O., Ahiakpa, J. K., et al. (2022). *GMOs in Africa: Status, adoption and public acceptance*. Food Control.109193.

Giller, K. E., Delaune, T., Silva, J. V., Descheemaeker, K., van de Ven, G., Schut, A. G., et al. (2021). The future of farming: Who will produce our food? *Food Secur.* 13 (5), 1073–1099. doi:10.1007/s12571-021-01184-6

Glass, J. A. (2000). The merits of ratifying and implementing the Cartagena Protocol on Biosafety. Nw. *J. Int'l L. Bus.* 21, 491.

Glover, B., Akinbo, O., Savadogo, M., Timpo, S., Lemgo, G., Sinebo, W., et al. (2018)., 12. BioMed Central, 11–28. doi:10.1186/s12919-018-0108-yStrengthening regulatory capacity for gene drives in Africa: Leveraging NEPAD's experience in establishing regulatory systems for medicines and GM crops in Africa*BMC Proc.*, No. 8

Goyal, P., and Gurtoo, S. (2011). Factors influencing public perception: Genetically modified organisms. *GMO Biosaf. Res.* 2. doi:10.5376/gmo.2011.02.0001

Groenewald, H. (2021). "Chapter 5: The future: Induced genetic variation technologies, GMOs and responsible governance," in *Masehela. et al. 2021, an initial assessment of impacts on biodiversity from GMOs released into the environment in South Africa* (Pretoria: South African National Biodiversity Institute, an entity of the Department of Forestry, Fisheries and the Environment), 84–91.

Helliwell, R., Hartley, S., Pearce, W., and O'Neill, L. (2017). Why are NGO s sceptical of genome editing? NGOs' opposition to agricultural biotechnologies is rooted in scepticism about the framing of problems and solutions, rather than just emotion and dogma. *EMBO Rep.* 18 (12), 2090–2093. doi:10.15252/embr.201744385

Hielscher, S., Pies, I., Valentinov, V., and Chatalova, L. (2016). Rationalizing the GMO debate: The ordonomic approach to addressing agricultural myths. *Int. J. Environ. Res. public health* 13 (5), 476. doi:10.3390/ijerph13050476

Hlatshwayo, S. I., Modi, A. T., Hlahla, S., Ngidi, M., and Mabhaudhi, T. (2021). Usefulness of seed systems for reviving smallholder agriculture: A South African perspective. *Afr. J. Food, Agric. Nutr. Dev.* 21 (2), 17581–17603. doi:10.18697/ajfand.97.19480

Hokanson, K. E. (2019). When policy meets practice: The dilemma for guidance on risk assessment under the Cartagena protocol on biosafety. *Front. Bioeng. Biotechnol.* 7, 82. doi:10.3389/fbioe.2019.00082

Jones, R. B., Audi, P. A., and Tripp, R. (2001). The role of informal seed systems in disseminating modern varieties. The example of pigeonpea from a semi-arid area of Kenya. *Exp. Agric.* 37 (4), 539–548. doi:10.1017/s0014479701000461

Juma, C. (2015). *The new harvest: Agricultural innovation in Africa*. Oxford University Press.

Kameri-Mbote, P. (2002). The development of biosafety regulation in Africa in the context of the Cartagena protocol: Legal and administrative issues. *Rev. Eur. Comp. Int'l Envtl. L.* 11, 62–73. doi:10.1111/1467-9388.00303

Kamthan, A., Chaudhuri, A., Kamthan, M., and Datta, A. (2016). Genetically modified (GM) crops: Milestones and new advances in crop improvement. *Theor. Appl. Genet.* 129, 1639–1655. doi:10.1007/s00122-016-2747-6

Karembu, M. (2021). *Genome editing in Africa's agriculture 2021: An early take-off*. Nairobi, Kenya: International Service for the Acquisition of Agri-biotech Applications (ISAAA AfriCenter.

Kedir, A. M., and Kararach, G. (2019). "Rethinking agricultural transformation and food security in Africa," in *Hunger and malnutrition as major challenges of the 21st century*, 373–410.

Khan, F. A. (2014). *Biotechnology in medical sciences*. CRC Press.

Klümper, W., and Qaim, M. (2014). A meta-analysis of the impacts of genetically modified crops. *PloS one* 9 (11), e111629. doi:10.1371/journal.pone.0111629

Komen, J., Tripathi, L., Mkoko, B., Ofosu, D. O., Oloka, H., and Wangari, D. (2020). Biosafety regulatory reviews and leeway to operate: Case studies from sub-sahara Africa. *Front. Plant Sci.* 11, 130. doi:10.3389/fpls.2020.00130

Langat, A. (2022). Kenya lifts ban on genetically modified foods despite strong opposition. Online at: https://www.devex.com/news/kenya-lifts-ban-on-genetically-modified-foods-despite-strong-opposition-104170#:~:text=Kenya%20lifts%20ban%20on%20genetically%20modified%20foods%20despite%20strong%20opposition,-By%20Anthony%20Langat&text=Genetically%20modified%20foods%20have%20been,country%20facing%20runaway%20food%20shortages.

Lassoued, R., Phillips, P. W., Macall, D. M., Hesseln, H., and Smyth, S. J. (2021). Expert opinions on the regulation of plant genome editing. *Plant Biotechnol. J.* 19 (6), 1104–1109. doi:10.1111/pbi.13597

Lloyd, J. R., Beger, D., and Pillay, P. (2022). South Africa should rethink regulations on genetically modified plants. Online at: https://theconversation.com/south-africa-should-rethink-regulations-on-genetically-modified-plants-176254.

Lucht, J. M. (2015). Public acceptance of plant biotechnology and GM crops. *Viruses* 7 (8), 4254–4281. doi:10.3390/v7082819

Ma, Q. P. (2021). *Technological breakthroughs and future business opportunities in education, health, and outer space*, 112–132.Biotechnology: Recent developments, emerging trends, and implications for business

Machuka, J. (2001). Agricultural biotechnology for Africa. African scientists and farmers must feed their own people. *Plant physiol.* 126 (1), 16–19. doi:10.1104/pp.126.1.16

Makinde, D., Mumba, L., and Ambali, A. (2009). Status of biotechnology in Africa: Challenges and opportunities. *Asian Biotechnol. Dev. Rev.* 11 (3), 1–10.

Mark, P. (2020). African Scientists Directory fosters collaboration, counters populism. Online at: https://www.universityworldnews.com/post.php?story=20201027033045494.

Marris, C. (2001). Public views on GMOs: Deconstructing the myths. *EMBO Rep.* 2 (7), 545–548. doi:10.1093/embo-reports/kve142

Masehela, T. S., and Gouse, M. (2021). "Chapter 3: Status and trends of GMO trials and general releases in South Africa," in *An initial assessment of impacts on biodiversity from GMOs released into the environment in South Africa*. (Pretoria: South African National Biodiversity Institute, an entity of the Department of Forestry, Fisheries and the Environment), 35–53.

Masehela, T. S., Rhodes, J. I., Groenewald, H., Poole, C. J., Van den Berg, J., Gouse, M., et al. (2021). *An initial assessment of impacts on biodiversity from GMOs released into the environment in South Africa*. Pretoria: South African National Biodiversity Institute, an entity of the Department of Forestry, Fisheries and the Environment.

Masson, P., Tonello, C., and Balny, C. (2001). High-pressure biotechnology in medicine and pharmaceutical science. *J. Biomed. Biotechnol.* 1 (2), 85–88. doi:10.1155/s1110724301000158

McLean, M., Foley, M. E., and Pehu, E. (2012). *The status and impact of bio safety regulation in developing economies since ratification of the Cartagena protocol*.

Mfutso-Bengo, J. M., and Muula, A. S. (2007). Potential benefits and harm of biotechnology in developing countries: The ethics and social dimensions. *Afr. J. Med. Med. Sci.* 36, 63–67.

Montecillo, J. A. V., Chu, L. L., and Bae, H. (2020). CRISPR-Cas9 system for plant genome editing: Current approaches and emerging developments. *Agronomy* 10 (7), 1033. doi:10.3390/agronomy10071033

Nitin, K. S., Masehela, T. S., Chakravarthy, A. K., and Geerts, S. (2022). "Management of pests using genetic tools in Africa," in *Genetic methods and tools for managing crop pests* (Singapore: Springer Nature Singapore), 303–326.

Oloo, B. O. (2022). Kenya has lifted its ban on genetically modified crops: The risks and opportunities. Online at: https://theconversation.com/kenya-has-lifted-its-ban-on-genetically-modified-crops-the-risks-and-opportunities-192636.

Paarlberg, R. (2009). *Starved for science: How biotechnology is being kept out of Africa*. Harvard University Press.

Pertry, I., Sabbadini, S., Goormachtig, S., Lokko, Y., Gheysen, G., Burssens, S., et al. (2014). Biosafety capacity building: Experiences and challenges from a distance learning approach. *New Biotechnol.* 31 (1), 64–68. doi:10.1016/j.nbt.2013.08.008

Pham, P. V. (2018). "Medical biotechnology: Techniques and applications," in *Omics technologies and bio-engineering* (Academic Press), 449–469.

Phillips, T. (2008). Genetically modified organisms (GMOs): Transgenic crops and recombinant DNA technology. *Nat. Educ.* 1 (1), 213.

Pinstrup-Andersen, P., and Watson, D. D., II (2011). *Food policy for developing countries: The role of government in global, national, and local food systems*. Cornell University Press.

A. Pratap and S. Gupta (Editors) (2020). *The beans and the peas: From orphan to mainstream crops* (Woodhead Publishing).

Rao, M. J., and Wang, L. (2021). CRISPR/Cas9 technology for improving agronomic traits and future prospective in agriculture. *Planta* 254, 68–16. doi:10.1007/s00425-021-03716-y

Sammut, S. M. (2021). The role of the biotechnology industry in addressing health inequities in Africa: Strengthening the entire health care value chain. *J. Commer. Biotechnol.* 26 (4). doi:10.5912/jcb1008

Savadogo, M. (2022). *Capacity strengthening for risk assessment of new biotechnologies in Africa – building on experiences with genetic biocontrol*. Role of AUDA NEPAD. Side Event 4467, COP15, Montreal, Canada, December 9, 2022.

A. Shibata (Editor) (2014). *International liability regime for biodiversity damage: The nagoya-kuala lumpur supplementary protocol* (Routledge).

Smyth, S. J. (2020). The human health benefits from GM crops. *Plant Biotechnol. J.* 18 (4), 887–888. doi:10.1111/pbi.13261

Sprink, T., Wilhelm, R., and Hartung, F. (2022). Genome editing around the globe: An update on policies and perceptions. *Plant Physiol.* 190 (3), 1579–1587. doi:10.1093/plphys/kiac359

Stone, G. D., and Glover, D. (2017). Disembedding grain: Golden rice, the green revolution, and heirloom seeds in the Philippines. *Agric. Hum. Values* 34 (1), 87–102. doi:10.1007/s10460-016-9696-1

Stone, G. D., and Glover, D. (2011). Genetically modified crops and the 'food crisis': Discourse and material impacts. *Dev. Pract.* 21 (4-5), 509–516. doi:10.1080/09614524.2011.562876

The East African (2022). East Africa divided on GM foods as Kenya lifts ban. Online at: http://www.channelafrica.co.za/sabc/home/channelafrica/news/details?id=fb608914-7958-49d7-b6be-e5f9b8aac601&title=East%20Africa%20divided%20on%20GM%20foods%20as%20Kenya%20lifts%20ban.

Tran, L. S. P., Nishiyama, R., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2010). Potential utilization of NAC transcription factors to enhance abiotic stress tolerance in plants by biotechnological approach. *Gm. crops* 1 (1), 32–39. doi:10.4161/gmcr.1.1.10569

Tripathi, L., Dhugga, K. S., Ntui, V. O., Runo, S., Syombua, E. D., Muiruri, S., et al. (2022). Genome editing for sustainable agriculture in Africa. *Front. Genome Ed.* 4, 876697. doi:10.3389/fgeed.2022.876697

Trump, B., Cummings, C., Klasa, K., Galaitsi, S., and Linkov, I. (2022). Governing biotechnology to provide safety and security and address ethical, legal, and social implications. *Front. Genet.* 13, 1052371. doi:10.3389/fgene.2022.1052371

Turnbull, C., Lillemo, M., and Hvoslef-Eide, T. A. (2021). Global regulation of genetically modified crops amid the gene edited crop boom–a review. *Front. Plant Sci.* 12, 630396. doi:10.3389/fpls.2021.630396

van Niekerk, J., and Wynberg, R. (2017). Traditional seed and exchange systems cement social relations and provide a safety net: A case study from KwaZulu-natal, South Africa. *Agroecol. Sustain. Food Syst.* 41 (9-10), 1–25. doi:10.1080/21683565.2017.1359738

Wambugu, F. (1999). Why Africa needs agricultural biotech. *Nature* 400 (6739), 15–16. doi:10.1038/21771

Wickson, F., Preston, C., Binimelis, R., Herrero, A., Hartley, S., Wynberg, R., et al. (2017). Addressing socio-economic and ethical considerations in biotechnology governance: The potential of a new politics of care. *Food ethics* 1, 193–199. doi:10.1007/s41055-017-0014-4

Zarrilli, S. (2007). *Global perspective on production of biotechnology-based bioenergy and major trends"*. Rome: FAO Biotechnology and Bioenergy Seminars, FAO.

Zerbe, N. (2008). Sowing the seeds of progress: The agricultural biotechnology debate in Africa. *Hist. Compass* 6 (2), 404–425. doi:10.1111/j.1478-0542.2008.00512.x

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Review
PUBLISHED 08 June 2023
DOI 10.3389/fbioe.2023.1205865

OPEN ACCESS

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RECEIVED 14 April 2023
ACCEPTED 25 May 2023
PUBLISHED 08 June 2023

CITATION
James SL, Quemada H, Benedict MQ and Dass B (2023), Requirements for market entry of gene drive-modified mosquitoes for control of vector-borne diseases: analogies to other biologic and biotechnology products.
Front. Bioeng. Biotechnol. 11:1205865.
doi: 10.3389/fbioe.2023.1205865

Requirements for market entry of gene drive-modified mosquitoes for control of vector-borne diseases: analogies to other biologic and biotechnology products

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Gene drive-modified mosquitoes (GDMMs) are proposed as new tools for control and elimination of malaria and other mosquito-borne diseases, and promising results have been observed from testing conducted in containment. Although still at an early stage of development, it is important to begin now to consider approval procedures and market entry strategies for the eventual implementation of GDMMs in the context of disease control programs, as these could impact future research plans. It is expected that, as for other types of new products, those seeking to bring GDMMs to market will be required to provide sufficient information to allow the regulator(s) to determine whether the product is safe and effective for its proposed use. There already has been much emphasis on developing requirements for the biosafety components of the "safe and effective" benchmark, largely concerned with their regulation as genetically modified organisms. Other potential approval requirements have received little attention, however. Although GDMMs are expected to be implemented primarily in the context of public health programs, any regulatory analogies to other public health products, such as pharmaceuticals, vaccines, or chemical pesticides, must take into account the characteristics of live mosquito products. Typical manufacturing standards related to product identity, potency or quality will need to be adapted to GDMMs. Valuable lessons can be drawn from the regulatory approval processes for other whole organism and genetically modified (GM) organism products. Supply chain requirements, such as scale of production, location and design of production facilities, and methods of distribution and delivery, will be dependent upon the characteristics of the particular GDMM product, the conditions of use, and the region to be served. Plans for fulfilling supply chain needs can build upon experience in the development of other live insect products for use in public health and agriculture. Implementation of GDMMs would benefit from additional research on enabling technologies for long-term storage of mosquito life stages, efficient mass production, and area-wide delivery of GDMMs. Early consideration of these practical requirements for market entry will help to mitigate downstream delays in the development of these promising new technologies.

KEYWORDS

malaria, mosquito, genetic modification, gene drive, market entry, regulatory approval, supply chain, manufacturing standards

Introduction

The World Health Organization (WHO) estimates that over 700,000 deaths occur annually from parasitic, bacterial, and viral diseases transmitted by insect or other invertebrate vectors (World Health Organization, 2023a). Malaria alone, a parasitic disease transmitted by anopheline mosquitoes, was reported to cause some 619,000 deaths worldwide in 2021, approximately 96% of which occurred in Africa (World Health Organization, 2022a). There have been multiple calls to improve methods for malaria treatment and prevention (e.g., Rabinovich et al., 2017; Feachem et al., 2019; World Health Organization, 2019). These include calls for innovative vector control tools.

Gene drive-modified mosquitoes (GDMMs) have been recognized as potentially transformative new tools for control and elimination of malaria and other mosquito-borne diseases (World Health Organization, 2020a). Although other species also transmit malaria in Africa, gene drive research currently is most advanced in mosquitoes of the *Anopheles gambiae* species complex, which historically have been important malaria vectors in that region (Sinka, 2012; Global Health Network, 2023).

WHO guidance on the research and development pathway for GDMMs (World Health Organization, 2021) calls for testing initially to be conducted under physical confinement, as in insectaries or large cages. The WHO guidance recommends that during early confined testing measurable efficacy and biosafety surrogate indicators should be identified that can be expected to correlate with the ability of the GDMM product to accomplish the intended use in the field (the product claim). Reports of success already are coming from such contained testing, with results based on defined endpoints supporting the investigational claim (Kyrou et al., 2018; Pham et al., 2019; Carballar-Lejarazu et al., 2020; Hammond et al., 2021; Ellis et al., 2022). The identified efficacy and safety correlates, as well as the intended use, will be captured in a product-specific Target Product Profile, and will inform the proposed testing endpoints in regulatory applications submitted by the developer and evaluated by national regulators as the basis for advancing through open field releases of increasing size and scope. For products making a disease reduction claim, field releases eventually would encompass a large enough area to allow for assessment of any resulting reduction in malaria transmission (World Health Organization, 2021). Efficacy and safety data and information from appropriately designed field trials will provide the scientific evidence supporting the product claim and intended use as a vector control and/or malaria control tool in an application for approval for market entry, which will be evaluated by the appropriate regulatory authorities. GDMM products making a vector control claim could be regulated differently from those making a public health claim (James et al., 2023). Market entry is defined here to mean that the product has undergone regulatory approval and is being made available to end users, whether in return for payment or free of charge. No form of GDMMs has yet progressed to field testing. Nevertheless, in order to plan for success it is not too early to begin considering the needs for bringing GDMMs to market as public health tools.

Implementation considerations

The GDMM product previously has been defined as any life stage of the transgenic mosquitoes that is produced under controlled conditions for deliberate release (James et al., 2018; James et al., 2020; James et al., 2023). If approved for market entry, those responsible for national or regional disease control priorities will decide whether the GDMM product should move into wider and more systematic releases as part of a national or regional malaria control program. The implementation phase is the post-investigational use of GDMMs, following satisfactory demonstration of safety, efficacy and acceptability in field trials and a decision to initiate widescale releases (World Health Organization, 2021). This phase can be considered analogous to commercialization of more familiar biotechnology and public health products such as drugs, vaccines, insecticides, and crops. Commercialization is broadly defined as a process for bringing new products or services to market (as defined above). The scope of the commercialization process generally includes regulatory approval (including fulfillment of agreed-upon post-approval requirements), production, distribution, marketing, and other key functions that will be as critical for success of a GDMM product as they are for other products. Yet these practical aspects of operationalizing GDMMS have received little attention to date.

Rearing of GDMMs shares many characteristics with manufacturing of other types of public health products. Implementation of GDMMs will require provision of a consistent product at the necessary scale, as well as its delivery in a way that is designed to reliably achieve the claimed vector control or other public health effect. Plans for achieving area-wide protection by GDMMs are likely to be more context specific than is the case for medical or public health products aimed at individual or household use. Thus, specifics of a release plan, including the location of release sites, how many GDMMs will be released at each site, and how often these releases will occur at each site, may differ for each product in a particular setting. This will depend upon factors such as the type of gene drive system and heritability of the transgenic construct (Box 1), the population size of the targeted mosquito species at the site, and biological traits of the released male GDMMs such as mating competitiveness with respect to wild males (e.g., North et al., 2019; Kaiser et al., 2021). In general, self-sustaining gene drive products are expected to require release of lower numbers of GDMMs over a shorter period of time to yield a long-term effect. Because they are expected to require relatively larger and/or more frequent releases to sustain effectiveness in the region of interest, self-limiting GDMM approaches are likely to require greater capacity for production and delivery. Moreover, this level of production and delivery may need to be maintained long-term, since it has been found with other genetic biocontrol methods that re-invasion by the targeted species can occur rapidly following

cessation of the control measure (e.g., Meyer et al., 2016). Localizing GDMMs are likely to require more extensive releases to provide widespread coverage, which also will have production and delivery ramifications.

BOX 1 | A primer on gene drive systems

Genetically modified (GM) mosquitoes (also called genetically engineered, transgenic, or living modified mosquitoes) demonstrate traits that are introduced through use of recombinant DNA technology. Gene drive refers to a process, either naturally occurring or resulting through use of recombinant DNA technology, whereby a particular gene or genetic construct is able to enhance its own inheritance so that it becomes more prevalent in the population over successive generations (Alphey et al., 2020). Engineered gene drive systems can be used to introduce new and potentially beneficial traits rapidly into a population. Gene drive-modified mosquitoes (GDMMs) are a subset of GM mosquitoes that contain an engineered gene drive system.

Several different gene drive systems have been proposed for use in preventing transmission of mosquito-borne diseases, and others likely will be developed in the future (reviewed in World Health Organization, 2021). These systems currently aim either to reduce the size of the vector population by inhibiting their reproduction or survival (a strategy termed population suppression or reduction) or to modify the mosquitoes to make them less competent to transmit a pathogen (variously termed population replacement, modification, conversion, or alteration). Self-sustaining drives are intended to persist, passing the modification on through subsequent generations indefinitely. Because of this persistence, many self-sustaining drives are expected to spread widely within interbreeding mosquito populations. Low threshold drives are a type of self-sustaining system in which this spread can be initiated by release of relatively few modified mosquitoes. Other types of gene drive systems aim to impose either temporal (self-limiting drives) or spatial (localizing or confined drives) restrictions on the spread of the modification. Self-limiting drives will eventually disappear from the target mosquito population, and may effectively be localizing because they do not persist long enough to spread widely. Localizing systems may be either self-limiting or self-sustaining.

Certain challenges are anticipated for large scale production and delivery as required for implementation in the context of national or regional disease control programs. Basic elements of the supply chain for conventional public health products such as drugs, pesticides and vaccines, from production through distribution, are generally established through commercial manufacturers and national health authorities or private sector vendors. In these cases, supply chain experience already exists and the requirements have been thoroughly studied, even though all elements may not be in place for a new product (Brown and Bollyky, 2021) and the supply chain may be fragmentary in inadequately resourced regions (U.S. Agency for International Development, 2011; Yadav, 2015). At least initially, GDMMs may face these same hurdles, as well as additional challenges arising from lack of understanding, experience, or pre-existing infrastructure for this new product class. For example, commercial entities and national health authorities have limited experience with live mosquito products, many current GDMM developers are laboratorians without substantial product development expertise, and regulatory frameworks for GM insects have not been clarified in many countries. Thus, early planning would greatly ease the process of market entry of GDMMs and would best begin in time to allow these challenges to be sufficiently addressed. Regulatory and policy considerations for GDMMs have been detailed elsewhere (e.g., World Health Organization, 2021; James et al., 2023). Here we consider manufacturing standards, production requirements, and delivery mechanisms for GDMMs, and highlight issues that require particular attention to inform planning for commercialization and incorporation into national malaria control programs. Post-implementation monitoring procedures are not considered within the scope of this analysis.

Manufacturing standards

For public health products such as pharmaceuticals that will be administered to individuals, those seeking to bring new products to market are required to provide sufficient information to allow the regulator(s) to determine whether the product is both safe and effective for the proposed health condition (the product claim) when administered according to the specified conditions of use, and whether the manufacturing, packaging, and storage methods will be able to maintain the integrity of the product across different release lots. These requirements generally involve specifications for product identity, potency, purity, and quality (e.g., U.S. Food and Drug Administration, 1999; European Medicines Agency, 1999; Code of Federal Regulations, 2023). For conventional drugs, assessment typically focuses on analysis of chemical composition, including concentration and stability of the active ingredient and presence of any contaminating components. Registration considerations for pesticides are largely risk-based, but like medicines also require description of all chemicals in the product and proof that the manufacturing process is reliable (e.g., U.S. Environmental Protection Agency, 2023). For biological products such as cell-derived or cell-based therapies, which are manufactured via serial passage and whose active ingredients cannot be straightforwardly chemically characterized, more appropriate types of tests have been developed to confirm that the product meets the claimed identity, strength and quality characteristics (e.g., Rayment and Williams, 2010; Carman et al., 2012). This provides a good example of how manufacturing standards can be adapted to the characteristics of new types of products. GDMMs will be whole organism products. Thus, regulatory approval requirements for products that also are live organisms, including other insect products or GM plants and animals, provide particularly relevant precedents for manufacturing standards for GDMMs intended as public health products (Romeis et al., 2020). However, these requirements may not be as familiar to health regulators.

Requirements for the biosafety components of the "safe and effective" benchmark for GDMMs are actively being addressed. Considerations for safety testing of other GM organisms have been a topic of extensive discussion, both from a human and animal (food) safety as well as an environmental safety perspective (e.g., Codex Alimentarius, 2003; U.S. Food and Drug Administration, 1997; Convention on Biological Diversity, 2000; European Food Safety Authority, 2010; European Food Safety Authority, 2011; European Food Safety Authority, 2013; Organization for Economic Cooperation and Development, 2023). These discussions also have extended to GDMMs and are ongoing (e.g., Convention on Biological Diversity, 2020; European Food Safety Authority, 2020; World Health Organization, 2021). Therefore, we focus here on other standards typically addressed in

regulatory approval procedures that have not been similarly examined. These standards are concerned with product quality and consistency rather than safety *per se*, and could involve regulatory agencies other than those responsible for safety assessments (James et al., 2023).

Lessons learned from other live mosquito products that have obtained regulatory approval for large-scale deployment can be especially informative for all aspects of market entry for GDMMs. Perhaps the most well-documented precedent for standardizing production of GDMMs would be practices common to live insect products used in the Sterile Insect Technique (SIT) method for population suppression. SIT has been widely and successfully used to control a variety of agricultural pest insects and is being adapted to mosquitoes. It involves the release of male insects, which have been sterilized by exposure to ionizing radiation, in sufficient numbers to out-compete fertile wild males for mating with wild females. Mating of sterile males with wild females reduces the number of viable progeny, resulting in a substantial decline in the overall size of the targeted local pest population (Bourtzis and Vreysen, 2021). Release of male insects has been found to be most cost-efficient in SIT programs (Lutrat et al., 2019), and generally raises fewer risk concerns. There exist accepted processes and protocols for SIT, including methods for *Aedes aegypti* mosquitoes (World Health Organization, 2020b; Dyck et al., 2021; International Atomic Energy Agency, 2023). Cage and small scale field trials of SIT for *Anopheles* species also have been initiated in Africa (Helinski et al., 2008; Republic of South Africa, 2018).

Another relevant precedent is Oxitec's GM, but non-driving, OX5034 product for *Aedes aegypti* population suppression, which has been approved for commercial release in Brazil and is undergoing field testing in the United States (Government of Brazil, 2020; U.S. Environmental Protection Agency, 2020; Oxitec, 2023a). Rearing procedures for GM *Aedes aegypti* have been published (Carvalho et al., 2014). Other programs also are pursuing population suppression or population replacement strategies based on the release of live *Aedes* mosquitoes infected with *Wolbachia* bacteria (Zeng et al., 2022; Consolidated Mosquito Abatement District, 2023; MosquitoMate, 2023; National Environment Agency Singapore, 2023; World Mosquito Program, 2023).

Identity

The standard of identity commonly describes the components a product must contain as well as those it may contain. In the manufacturing of GDMMs, methods will be required for routine authentication to confirm that the product retains the essential characteristics specified for the original regulatory approval for release to market (Benedict et al., 2018a). However, any identity standard for GDMMs must take into account inherent requirements of mosquito strain maintenance.

The practice of maintaining a well-characterized and protected master cell bank or seed stock is widely utilized in the manufacture of biological products to assure an ongoing supply of the originally characterized material, and cryopreservation is the method usually used for long-term storage (Hay, 1988; European Medicines Agency, 1998; U.S. Food and Drug Administration, 2010). Such a storage method is currently impractical for mosquitoes, however. Cryopreservation of mosquitoes generally has proven difficult due largely to characteristics of membrane permeability and chill sensitivity, and no reliable method for cryopreservation of any *An. gambiae* life stage is yet available (Gallichote et al., 2023). Cryopreservation remains an area of active research and a recent report of success with *An. stephensi* could hold promise (James et al., 2022).

The current inability to cryobank seed stock of *An. gambiae* GDMMs results in a need for transgenic lines to be continuously maintained through all life stages. This is not an unusual feature for insect products, and also is routinely the case for insects used in SIT. Mass production of GDMMs is expected to begin with establishment of a mother colony (U.S. Centers for Disease Control and Prevention, 2011; U.N. Food and Agriculture Organization, 2017). Maintenance of mosquito lines is more complex than some other insects in that it involves providing for both water-dwelling larval stages and blood-feeding adult stages. Colony failure at a production facility is a risk for any insect product, but may be particularly onerous for *Anopheles* GDMMs since the inability to cryobank seed stock could result in the need to *de novo* recreate the strain. It is not known how different regulatory authorities might choose to interpret the scientific equivalence of a *de novo* rederived line versus the initial line upon which approval of the product was based. The possibility that such an event might require new testing or new approval makes it prudent to maintain the strain at multiple sites to assure a dependable source of the GDMM stock in case of colony failure at one production facility.

Minor variations in the genetic background may be introduced in the course of protracted maintenance of the original GDMM strain as a result of the accumulation of random, or gene drive-system-induced, mutations as well as strain evolution in the laboratory or insectary. Avoidance of adaptation to insectary environments, potentially resulting in reduced fitness and diminished effectiveness, may necessitate occasional refreshing of the GDMM colony through crossing with wild-type mosquitoes. Although such refreshing introduces the possibility of changes in the genetic background of the GDMMs over time, this issue is common to all live insect products as well as other colony-managed animals. Indeed, needs for strain maintenance and replacement have been extensively addressed for SIT (Dyck et al., 2021). There also may be reasons to customize the GDMM product to local circumstances. For example, this could be desirable if the local vector population is substantially more insecticide resistant than the GDMM strain, which might put the GDMMs at a temporary disadvantage for establishment (Garcia et al., 2019). This possibility of local customization has likewise been suggested as a means to enhance mating compatibility with local mosquito populations or avoid inadvertently introducing new characteristics into the native population. If required, such customization could be done by introduction, through repeated backcrossing with mosquitoes of the local genetic background. Alternatively, it could be accomplished by transformation of local mosquitoes using the transgenic construct; in this case, however, the ramifications for product approval must be kept in mind (Connolly et al., 2023). Whether a new transformation event would be more likely to be considered a new product than one derived by introduction or

introgression, as well as the types of data and information required for decision-making in each of these cases, will be determined by regulatory authorities.

Since strain maintenance is likely to require ongoing interbreeding and possible occasional outbreeding, it has been recommended that GDMM authentication methods concentrate only on the most distinctive characteristics of the strain (Benedict et al., 2018a). Suggested identity criteria for GDMMs therefore focus on the description of the transgenic construct, including copy number and location in the mosquito genome, because precedent indicates that the transformation event is expected to be the regulated article and the expression product of the construct is responsible for the direct effect (James et al., 2023). For other GM animals, identity characterization also focuses largely on the transgenic construct and its stability across generations (U.S. Food and Drug Administration, 2015). The identity method should be sufficiently discriminatory to show that the construct remains as described in the application for approval. The lack of emphasis on details of the genetic background of the mosquito is consistent with the lack of a formal regulatory standard for identity of SIT products, where the norm is simply to use a local strain of the targeted pest species (J. Bouyer, personal communication) and to allow colony refresh through introduction of the wild type as necessary (Dyck et al., 2021).

Potency

For live mosquito products, the potency standard will equate to performance characteristics. Estimates of performance during production must be based on selected surrogate indicators that can be routinely measured in the insectary and are considered reflective of the product's ability to perform its claimed function. As an example, for SIT with *Ae. aegypti*, performance generally relates to fitness and behavioral characteristics, such as: "the male is capable of flying, surviving and dispersing in the environment; mixing with the wild population; competing with its wild counterparts in courting, mating with and inseminating wild females, thus reducing the probability of those females mating with fertile wild males" (World Health Organization, 2020b). However, assessment of some of these parameters requires highly involved and technically demanding field studies such as mark-release-recapture experiments (Benedict et al., 2018b) that are not amenable to routine operational application. Therefore, for SIT, measurement of emerging male mosquito flight capacity in insectary settings has been proposed as a useful performance test (Balestrino et al., 2017; Culbert et al., 2018; Culbert et al., 2020).

The most consequential surrogate indicators of performance may differ for different types of GDMM products. Parameters related to efficacy and safety of GM mosquitoes as described by the World Health Organization 2021 are likely possibilities from which to select key surrogate indicators on a case-specific basis. Proposed performance criteria for use in manufacturing include competitiveness of GDMM mosquitoes with regard to their wild counterparts, as well as strength of the gene drive construct, measured as spread through a population (James et al., 2023). Field testing will provide an important opportunity to evaluate proposed GDMM performance standards. Therefore, in planning for field trials, choice of indicators should take into consideration their future utility and cost as routine quality control surrogates for the final marketable product.

Setting minimal performance requirements for a live GDMM product will be more nuanced than setting potency standards for a chemical entity. For example, deficits in fitness can be overcome by release of increased numbers of GDMMs, deflecting the question of efficacy more toward cost and logistical issues. The extent to which such flexibility is allowable likely will depend upon the wording of the product claim. In the particular case of GDMMs for malaria control, the efficacy required of a viable product also may be dependent on the disease transmission level at the treatment site and therefore variable according to local conditions, including availability and effectiveness of other control methods. Pre-approval testing may need to explore these variables so that the label language can adequately represent the potential variety of product uses.

Generally, performance standards are set by manufacturers according to the product claim and use case. With SIT for other insects, for example, performance requirements regarding flight ability or survival are not imposed by regulators but are a matter of negotiation between the manufacturer and the user (J. Bouyer, personal communication). Likewise for GDMMs, performance standards would best be established by the manufacturer according to local conditions and user requirements. Regulators will, however, require data demonstrating that performance supports the product claim.

Quality

For other live mosquito products, as well as GM crops, demonstration of the maintenance of product quality is the responsibility of the manufacturer (J. Bouyer, S. O'Neill, personal communication). Manufacturers will need to be proactive in establishing their own quality standards to protect the reputation of the product, and discussing these with the regulators. Considerations likely would include characteristics associated with ability to achieve the product claim, such as fitness and maintenance of the transgenic construct in a functional form, presence of any contaminating mosquito species, or, for products which involve male-only releases, percentage presence of female mosquitoes from the product line. Quality management systems will be needed at production facilities to ensure that the GDMM product consistently and reliably meets the applicable identity and performance requirements. While good manufacturing practice is not defined for GDMMs, certain common principles (Sarvari et al., 2020) that would be pertinent to GDMM production include: designing and constructing the facilities and equipment properly; writing sufficiently detailed, comprehensible, standard operating procedures (SOPs) and instructions, with updating as needed; confirming these processes; following written procedures and evaluating and maintaining records of staff performance and training; establishing a record-keeping system and documenting work; monitoring and regularly inspecting facilities and calibrating

and maintaining equipment; ensuring the quality of materials and protecting against contamination; conducting planned and periodic audits that help to recognize any errors and correct noncompliance; and, promoting workplace quality and safety.

For SIT programs (Dyck et al., 2021), in addition to ongoing performance evaluation as discussed above, other quality considerations have been classified as: production control (defined as monitoring all aspects of insect rearing, including materials and equipment used, personnel and environment); and process control (defined as measuring how things are done to identify possible sources of variability). SIT programs, especially those developing methods for *Ae. aegypti* mosquitoes, can provide context for both production and process control. The specifics of facility design, colonization, rearing, handling and strain maintenance have been well described (Food and Agriculture Organization, 2012; International Atomic Energy Agency, 2017; Dyck et al., 2021). Certification of rearing facilities, as required by Good Laboratory Practices or ISO 9000 standards, has not generally been considered necessary, although this may vary by country. A need for external inspection, qualification and permitting of the production facility, e.g., by national authorities and/or WHO Prequalification Inspection Services, can be expected (James et al., 2018; World Health Organization, 2020b; Dyck et al., 2021; World Health Organization, 2023b).

Quality control considerations will include avoiding colony contamination by: verifying the species of any locally-derived mosquitoes introduced into the colony; ensuring the absence of human or animal disease agents known to be transmitted by the mosquito species; and, safe-guarding that appropriate containment procedures are in place to prevent cross-contamination among different GDMM strains (Benedict et al., 2018a; American Committee of Medical Entomology, 2022). Housing and feeding of both aquatic and adult life stages will require materials, such as larval water and food source and adult sugar and blood source, that meet applicable regulatory requirements.

Ability to detect non-conformance with agreed upon identity and performance expectations and to initiate appropriate corrective actions within the manufacturing process is an important component of quality management. Appropriate data management systems will be key, enabling assimilation and assessment of quality metrics over time and quick identification of characteristics that are out of specification so that rapid remedial action can be taken (e.g., International Atomic Energy Agency, 2018). Some form of regular auditing can help to ensure the ongoing identity, performance and quality of the GDMM strains. Reports from internal audits may be required by the regulator, but it is likely that the regulator also will perform occasional audits. A report of product failure in the field arising from post-implementation monitoring could trigger an audit.

Market entry

The market for GDMMs has yet to be explored since no product has to date been put forward. As with other aspects of the GDMM development pathway, processes established for market entry of conventional public health products are unlikely to be entirely applicable (James et al., 2018). Thus, complementary or additional mechanisms that may need to be put in place for operationalizing GDMM products should be considered. This involves identifying the potential customers and understanding their interests. While not excluding other possible uses, it has generally been assumed that GDMMs largely will be used by disease control programs in the context of their vector management activities and therefore the most likely customers will be health-related government agencies at the national, provincial and/or local level. Consideration of potential business models should recognize that government agencies may wish to establish their own GDMM manufacturing and delivery capabilities, acquire the GDMM product to deliver themselves, or contract for the GDMM product and all activities necessary to deliver it. Revisiting the SIT precedent, options may exist for both government-run programs and private suppliers, or a mix of both (Dyck et al., 2021). Early analysis of the market will support development of a product or service that is relevant to customer needs and help to clarify appropriate business models. It also will facilitate understanding among potential users of GDMMs as a new and possibly valuable tool for meeting their public health goals.

Production facilities

Procedures for establishing SIT production facilities can provide useful guidance for scale-up production of GDMMs (Dyck et al., 2021). These identify factors for determining optimal location of individual production facilities, such as logistical access, availability of necessary resources (e.g., power and water), generally enabling government requirements, local acceptance, and labor availability. Establishment of production facilities will require adequately designed and equipped manufacturing facilities, well trained staff, and SOPs for mosquito husbandry, quality management, and documentation. Optimization of each element of the rearing process will underpin efficient scale-up production and reliable delivery of high-quality products. Production of GDMMs may involve additional provisions for the biosafety for GMOs that are not ordinarily encountered by SIT programs (e.g., Australian Government, 2011; U.S. Food and Drug Administration, 2015; U.S. Centers for Disease Control and Prevention, 2020; World Health Organization, 2020c). While containment considerations for research on GDMMs have been addressed elsewhere (American Committee of Medical Entomology, 2022), the appropriate containment measures for post-investigational manufacturing and distribution will need to be determined by regulatory authorities. It is possible that a favorable decision for wide-scale implementation will be accompanied by more relaxed containment requirements during production and transport. As mentioned earlier, if different GDMM strains will be maintained within the same production facility, facility design must anticipate a need for appropriate segregation of the separate strains, as well as ongoing testing to ensure that no mixing has occurred and a remediation plan in the event that cross-contamination is detected (Benedict et al., 2018a).

Launching qualified production sites for even small-scale production of *An. gambiae* GDMMs in Africa could well be an intensive multiyear process (Guissou et al., 2022). Planning for where these facilities should be located, how they must be

designed, how they will be funded, and what quality management systems must be put in place should be considered as early as possible, as this will be vital for reducing what could amount to substantial lag time between a decision to implement and the actual ability to implement.

Facility location

Other types of products are often manufactured in centralized facilities to take advantage of economy of scale (increased efficiency based on access to well-characterized equipment and processes and well-trained staff). This allows for maximum control of critical factors influencing product quality (Medcalf, 2016). Based on the SIT example, manufacturing of live insect products within a centralized mass rearing facility also has the benefit of simplifying quality assurance and reducing cost of production. Both of these issues are important determinants of product uptake. The applicability of a centralized production model for *An. gambiae* GDMMs, however, depends on a number of issues encompassing both technical and political factors.

While similarly an issue for SIT and other live mosquito products, distance to the release area and limitations on shipping and delivery options pose particular complexities for *An. gambiae* GDMMs. *Anopheles gambiae* differ substantially from *Ae. aegypti*, which is the subject of most current work on live mosquito products. *Aedes aegypti* eggs maintain viability after extended periods of desiccation (Faull and Williams, 2015). This has allowed a centralized production model whereby eggs are shipped from a remote facility to field sites for short-term storage, production and distribution of other life stages, or even direct delivery to the field (Oxitec, 2023a; Oxitec, 2023b). However, the fact that cryopreservation is not yet reliable and that *An gambiae* eggs rapidly lose viability, even at low temperatures (Ebrahimi et al., 2014; Mazigo et al., 2019), may limit the prospects for routine long-range supply in quantities required for implementation. Continued research may identify ways to overcome these limitations in the future. For example, certain compacting and chilling conditions have allowed for short-term transport of adult male *An. arabiensis* up to 24 h (Culbert et al., 2017). However, at present, this limitation may dictate distance of the production facility from the release sites to allow for delivery of viable GDMMs with the necessary performance characteristics. This concern should be clarified during pre-approval testing, and simple assays for performance of transported GDMMs upon arrival at field sites should be determined at that time.

A distributed manufacturing model could help to address these delivery limitations. In the case of GDMMs for control of malaria in Africa, a distributed model could also support more local autonomy and entrepreneurial opportunities that would be attractive to government and public end users. This might take the form of regional, national, or even local production sites. Challenges associated with a highly distributed model include assuring that all individual production facilities meet applicable regulatory requirements, and that their GDMM products meet quality requirements and are equivalent functionally. In one possible version of the distributed model, transgenic mosquitos might be produced *de novo* in a facility near the release area by injection of the transgene DNA into local mosquitoes. As mentioned above however, this could have important regulatory implications concerning whether each new transformation event conducted in a different facility would be considered a new product (Connolly, 2023; James et al., 2023). Regional production could provide some of the advantages of a centralized model while still reducing distance between manufacturing and release sites.

Any form of centralized or regional production likely would benefit from agreement among involved countries to allow a GDMM product manufactured elsewhere to be introduced into their country (James et al., 2023). This might require market entry plans to be broached with potentially involved countries early in the development process to foster understanding of the expected benefits, identify concerns, and understand how any importation issues can be addressed. An international framework to facilitate transboundary shipments of sterile insects has been proposed (Enkerlin and Pereira, 2022), and mechanisms for harmonization of regulatory requirements for GDMMs are being explored by the African Union (African Union Development Agency-NEPAD, 2023).

Production processes

For a particular GDMM product, the scale of production required for implementation will be determined by the desired public health effect, which dictates a release plan to achieve the expected reduction in vector numbers and/or disease transmission over a specified area, perhaps within a specified timeframe. Although the scale of production required for self-limiting or localizing GDMMs is generally expected to be greater than for those that are self-sustaining, the more extensive the releases of self-sustaining GDMMS are the more quickly positive epidemiologic results can be expected. For both self-sustaining and self-limiting GDMMs, there may be limitations on the timing of releases with respect to seasonality that could require periodic surges in production. The range of anticipated release numbers, pattern and frequency of GDMM releases necessary to achieve the desired effect should be clarified via pre-approval field studies, although these may need to be further adjusted to meet the real world challenges of operationalizing the technology for implementation at scale.

Large-scale production of GDMMs will benefit from mechanization, which could help both to maintain quality and create a more affordable product. For other biotechnology products using a distributed production model, automation has been proposed as an important contributor to ensuring consistent product quality across different manufacturing locations (Medcalf, 2016). Additionally, for GDMMs designed for population suppression in which no means is available to suppress transgene effector expression and maintain the strain in homozygous form, complex rearing procedures may be necessary that involve backcrossing each generation with non-transgenic mosquitoes to maintain the transgenic element with screening and segregation by sex and transgene status at each generation. Mechanization of these highly labor-intensive steps would facilitate scaling of GDMMs for operational use even if release numbers are substantially lower than for conventional SIT approaches.

Depending on the insect species being produced for SIT, some level of mechanization has been achieved in almost all stages of the rearing process. Because of their aquatic stage, mosquito rearing is more complex and labor-intensive than rearing of certain other insects targeted for SIT. Studies on mass rearing of *An. arabiensis* underway in Sudan and South Africa (Maiga et al., 2020) and of *An. gambiae* s.l. in West Africa (Zubair et al., 2021) are yielding insights into mass rearing requirements for anophelines. Oxitec also is working to develop its GM Friendly™ mosquito technology in *An. stephensi* and *An. albimanus,* although this work is at an early stage (Oxitec, 2023c). While more advanced, mass production methods for *Aedes* currently still are adequate only at a relatively limited geographic scale (e.g., city-wide). However, sophisticated automation processes employing robotics for mass rearing are being tested (Crawford et al., 2020).

It has generally been assumed that releases of GDMMs into the field will consist of males, which would minimize any nuisance or risk posed by the possibility that released females could bite humans. As mentioned above, this precedent has been set by self-limiting genetic biocontrol approaches aimed at population suppression, in which large numbers of mosquitoes must be released on a continuous basis. With population replacement drives where the potential for disease transmission is greatly reduced and/or self-sustaining gene drives in which only low numbers of mosquitoes will be released, it is possible that any risks related to release of females will be judged acceptable. Precedent for mixed sex releases exists with the *Wolbachia*-mediated population replacement technology in *Aedes aegypti* (World Mosquito Program, 2023). If the intention is to release only male GDMMs, more facile methods to separate the sexes also could increase efficiency and reduce production costs. A variety of techniques have been used to separate male from female mosquitoes (Lutrat et al., 2019). For *Aedes* species, it is common to separate the sexes at the pupal or adult stages based on morphology, typically using sieves and/or plate separators (Carvalho et al., 2014), which is a labor and cost intensive process and can result in a small percentage of females remaining in the release batches. Recently, an automated process has been developed that separates out females based first on pupal body size and then by visual recognition of adult body parts (Crawford et al., 2020). Currently, pupal sex segregation methods developed for *Aedes* are unsuitable for *An. gambiae*, where there are not distinct differences in size between male and female pupae. Sex separation remains an obstacle to scaling up of *Anopheles* GDMMs as current methods rely on individual sorting at pupae or adult stage by trained technicians. A sex-specific transgenic fluorescent marker has been used successfully to separate male from female larval stages by flow cytometry (e.g., Cateruccia et al., 2005; Marois et al., 2012) and efforts are underway to make this method amenable to field use. It may eventually be possible to adapt some aspects of the automated sorting systems based on image recognition of adults developed for *Aedes* (Crawford et al., 2020; Senecio, 2023a) to *Anopheles*, but the sophisticated equipment required may be difficult to obtain and maintain in a developing country setting and/or, for a distributed manufacturing model, in multiple locations. Other alternatives for sex sorting also are being explored (Lutrat et al., 2019). These include spiking the blood meal with mosquito toxicants to kill blood feeding females (Yamada et al., 2013; Gunathilaka et al., 2019), using RNAi-based sex distorter systems to deplete females (Hoang et al., 2016; Taracena et al., 2019), as well as genetic sexing mechanisms (Meza et al., 2018; Mysore et al., 2021; Spinner et al., 2022).

Distribution and delivery

Plans for market entry of GDMMs as a malaria control tool will need to include efficient methods for achieving the necessary level of area-wide coverage. Programs utilizing GDMMs to prevent malaria in Africa may have goals that range in ambition from control of transmission in an urban/peri-urban area (Doumbe-Belisse et al., 2021; Tadesse et al., 2021; World Health Organization, 2022b) to reduction of transmission in largely rural regions (World Health Organization, 2015; World Health Organization, 2020d; World Health Organization, 2022a) to malaria eradication across the continent (Feachem et al., 2019; World Health Organization, 2019). These different goals translate to vastly different coverage requirements. Depending upon the location of production facilities, it may be possible to perform releases directly or there may be a need for some form of intermediary staging facility. For example, some SIT programs have established a model of centralized production combined with local emergence and release facilities (Dyck et al., 2021). In this case, distribution of GDMM products would be a two-step process that involves distribution from a centralized or regional production facility to local staging facilities followed by delivery to more widely dispersed release sites.

In any situation where the production facility is remote from the release sites, protocols for transportation of GDMMs, such as storage conditions, temperature monitoring, tracking, labelling, disposition of shipping materials, and record keeping, will need to be prepared and tested in advance (e.g., World Organization for Animal Health, 2022; IATA, 2023). For centralized or regional facilities, involving transport across national boundaries, aspects of international shipping of live mosquito products include regulatory permit and health inspection requirements, containment and chain of custody issues, and challenges of ensuring product integrity/quality during handling. Certain of these requirements may differ according to the life stage that is shipped/transported. International guidelines have been developed for transboundary shipment of irradiated sterile insects (U.N. Food and Agriculture Organization, 2022) and for biological control agents more broadly (U.N. Food and Agriculture Organization, 2005). Gene drive modifications may require the imposition of additional conditions for shipping, not only to provide a level of containment necessary to avoid inadvertent release in transit that might result in unauthorized establishment of the mosquito but also to satisfy notification requirements for transboundary movement of GMOs if applicable Clearing House, Biosafety (2023). Conditions of the shipping route, i.e., how many stops are involved, how much time is required for transit, possibility of seasonal temperature effects, and regulatory requirements of transit countries, will be an important consideration for location of the manufacturing facility. Any particular requirements or restrictions on international transport of GDMMs should be explored before decisions about facility location(s) are reached.

More work is needed to develop efficient mechanisms for delivery of GDMMs to widespread release sites. Agricultural SIT programs have identified three basic mechanisms for insect release:

ground-based containers, mobile ground-based vehicles, and aerial vehicles. The advantages and disadvantages of each approach have been extensively described (Dyck et al., 2021). To summarize, placement of ground-based receptacles containing pupae or eggs is labor intensive and subject to limited access, weather, human and animal intervention, and predation. Mobile ground release of adults, for example, from trucks or vans, requires fewer workers and can treat a larger though still limited area, but distribution remains subject to access (roads and terrain, weather). The expected ability of gene drive modifications to spread beyond the site of release may, however, reduce the challenges of limited access with ground-based approaches that has been experienced with other live mosquito products. Aerial release of adults can cover larger areas regardless of terrain, with the distance as well as quantity and viability of the payload being determined by the type of vehicle (e.g., fixed wing or rotary aircraft, various types of unmanned aerial vehicles (UAVs)), the packaging, and the release mechanism. However, aerial delivery can require expensive equipment and trained operators, is subject to weather, and may impose survival or fitness costs.

To date, programs releasing living *Ae. aegypti* mosquitoes have aimed for coverage over limited areas (towns, cities or suburbs). This has most often involved collection of adults or eggs from a local production facility and same day delivery by van or truck to release sites. Release methods have included manual placement of egg-containing boxes at relatively protected sites or discharge of adults from various types of cartons or tubes in yards or streets. However, methods for longer-term transport of irradiated *Ae. aegypti* adults are improving, which may expand opportunities for SIT programs (Maiga et al., 2023). Limitations to the area that can be covered by these manual methods have led to the exploration of alternative mechanisms to facilitate broader access. Such ideas include the use of mini-mobile laboratories for production (Public Broadcasting System, 2018) and new concepts for automated ground release or releases from UAVs or fixed wing aircraft (e.g., Bouyer et al., 2020; Marina et al., 2022; Senecio, 2023b).

Release of *An. gambiae* eggs does not currently seem a feasible option, at least in rural areas, because of their limited viability as well as the abundance of predators expected under field conditions. Although chilling techniques have been adapted to adult mosquitoes that prolong their viability (Bailey et al., 1979; Culbert et al., 2017), the distance that potentially can be covered by transporting *An. gambiae* adults in ground-based vehicles to release sites is likely to be restricted even in the presence of accessible roads, which are not always a given. While aerial transport and release across large areas using fixed or rotary wing aircraft has become standard for SIT against certain insect pests, similar options generally have been limited by the inherent fragility of adult mosquitoes. As reported to date for mosquito release, UAV transport has been piloted within fairly limited areas (e.g., Francaise, 2021; Bouyer et al., 2020). Experimentation to expand options for UAV transport is being actively pursued, however (Mechan et al., 2023).

Discussion

GDMMs are being proposed as new tools to control and eliminate malaria in Africa because currently available control methods thus far have proven insufficient to achieve global goals and disease incidence has recently shown signs of rebounding (World Health Organization, 2022a). GDMMs have a number of theoretical advantages for preventing malaria transmission, including their utility against difficult-to-reach mosquito populations, the equitability of their effect regardless of socioeconomic conditions, and their ability to function in situations where other control methods have been disrupted (World Health Organization, 2021). Promising results from caged testing of GDMMs thus far support the potential of these technologies (e.g., Kyrou et al., 2018; Carballar-Lejarazu et al., 2020; Hammond et al., 2021; Ellis et al., 2022). However, challenges are expected with operationalizing GDMMs for malaria control. Challenges relating to risk analysis, regulatory and policy frameworks, as well as ethical concerns, are topics of ongoing discussion, and work is actively underway to address them (e.g., World Health Organization, 2021; James et al., 2023). Planning for other aspects of implementation, including manufacturing and delivery requirements, has to date been less of a priority. Nonetheless, several issues remain to be addressed to prepare for eventual market entry of these new products and new or updated mechanisms may need to be put in place, which could require substantial planning, time, and coordination. The best fit for handling live mosquito products will have to be determined for each country or region where these products will be deployed.

A major challenge relates simply to raising awareness of GDMMs among regulators and other decision-makers in disease endemic countries. Although GDMMs are expected largely to be implemented for public health benefit in the context of disease control programs, they differ from conventional public health tools such as drugs, vaccines and insecticides, in important ways. GDMMs are classified as living modified organisms (Convention on Biological Diversity, 2017), and will be regulated for biosafety according to mechanisms described under the Cartagena Protocol for Biodiversity (Convention on Biological Diversity, 2000) in the 173 countries that are signatories to the Protocol. According to these mechanisms, multiple ministries are likely to participate in biosafety decision-making (James et al., 2023). Ministries of Health likely will be interested in the effectiveness of GDMMs for disease prevention as well as product safety. Health regulators may be most familiar with effectiveness criteria developed for conventional products that can be chemically or physiologically characterized and are intended for individual or household use. However, typical manufacturing standards focused on product identity, potency or quality will need to be adapted for GDMMs. This will require health regulators to become familiar with the characteristics of live mosquito products. For example, any identity requirement is best focused on the transgenic construct rather than the mosquito genetic background, since standard practices of mosquito husbandry are likely to introduce changes in the overall genetic makeup of the GDMM line over time. Setting a performance standard for a live GDMM product also will be less straightforward than setting potency standards for a chemical entity, since performance requirements will be influenced by the nature of the GDMM (e.g., spread and persistence), the release plan (e.g., size and frequency of GDMM releases), and local disease transmission conditions (e.g., size of the local vector population and use of other control measures). Thus, certain requirements will be best

negotiated between the manufacturer and the user (presumably the national disease control program) rather than imposed by regulators, with the manufacturer being responsible for ensuring that these requirements are consistently met. Oversight of the product can be maintained through auditing during manufacturing as well as ongoing post-implementation efficacy monitoring. Regulatory processes applied for market entry of other whole organism products, such as agricultural SIT programs, GM crops and animals, or other modified mosquito products, will be informative in this regard.

Appropriate business models are only beginning to be explored. It is not too early to begin the necessary outreach to understand the potential market for GDMMs, as this information may shape some crucial decisions in the research and development pathway. The decision to incorporate any type of GDMMs into a national control program likely will be dependent upon perceived cost and differential advantage with respect to other malaria control tools that must be readministered with some degree of regularity (such as insecticide-treated nets, indoor residual spraying, chemotherapy, or the current RTS,S vaccine (World Health Organization, 2022a)). Sustainability is a critical issue for GDMM programs (Haakenstad et al., 2019; World Health Organization, 2022a). If GDMMs are able to provide more durable and low cost protection as predicted, this could result in a substantial advantage that should be attractive to national governments and other funders. Likewise, the value proposition will take into account the scale of the public health goal. The vast area of the malaria belt in Africa (Institute of Tropical Medicine Antwerp, 2022; World Bank, 2023) and the rural nature of much of this region could favor an *An. gambiae* GDMM designed to spread and persist to contribute to a malaria eradication goal (Feachem et al., 2019). More focal malaria control goals may be amenable to self-limiting and/or localizing products.

For other types of products, centralized production is known to provide cost advantages of economy of scale. Some *Ae. aegypti* live mosquito products are employing a centralized production approach, which is made possible because of the ability to ship their eggs over long distances. However, *An. gambiae* are known to be fragile in transport. Moreover, country regulations governing introduction of GM organisms within their respective boundaries and containment requirements related to the presence of driving transgenes may further complicate international distribution and delivery of GDMMs. These limitations, if not overcome, could favor a more distributed model with multiple production facilities in locations appropriate to attain the level of coverage necessary to achieve the public health goal. A distributed model may also offer advantages for local autonomy. Production requirements, and therefore the business model, also will be influenced by the nature of the GDMM product, whether self-sustaining, self-limiting or localizing. It is possible that the production scale required for implementation of low threshold self-sustaining GDMMs will not be large since the modification is intended to spread autonomously from small releases into interbreeding populations by mating. Self-limiting or localizing GDMM approaches, anticipated to require relatively more frequent or larger releases to maintain broad effectiveness, are expected to face greater challenges to the ability to produce GDMMs at the necessary scale. Production requirements also may have implications for quality control, which likely will be easier to maintain for ongoing versus intermittent manufacturing. Thus, while the possibility that self-sustaining GDMMs will need to be released in lower numbers and less frequently to maintain efficacy could translate to a production advantage, it also might raise the practical issue of how to maintain a robust infrastructure for only sporadic production. This issue might be addressed through a centralized approach, with sustained production of the GDMM product for multiple markets. In the distributed model, it could be addressed by manufacturing within the same facility of several types of GDMMs (for example, using the same construct in different *Anopheles* species) for alternating implementation campaigns.

Manufacturing efficiency and quality control could be enhanced by the further development of several enabling technologies. Mechanization of key production steps would benefit large-scale production and quality control of all types of GDMMs. Improvement of capabilities for mass rearing of *Anopheles*, including development of automation technologies that will be affordable and sustainable in developing countries, is an area ripe for further research. Other enabling activities that developers should consider during early research include methods for preservation of GDMM product seed stock and identification of high-throughput mechanisms for assessing product identity and performance that will be suitable for routine use in manufacturing and monitoring.

Release mechanisms currently in use offer a relatively limited area of coverage. This has been a hurdle for scale-up of other live mosquito products and likely will present a disadvantage for self-limiting GDMM approaches. Self-sustaining approaches may be substantially better able to overcome this challenge due to their ability to spread the modification by mating, but this will depend on the degree of connectedness of *An. gambiae* populations as well as the timeframe over which disease reduction is expected. Current coverage limitations for release of live mosquito products may be overcome by newer delivery possibilities, such as aerial mechanisms, if these can be made cost-effective.

Coordination with other vector control methods will be important for successful GDMM implementation. Delivery and release of GDMMs could be performed by staff of the national vector control program and/or other government programs or by their contracted agents. In this case, the timing of GDMM delivery with respect to insecticide-based vector control programs, such as indoor residual spraying, must be planned from the perspective of staff availability as well as to ensure that newly-released GDMMs are not depleted before the modification can become established within the local vector population, although this might be expected to have little effect on male mosquitoes relative to indoor-feeding females. While not specifically discussed here, post-release monitoring for safety and efficacy also will be an important aspect of GDMM implementation (World Health Organization, 2021). The more delivery and monitoring can be integrated with other activities routinely conducted by national disease control programs, the lower the additional effort and cost that might be expected for GDMM implementation.

Here we have considered some current practical challenges related to market entry for GDMMs, with a focus on those under development for malaria control in Africa (Box 2). Some of these activities are broadly applicable to all GDMM types, some are more relevant for one type of GDMM than another, and others will be

specific to a particular GDMM product. While only a beginning, it is hoped that this initial analysis will focus attention on currently unresolved issues that are important for the ultimate success of GDMM products, and stimulate further planning and investment to address these issues. Those presently engaged in more upstream research on GDMMs may consider these analyses premature, yet beginning to tackle them now could help them avoid some costly mistakes later in the development pathway.

BOX 2 | Enabling activities for market entry of new GDMM products for malaria

- Increased understanding of GDMM technologies among regulators and other relevant government authorities
- Market analysis and clarification of the value proposition for each product
- Development of the business model for each product
- Clarification of regulatory approval requirements and new product approval
- Identification of indicators to be used for quality management in manufacturing each product
- Improved methods for long-term storage and preservation of mosquito strains
- Improved methods for mechanization of rearing and sex separation processes that are both high-throughput and suitable for use in developing countries
- Development of efficient mechanisms for transportation and delivery to release sites
- Identification of requirements for integration of GDMMs with other malaria control methods

Author contributions

SJ, HQ, MB, and BD conducted research and interviews on which the manuscript is based. SJ wrote the manuscript, with substantial input and review by HQ, MB, and BD. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by a grant from the Bill & Melinda Gates Foundation (INV-008525).

Acknowledgments

The authors would like to thank Drs. Jeremy Bouyer, Andrew McKemey, Scott O'Neill, and Nathan Rose for helpful discussions that contributed greatly to the development of this manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

African Union Development Agency-NEPAD (2023). Integrated vector management. Available at: https://www.nepad.org/microsite/integrated-vector-management-ivm#:~:text=The%20purpose%20of%20the%20IVM,order%20to%20effectively%20control%20vectors (Accessed March 15, 2023).

Alimentarius (2003). CAC/GL 45-2003. Guideline for the conduct of food safety assessment of foods derived from recombinant DNA plants. Available at: https://www.fao.org/fao-who-codexalimentarius/codex-texts/guidelines/pt/(Accessed March 20, 2023).

Alphey, L. S., Crisanti, A., Randazzo, F., and Akbari, O. S. (2020). Standardizing the definition of gene drive. *Proc. Natl. Acad. Sci. U. S. A.* 117, 30864–30867. doi:10.1073/pnas.2020417117

American Committee of Medical EntomologyAmerican Society of Tropical Medicine and Hygiene (2022). Containment practices for arthropods modified with engineered transgenes capable of gene drive addendum 1 to the arthropod containment guidelines, version 3.2. *Vector-Borne Zoonotic Dis.* 22, 3–17. doi:10.1089/vbz.2021.0035

Australian Government (2011). Guidelines for the transport, storage and disposal of GMOs. Available at: https://www.ogtr.gov.au/sites/default/files/files/2021-07/guidelines_for_the_transport_storage_and_disposal_of_gmos.pdf (Accessed March 14, 2023).

Bailey, D. L., Thomas, J. A., Munroe, W. I., and Dame, D. A. (1979). Viability of eggs of *Anopheles albimanus* and *Anopheles quadrimaculatus* when dried and stored at various temperatures. *Mosq. News* 39, 113–116. Available at: https://ia600500.us.archive.org/6/items/cbarchive_117307_viabilityofeggsofanophelesalbi1979/MN_V39_N1_P113-116_text.pdf.

Balestrino, F., Puggioli, A., Carrieri, M., Bouyer, J., and Bellini, R. (2017). Quality control methods for *Aedes* albopictus sterile male production. *PLoS Negl. Trop. Dis.* 11, e0005881. doi:10.1371/journal.pntd.0005881

Benedict, M. Q., Burt, A., Capurro, M. L., De Barro, P., Handler, A. M., Hayes, K. L., et al. (2018a). Recommendations for laboratory containment and management of gene drive systems in arthropods. *Vector-borne Zoonotic Dis.* 18, 2–13. doi:10.1089/vbz.2017.2121

Benedict, M. Q., Charlwood, D., Harrington, L. C., Lounibos, P., Reisen, W. K., and Tabachnick, W. J. (2018b). Guidance for evaluating the safety of experimental releases of mosquitoes, emphasizing mark-release-recapture techniques. *Vector-borne Zoonotic Dis.* 18, 39–48. doi:10.1089/vbz.2017.2152

Bourtzis, K., and Vreysen, M. J. B. (2021). Sterile insect technique (SIT) and its applications. *Insects* 12, 638. doi:10.3390/insects12070638

Bouyer, J., Culbert, N. J., Dicko, A. H., Pacheco, M. G., Virginio, J., Pedrosa, M. C., et al. (2020). Field performance of sterile male mosquitoes released from an uncrewed aerial vehicle. *Sci. Robot.* 5, eaba6251. doi:10.1126/scirobotics.aba6251

Brown, C. P., and Bollyky, T. J. (2021). How COVID-19 vaccine supply chains emerged in the midst of a pandemic. Available at: https://www.piie.com/sites/default/files/documents/wp21-12.pdf (Accessed March 13, 2023).

Carballar-Lejarazu, R., Ogaugwu, C., Tushar, T., Kelsey, A., Pham, T. B., Murphy, J., et al. (2020). Next-generation gene drive for population modification of the malaria vector mosquito *Anopheles gambiae*. *Proc. Natl. Acad. Sci.* 117, 22805–22814. doi:10.1073/pnas.2010214117

Carmen, J., Burger, S. R., McCaman, M., and Rowley, J. A. (2012). Developing assays to address identity, potency, purity and safety: Cell characterization in cell therapy process development. *Regen. Med.* 7, 85–100. doi:10.2217/rme.11.105

Carvalho, D. O., Nimmo, D., Naish, N., McKemey, A. R., Gray, P., Wilke, A. B. B., et al. (2014). Mass production of genetically modified *Aedes aegypti* for field releases in Brazil. *J. Vis. Exp.* 83, 3579. doi:10.3791/3579

Cateruccia, F., Benton, J. P., and Crisanti, A. (2005). An *Anopheles* transgenic sexing strain for vector control. *Nat. Biotechnol.* 23, 1414–1417. doi:10.1038/nbt1152

Clearing House, Biosafety (2023). The advance informed agreement (AIA) procedure for LMOs for intentional introduction into the environment. Available at: https://bch.cbd.int/help/topics/en/The_Advance_Informed_Agreement_procedure.html (Accessed March 15, 2023).

Code of Federal Regulations (2023). Title 21. Part 314. Applications for FDA approval to market a new drug. Available at: https://www.ecfr.gov/current/title-21/chapter-I/subchapter-D/part-314 (Accessed March 23, 2023).

Connolly, J. B. (2023). Defining transformation events for gene drive in species complexes. Modern biotechnology in integrated plant production. *IOBC-WPRS Bull.* 163, 8–20. Available at: https://iobc-wprs.org/product/defining-transformation-events-for-gene-drive-in-species-complexes/.

Connolly, J. B., Romeis, J., Devos, Y., Gandorf, D. C. M., Turner, G., Coulibaly, M. B., et al. (2023). Gene drive in species complexes: Defining target organisms. *Trends Biotechnol.* 41, 154–164. doi:10.1016/j.tibtech.2022.06.013

Consolidated Mosquito Abatement District (2023). Sterile insect technique. Available at: https://www.arcgis.com/apps/MapJournal/index.html?appid=0586437f5b69477fbad9f3774047420a (Accessed March 14, 2023).

Convention on Biological Diversity (2000). Cartagena protocol on biosafety to the convention on biological diversity. Available at: https://bch.cbd.int/protocol/text/ (Accessed March 20, 2023).

Convention on Biological Diversity (2017). Report of the ad hoc technical expert group on synthetic biology. Available at: https://www.cbd.int/doc/c/aa10/9160/6c3fcedf265dbee686715016/synbio-ahteg-2017-01-03-en.pdf (Accessed March 20, 2023).

Convention on Biological Diversity. (2020). Study on risk assessment: Application of annex I on decision CP-9/13 to living modified organisms containing engineered gene drives. Available at: https://bch.cbd.int/protocol/risk_assessment/cp-ra-ahteg-2020-01-04-en-2.pdf.

Crawford, J., Clarke, D. W., Criswell, V., Desnoyer, M., Cornel, D., Deegan, B., et al. (2020). Efficient production of male *Wolbachia*-infected *Aedes aegypti* mosquitoes enables large-scale suppression of wild populations. *Nat. Biotechnol.* 38, 482–492. doi:10.1038/s41587-020-0471-x

Culbert, N. J., Balestrino, F., Dor, A., Herranz, G. S., Yamada, H., Wallner, T., et al. (2018). A rapid quality control test to foster the development of genetic control in mosquitoes. *Sci. Rep.* 8, 16179. doi:10.1038/s41598-018-34469-6

Culbert, N. J., Lees, R. S., Vreysen, M. J. B., Darby, A. C., and Gilles, J. R. L. (2017). Optimised conditions for handling and transport of male *Anopheles arabiensis*: Effects of low temperature, compaction, and ventilation on male quality. *Ent. Exp. Appl.* 164, 276–283. doi:10.1111/eea.12610

Culbert, N. J., Somda, N. S. B., Hamidou, M., Soma, D. D., Caravantes, S., Wallner, T., et al. (2020). A rapid quality control test to foster the development of the sterile insect technique against *Anopheles arabiensis*. *Malar. J.* 19, 44. doi:10.1186/s12936-020-3125-z

Doumbe-Belisse, P., Kopya, E., Ngadjeu, C. S., Sonhafouo-Ciana, N., Talipouo, A., Djamouko-Djonkam, L., et al. (2021). Urban malaria in sub-saharan Africa: Dynamic of the vectorial system and the entomological inoculation rate. *Malar. J.* 20, 364. doi:10.1186/s12936-021-03891-z

Dyck, V. A., Hendrichs, J., and Robinson, A. S. (2021). *Sterile insect technique*. Boca Raton: CRC Press.

Ebrahimi, B., Shakibi, S., and Foster, W. A. (2014). Delayed egg hatching of *Anopheles gambiae* (Diptera: Culicidae) pending water agitation. *J. Med. Ent.* 51, 580–590. doi:10.1603/ME13100

Ellis, D. A., Avraam, G., Hoermann, A., Wyer, C. A. S., Ong, Y. X., Christophides, G. K., et al. (2022). Testing non-autonomous antimalarial gene drive effectors using self-eliminating drivers in the African mosquito vector *Anopheles gambiae*. *PLoS Genet.* 18, e1010244. doi:10.1371/journal.pgen.1010244

Enkerlin, W. R., and Pereira, R. (2022). The sterile insect technique: An international framework to facilitate transboundary shipments of sterile insects: -EN- the sterile insect technique: An international framework to facilitate transboundary shipments of sterile insects -fr- La technique de l'insecte stérile: Un cadre international pour faciliter les transports transfrontaliers d'insectes stériles -es- técnica del insecto estéril y ordenamiento internacional para facilitar los envíos transfronterizos de insectos estériles. *Rev. Sci. Tech. Off. Int. Epiz.* 41, 66–74. doi:10.20506/rst.41.1.3303

European Food Safety Authority (2013). Guidance on the environmental risk assessment of genetically modified animals. *EFSA J.* 11, 3200. doi:10.2903/j.efsa.2013.3200

European Food Safety Authority (2010). Guidance on the environmental risk assessment of genetically modified plants. *EFSA J.* 8, 1879. doi:10.2903/j.efsa.2010.1879

European Food Safety Authority (2011). Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. *EFSA J.* 9, 2193. doi:10.2903/j.efsa.2011.2193

European Medicines Agency (1999). ICH topic A6B specifications: Test procedures and acceptance criteria for biotechnological/biological products. Available at: https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-6-b-test-procedures-acceptance-criteria-biotechnological/biological-products-step-5_en.pdf (Accessed March 13, 2023).

European Medicines Agency (1998). ICH topic Q5D quality of biotechnological products: Derivation and characterization of cell substrates used for production of biotechnological/biological products. Available at: https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-5-d-derivation-characterisation-cell-substrates-used-production-biotechnological/biological-products-step-5_en.pdf (Accessed March 14, 2023).

Faull, K. J., and Williams, C. R. (2015). Intraspecific variation in desiccation survival time of *Aedes aegypti* (L) mosquito eggs of Australian origin. *J. Vector Ecol.* 40, 292–300. doi:10.1111/jvec.12167

Feachem, R. G. A., Chen, I., Akbari, O., Bertozzi-Villa, A., Bhatt, S., Binka, F., et al. (2019). Malaria eradication within a generation: Ambitious, achievable, and necessary. *Lancet* 394, 1056–1112. doi:10.1016/S0140-6736(19)31139-0

Food and Agriculture Organization (2017). Guidelines for standardized mass rearing of *Anopheles* mosquitoes. Available at: https://www.iaea.org/sites/default/files/21/06/nafa-ipc-manual-guidelines-for-standardised-mass-rearing-of-anopheles-mosquitoes-v1.0.pdf (Accessed March 14, 2023).

Food and Agriculture Organization (2022). International guideline for transboundary shipments of irradiated sterile insects. Available at: https://www.iaea.org/sites/default/files/2022.transboundary_shipments_of_sterile_insects.pdf (Accessed March 21, 2023).

Food and Agriculture Organization (2005). ISPM3. Guidelines for the export, shipment, import and release of biological control agents and other beneficial organisms. Available at: https://www.fao.org/3/j5365e/j5365e.pdf (Accessed April 10, 2023).

Food and Agriculture Organization (2012). The FAO/IAEA spreadsheet for designing and operation of insect mass rearing facilities. Available at: https://www.iaea.org/resources/manual/the-fao/iaea-spreadsheet-for-designing-and-operation-of-insect-mass-rearing-facilities (Accessed March 14, 2023).

Francaise (2021). Dengue control: The first drone release of sterile mosquitoes in reunion. Available at: https://www.cirad.fr/en/press-area/press-releases/2021/drone-release-of-sterile-mosquitoes (Accessed March 15, 2023).

Gallichote, E. N., Dobos, K. M., Ebel, G. D., Hagedorn, M., Rasgon, J. L., Richardson, J. H., et al. (2023). Towards a method for cryopreservation of mosquito vectors of human pathogens. *Cryobiology* 99, 1–10. doi:10.1016/j.cryobiol.2021.02.001

Garcia, G. de A., Sylvestre, G., Aguiar, R., da Costa, G. B., Marins, A. J., Lima, J. B. P., et al. (2019). Matching the genetics of released and local *Aedes aegypti* populations is critical to assure *Wolbachia* invasion. *PLoS Neglected Trop. Dis.* 13, e0007023. doi:10.1371/journal.pntd.0007023

Global Health Network (2023). Global vector hub. Available at: https://globalvectorhub.tghn.org/vector-species/anopheles-mosquitoes/(Accessed March 13, 2023).

Government of Brazil. (2020). Official diary of the union. Extract from Technical Opinion No. 6,946/2020. Available at: . https://www.in.gov.br/web/dou/-/extrato-de-parecer-tecnico-n-6.946/2020-258262552

Guissou, C., Quinlan, M. M., Sanou, R., Ouedraogo, R. K., Namountougou, M., and Diabate, A. (2022). Preparing an insectary in Burkina Faso to support research in genetic technologies for malaria control. *Vector-borne Zonotic Dis.* 22, 18–28. doi:10.1089/vbz.2021.0041

Gunathilaka, N., Ranathunge, T., Udayanga, L., Wijegunawardena, A., Gilles, J. R. L., and Abeyewickreme, W. (2019). Use of mechanical and behavioural methods to eliminate female *Aedes aegypti* and *Aedes albopictus* for sterile insect technique and incompatible insect technique applications. *Parasites Vectors* 12, 148. doi:10.1186/s13071-019-3398-7

Haakenstad, A., Harle, A. C., Tsakalos, G., Micah, A. E., Tao, T., Anjomshjoa, M., et al. (2019). Tracking spending on malaria by source in 106 countries, 2000–16: An economic modelling study. *Lancet* 19, 703–716. doi:10.1016/S1473-3099(19)30165-3

Hammond, A., Polgioni, P., Persampieri, T., North, A., Minuz, R., Trusso, A., et al. (2021). Gene-drive suppression of mosquito populations in large cages as a bridge between lab and field. *Nat. Commun.* 12, 4589. doi:10.1038/s41467-021-24790-6

Hay, R. J. (1988). The seed stock concept and quality control for cell lines. *Anal. Biochem.* 17, 225–237. doi:10.1016/0003-2697(88)90480-0

Helinski, M. E. H., Hassan, M. M., El-Motasim, W. M., Malcolm, C. A., Knols, B. G. J., and El-Sayed, B. (2008). Towards a sterile insect field release of *Anopheles arabiensis* mosquitoes in Sudan: Irradiation, transportation, and field cage experimentation. *Malar. J.* 7, 65. doi:10.1186/1475-2875-7-65

Hoang, K. P., Teo, T. M., Ho, T. X., and Le, V. S. (2016). Mechanisms of sex determination and transmission ratio distortion in *Aedes aegypti*. *Parasit. Vectors* 9, 49. doi:10.1186/s13071-016-1331-x

IATA (2023). Questions and answers for shipping live animals by air. Available at: https://www.iata.org/en/publications/newsletters/iata-knowledge-hub/questions-and-answers-for-shipping-live-animals-by-air/(Accessed April 6, 2023).

Institute of Tropical Medicine Antwerp (2022). Malaria – map of Africa. Available at: https://www.wanda.be/en/a-z-index/malaria-kaart-afrika-/(Accessed March 15, 2023).

International Atomic Energy Agency (2017). Guidelines for routine colony maintenance of *Aedes* mosquito species. Available at: https://www.iaea.org/sites/default/files/21/06/nafa-ipc-manual-guidelines-for-routine-colony-maintenance-of-aedes-mosquito-species-v1.0.pdf (Accessed March 14, 2023).

International Atomic Energy Agency (2023). Manuals and protocols. Available at: https://www.iaea.org/topics/insect-pest-control/manuals-and-protocols (Accessed March 14, 2023).

International Atomic Energy Agency (2018). The dashboard for managers of sterile insect technique production facilities. Available at: https://www.iaea.org/resources/manual/the-dashboard-for-managers-of-sterile-insect-technique-production-facilities (Accessed March 14, 2023).

James, E. R., Wen, Y., Overby, J., Pluchino, K., McTighe, S., Matheny, S., et al. (2022). Cryopreservation of *Anopheles stephensi* embryos. *Sci. Rep.* 12, 43. doi:10.1038/s41598-021-04113-x

James, S., Collins, F. H., Welkhoff, P. A., Emerson, C., Godfray, H. C. J., Gottlieb, M., et al. (2018). Pathway to deployment of gene drive mosquitoes as a potential biocontrol tool for elimination of malaria in sub-saharan Africa: Recommendations of a scientific working group. *Am. J. Trop. Med. Hyg.* 98 (6), 1–49. doi:10.4269/ajtmh.18-0083

James, S. L., Dass, B., and Quemada, H. M. (2023). Regulatory and policy considerations for the implementation of gene drive-modified mosquitoes to prevent malaria transmission. *Transgenic Res.* 32, 17–32. doi:10.1007/s11248-023-00335-z

James, S. L., Marshall, J. M., Christophides, G. K., Okumu, F. O., and Nolan, T. (2020). Toward the definition of efficacy and safety criteria for advancing gene drive-modified mosquitoes to field testing. *Vector-Born Zoonot. Dis.* 20, 237–251. doi:10.1089/vbz.2019.2606

Kaiser, M. L., Wood, O. R., Damiens, D., Brooke, B. D., Koekmoer, L. L., and Munhenga, G. (2021). Estimates of the population size and dispersal range of *Anopheles arabiensis* in northern KwaZulu-natal, South Africa: Implications for a planned pilot programme to release sterile male mosquitoes. *Parasites Vectors* 14, 205. doi:10.1186/s13071-021-04674-w

Kyrou, K., Hammond, A. M., Galizi, R., Kranjc, N., Burt, A., Beaghton, A. K., et al. (2018). A CRISPR-Cas9 gene drive targeting *doublesex* causes complete population suppression in caged *Anopheles gambiae* mosquitoes. *Nat. Biotechnol.* 36, 1062–1066. doi:10.1038/nbt.4245

Lutrat, C., Glesbrecht, D., Marois, E., Whyard, S., Bakdet, T., and Bouyer, J. (2019). Sex sorting for pest control: it's raining men. *Trends Parasitol.* 35, 649–662. doi:10.1016/j.pt.2019.06.001

Maiga, H., Bakhoum, M. T., Mamai, W., Diouf, G., Somda, N., Wallner, T., et al. (2023). From the lab to the field: Long-distance transport of sterile *Aedes* mosquitoes. *Insects* 14, 207. doi:10.3390/insects14020207

Maiga, H., Mamai, W., Somda, N. S. B., Wallner, T., Poda, B. S., Salvador-Herranz, G., et al. (2020). Assessment of a novel adult mass-rearing cage for *Aedes albopictus* (Skuse) and *Anopheles arabiensis* (Patton). *Insects* 11, 801. doi:10.3390/insects11110801

Marina, C. F., Liedo, P., Bond, J. G., Osorio, A. R., Valle, J., Angulo-Kladt, R., et al. (2022). Comparison of ground releases and drone-mediated aerial release of *Aedes aegypti* sterile males in southern Mexico: Efficacy and challenges. *Insects* 12, 347. doi:10.3390/insects13040347

Marois, E., Scali, C., Soichot, J., Kappler, C., Levashina, E. A., and Catteruccia, F. (2012). High-throughput sorting of mosquito larvae for laboratory studies and for future vector control interventions. *Malar. J.* 11, 302. doi:10.1186/1475-2875-11-302

Mazigo, E., Kidima, W., Myamba, J., and Kweka, E. J. (2019). The impact of *Anopheles gambiae* egg storage for mass rearing and production success. *Malar. J.* 18, 52. doi:10.1186/s12936-019-2691-4

Mechan, F., Bartonicek, Z., Malone, D., and Lees, R. S. (2023). Unmanned aerial vehicles for surveillance and control of vectors of malaria and other vector-borne diseases. *Malar. J.* 22, 23. doi:10.1186/s12936-022-04414-0

Medcalf, N. (2016). Centralized or decentralized manufacturing? Key business model considerations for cell therapies. *Cell Gene Ther. Insights* 2, 95–109. Available at: https://www.insights.bio/cell-and-gene-therapy-insights/journal/article/519/Centralized-or-decentralized-manufacturing-Key-business-model-considerations-for-cell-therapies.

Meyer, A., Holt, H. R., Selby, R., and Guitian, J. (2016). Past and ongoing tsetse and animal trypanosomiasis control operations in five african countries: A systematic review. *PLoS Negl. Trop. Dis.* 10, e0005247. doi:10.1371/journal.pntd.0005247

Meza, J. S., ul Haq, I., Vreysen, M. J. B., Bourtzas, K., Kyritsis, G. A., and Caceres, C. (2018). Comparison of classical and transgenic genetic sexing strains of Mediterranean fruit fly (Diptera: Tephritidae) for application of the sterile insect technique. *PLoS ONE* 13, e0208880. doi:10.1371/journal.pone.0208880

MosquitoMate (2023). MosquitoMate: How it works. Available at: https://mosquitomate.com/how-it-works/(Accessed March 14, 2023).

Mysore, K., Sun, L., Rkoethele, J. B., Li, P., Igieded, J., Misenti, J. K., et al. (2021). A conserved female-specific larval requirement for MtnB function facilitates sex separation in multiple species of disease vector mosquitoes. *Parasit. Vectors* 14, 338. doi:10.1186/s13071-021-04844-w

European Food Safety AuthorityNaegeli, H., Bresson, J., Dalmay, T., Dewhurst, I. C., Epstein, M. M., et al. (2020). Adequacy and sufficiency evaluation of existing EFSA guidelines for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of genetically modified insects containing engineered gene drives. *EFSA J.* 18, 6297. doi:10.2903/j.efsa.2020.6297

National Environment Agency Singapore (2023). *Wolbachia-Aedes* mosquito suppression strategy. Available at: https://www.nea.gov.sg/corporate-functions/resources/research/wolbachia-aedes-mosquito-suppression-strategy (Accessed March 14, 2023).

North, A. R., Burt, A., and Godfray, C. J. (2019). Modelling the potential of genetic control of malaria mosquitoes at national scale. *BMC Biol.* 17, 26. doi:10.1186/s12915-019-0645-5

Organization for Economic Cooperation and Development. (2023). Harmonization of regulatory oversight in biotechnology. Available at: https://www.oecd-ilibrary.org/environment/harmonisation-of-regulatory-oversight-in-biotechnology_23114622 [Accessed March 2023].

Oxitec (2023a). *Aedes do Bem*. Available at: https://www.aedesdobem.com.br/ (Accessed May23, 2023).

Oxitec (2023c). Friendly™ *Anopheles stephensi*. Available at: https://www.oxitec.com/anopheles-stephensi (Accessed March 15, 2023).

Oxitec (2023b). Just add water: Oxitec's new FriendlyTM mosquito mini-capsule technology rapidly suppresses 95% of disease-spreading *Aedes aegypti* in Brazil trial. Available at: https://www.oxitec.com/en/news/just-add-water-oxitecs-new-friendly-mosquito-mini-capsule-technology-rapidly-suppresses-95-of-disease-spreading-aedes-aegypti-in-brazil-trial (Accessed March 14, 2023).

Pham, T. B., Phong, C. H., Bennett, J. B., Hwang, K., Jasinkiene, N., Parker, K., et al. (2019). Experimental population modification of the malaria vector mosquito *Anopheles stephensi*. *PLoS Genet.* 15, e1008440. doi:10.1371/journal.pgen.1008440

Public Broadcasting System (2018). The future of genetically modified mosquitoes could be in mini, moveable labs. Available at: https://www.pbs.org/newshour/science/the-future-of-genetically-modified-mosquitoes-could-be-in-mini-moveable-lab (Accessed March 15, 2023).

Rabinovich, R. N., Drakeley, C., Djimde, A. A., Hall, B. F., Hay, S. I., Hemingway, J., et al. (2017). malERA: An updated research agenda for malaria elimination and eradication. *PLoS Med.* 14, e1002456. doi:10.1371/journal.pmed.1002456

Rayment, E. A., and Williams, D. J. (2010). Concise review: Mind the gap: Challenges in characterizing and quantifying cell- and tissue-based therapies for clinical translation. *Stem Cells* 28, 996–1004. doi:10.1002/stem.416

Republic of South Africa (2018). Sterile insect technique field trials to eliminate malaria under way. Available at: https://www.dst.gov.za/index.php/media-room/latest-news/2662-sterile-insect-technique-field-trials-to-eliminate-malaria-under-way (Accessed March 15, 2023).

Romeis, J., Collatz, J., Glandorf, D. C. M., and Bonsall, M. B. (2020). The value of existing regulatory frameworks for the environmental risk assessment of agricultural pest control using gene drives. *Env. Sci. Policy* 108, 19–36. doi:10.1016/j.envsci.2020.02.016

Sarvari, M., Alavi-Moghadamm, S., Larijani, B., Rezazadeh, I., and Arjmand, B. (2020). in *Principles of good manufacturing practice in biomedical product development: Bench to bedside* B. Arjmand, M. Payab, and P. Goodarzi (Switzerland: Springer), 61–68. doi:10.1007/978-3-030-35626-2_6

Senecio (2023b). Release technologies. Available at: https://www.senecio-robotics.com/release-solutions (Accessed March 15, 2023).

Senecio (2023a). Senecio AI based mosquito sex sorting module. Available at: https://www.senecio-robotics.com/(Accessed March 15, 2023).

Sinka, M. E. (2012). Global distribution of the dominant vector species of malaria *Anopheles* mosquitoes – new insights into malaria vectors. doi:10.5772/54163

Spinner, S. A. M., Barnes, Z. H., Puinean, A. M., Gray, P., Dafa'alla, T., Phillips, C. E., et al. (2022). New self-sexing *Aedes aegypti* strain eliminates barriers to scalable and sustainable vector control for governments and communities in dengue-prone environments. *Front. Bioeng. Biotechnol.* 10, 975786. doi:10.3389/fbioe.2022.975786

Tadesse, F. G., Ashine, T., Teka, H., Esayas, E., Messenger, L. A., Chali, W., et al. (2021). *Anopheles stephensi* mosquitoes as vectors of Plasmodium vivax and falciparum, Horn of Africa, 2019. *Emerg. Infect. Dis.* 27, 603–607. doi:10.3201/eid2702.200019

Taracena, M. L., Hunt, C. M., Benedict, M. Q., Pennington, P. M., and Dotson, E. M. (2019). Downregulation of female doublesex expression by oral-mediated RNA interference reduces number and fitness of *Anopheles gambiae* adult females. *Parasit. Vectors* 12, 170. doi:10.1186/s13071-019-3437-4

U.S. Agency for International Development (2011). The logistics handbook. A practical guide for the supply chain management of health commodities. Available at: https://ghsupplychain.org/sites/default/files/2019-07/Logistics%20Handbook.pdf (Accessed March 13, 2023).

U.S. Centers for Disease Control and Prevention (2020). Biosafety in microbiological and biomedical laboratories. Available at: https://www.cdc.gov/labs/pdf/SF__19_308133-A_BMBL6_00-BOOK-WEB-final-3.pdf (Accessed March 14, 2023).

U.S. Centers for Disease Control and Prevention (2011). MR4 Methods in *Anopheles* research. Available at: https://www.yumpu.com/en/document/read/49409322/methods-in-anopheles-research-mr4 (Accessed March 14, 2023).

U.S. Environmental Protection Agency (2023). About pesticide registration. Available at: https://www.epa.gov/pesticide-registration/about-pesticide-registration (Accessed March 21, 2023).

U.S. Environmental Protection Agency (2020). EPA approves experimental use permit to test innovative biopesticide tool to better protect public health. Available at: https://www.epa.gov/pesticides/epa-approves-experimental-use-permit-test-innovative-biopesticide-tool-better-protect (Accessed March 14, 2023).

U.S. Food and Drug Administration (2010). Guidance for industry. Characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications. Available at: https://www.fda.gov/media/78428/download (Accessed March 14, 2023).

U.S. Food and Drug Administration (2015). Guidance for industry. Regulation of genetically engineered animals containing heritable recombinant DNA constructs. Available at: https://www.fda.gov/media/135115/download (Accessed March 23, 2023).

U.S. Food and Drug Administration (1997). HACCP principles and application guidelines. Available at: https://www.fda.gov/food/hazard-analysis-critical-control-point-haccp/haccp-principles-application-guidelines#execsum (Accessed March 13, 2023).

U.S. Food and Drug Administration (1999). Q6B specifications: Test procedures and acceptance criteria for biotechnological/biological products. Available at: https://www.fda.gov/media/71510/download (Accessed March 20, 2023).

World Bank (2023). Land area (sq. km) - sub-Saharan Africa. Available at: https://data.worldbank.org/indicator/AG.LND.TOTL.K2?locations=ZG (Accessed March 15, 2023).

World Health Organization (2020a). Evaluation of genetically modified mosquitoes for the control of vector-borne Diseases. Available at: https://apps.who.int/iris/bitstream/handle/10665/336031/9789240013155-eng.pdf (Accessed March 13, 2023).

World Health Organization (2022b). Global framework for the response to malaria in urban areas. Available at: https://www.who.int/publications/i/item/9789240061781 (Accessed March 15, 2023).

World Health Organization (2015). Global technical strategy for malaria 2016-2030. Available at: https://www.who.int/docs/default-source/documents/global-technical-strategy-for-malaria-2016-2030.pdf (Accessed March 15, 2023).

World Health Organization (2021). Guidance framework for testing genetically modified mosquitoes. Available at: https://www.who.int/publications/i/item/9789240025233 (Accessed March 13, 2023).

World Health Organization (2020b). Guidance framework for testing the sterile insect technique as a vector control tool against *Aedes*-borne diseases. Available at: https://www.who.int/publications/i/item/9789240002371 (Accessed March 14, 2023).

World Health Organization (2020c). Laboratory biosafety manual. Available at: https://www.who.int/publications/i/item/9789240011311 (Accessed March 14, 2023).

World Health Organization (2019). Malaria eradication: Benefits, future scenarios and feasibility. Executive summary, WHO strategic advisory group on malaria eradication. Available at: https://www.who.int/publications/i/item/WHO-CDS-GMP-2019.10 (Accessed March 13, 2023).

World Health Organization (2023a). Vector-borne diseases. Available at: https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases (Accessed March 13, 2023).

World Health Organization (2023b). Welcome to inspection services. Available at: https://extranet.who.int/pqweb/inspection-services (Accessed March 14, 2023).{

World Health Organization (2020d). World malaria report 2020. Available at: https://www.who.int/publications/i/item/9789240064898 (Accessed March 13, 2023).

World Health Organization (2022a). World malaria report 2022. Available at: https://www.who.int/publications/i/item/9789240064898 (Accessed March 13, 2023).

World Mosquito Program (2023). Our work. Available at: https://www.worldmosquitoprogram.org/en/work (Accessed March 14, 2023).

World Organization for Animal Health (2022). Safety, regulatory and environmental issues related to international trade of live insects. Available at: https://doc.woah.org/dyn/portal/index.xhtml?page=alo&aloId=42654 (Accessed April 6, 2023).

Yadav, P. (2015). Health product supply chains in developing countries: Diagnosis of the root causes of underperformance and an agenda for reform. *Health Syst. Reform* 1, 142–154. doi:10.4161/23288604.2014.968005

Yamada, H., Soliban, S. M., Vreysen, M. J., Chadee, D. D., and Gilles, J. R. (2013). Eliminating female *Anopheles arabiensis* by spiking blood meals with toxicants as a sex separation method in the context of sterile insect technique. *Parasites Vectors* 6, 197. doi:10.1186/1756-3305-6-197

Zeng, Q., She, L., Yuan, H., Luo, Y., Wang, R., Mao, W., et al. (2022). A standalone incompatible insect technique enables mosquito suppression in the urban subtropics. *Commun. Biol.* 5, 1419. doi:10.1038/s42003-022-04332-6

Zubair, Q., Matthews, H., Sougoufara, S., Mujeeb, F., Ashall, S., Aboagye-Antwi, F., et al. (2021). Bulk-up synchronization of successive larval cohorts of *Anopheles gambiae* and *Anopheles coluzzii* through temperature reduction at early larval stages: Effects on emergence rate, body size and mating success. *Malar. J.* 20, 67. doi:10.1186/s12936-021-03602-8

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Original Research
PUBLISHED 09 June 2023
DOI 10.3389/fbioe.2023.1209308

OPEN ACCESS

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RECEIVED 20 April 2023
ACCEPTED 26 May 2023
PUBLISHED 09 June 2023

CITATION
Zarate S, Cimadori I, Jones MS, Roca MM and Barnhill-Dilling SK (2023), Assessing agricultural gene editing regulation in Latin America: an analysis of how policy windows and policy entrepreneurs shape agricultural gene editing regulatory regimes.
Front. Bioeng. Biotechnol. 11:1209308.
doi: 10.3389/fbioe.2023.1209308

Assessing agricultural gene editing regulation in Latin America: an analysis of how policy windows and policy entrepreneurs shape agricultural gene editing regulatory regimes

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This article explores the new developments and challenges of agricultural Gene Editing (GED) regulation in primarily nine countries of Latin America and the Caribbean (LAC) Region: Argentina, Bolivia, Brazil, Colombia, Guatemala, Honduras, Mexico, Paraguay and Peru. As Gene Editing technology develops, Latin America and the Caribbean regulatory regimes struggle to keep pace. Developers and regulators face challenges such as consumer perceptions, intellectual property, R&D funding (private and public), training, environmental and social impact, and access to domestic and international markets. Some Latin America and the Caribbean countries (e.g., Argentina) interpret existing legislation to promulgate regulations for biotechnology and Genetically Modified Organisms (GMOs), while others (e.g., Brazil and Honduras) have specific legislation for Genetically Modified Organisms. In both those cases, often a case-by-case approach is chosen to determine whether a Gene Editing organism is subject to Genetically Modified Organisms regulations or not. Other countries such as Peru have opted to ban the technology due to its perceived resemblance to transgenic Genetically Modified Organisms. After presenting the regulatory landscape for agricultural Gene Editing in Latin America and the Caribbean, this article addresses some of the differences and similarities across the region. Some countries have had more foresight and have dedicated resources to increase capacity and develop regulations (e.g., Brazil, Argentina, Colombia, Guatemala, Honduras, Mexico before 2018) while others struggle with bureaucratic limitations and partisanship of policymaking (e.g., Paraguay, Bolivia, Peru, Mexico after 2018). We propose that the differences and similarities between these regulatory regimes have emerged in part as a result of policy entrepreneurs (influential individuals actively involved in policy making) taking advantage of policy windows (opportunities for shaping policy and regulation). The third and remaining sections of this study discuss our main findings. Based on 41 semi structured interviews with regulators, scientists, product developers, NGOs and activists, we arrived at three main findings. First, there seems to be a consensus among most regulators interviewed that having harmonized regimes is a positive step to

facilitate product development and deployment, leading to commercialization. Second, reducing bureaucracy (e.g., paper work) and increasing flexibility in regulation go hand in hand to expedite the acquisition of key lab materials required by developers in countries with less robust regimes such as Peru and Bolivia. Finally, developing public and private partnerships, fostering transparency, and increasing the involvement of marginalized groups may increase the legitimacy of Gene Editing regulation.

KEYWORDS

gene editing, Latin America, policy, regulation, agricultural biotechnology

1 Introduction

GED is a new set of technologies that allow for targeted DNA modifications, with the most recent discovery being Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). CRISPR is a bacterial immune system that has been repurposed to be used in eukaryotic cells of animals and plants (Innovative Genomic Institute website, 2022). By combining CRISPR with the Cas9 protein, it is possible to make a cut in the DNA at a desired location and add, delete, or alter one or more nucleotides (Shukla-Jones, Friedrichs, and Winickoff, 2018). Gene Editing (GED) has increasingly attracted attention from scientists, policymakers, and regulators due to its potential uses in agriculture and human health. In the case of agriculture, it can be used to increase production, address climate change, and foster sustainability.

GED is known to be more precise than Genetic Modification (GM). Genetically Modified Organisms (GMOs) are created by inserting genes in random and multiple locations in the genome (Kuzma, 2018), with a low level of efficiency. GM is mostly used to create transgenic organisms, which require the insertion of foreign species' DNA sequences in the modified organism. Instead, CRISPR can be used to create a cisgenic modification in which genes from within the same species are efficiently transferred through a single or set of base pair swap(s), or by performing a simple "knockout" or removing a sequence to alter an organism's function or form (Kuzma, 2018). However, CRISPR can be also used to create transgenic organisms. This would happen if a donor template is provided that contains genes belonging to another species (or that are synthetic).

Due to these complexities, regulating GED, and CRISPR technology in particular, has become a challenge. Domestic and international regulatory bodies struggle to keep pace with emerging technologies such as GED, with many countries yet to commit to a path for regulation (Pixley et al., 2022). There is an important ongoing global debate about whether or not GED and GM should be regulated under the same frameworks. This is because GED may or may not involve the transitory introduction of foreign DNA sequences, may or may not result in transgenic products, and may or may not generate products that are different from those created through conventional breeding (Pixley et al., 2022). As a result, countries around the world have chosen different approaches on how to regulate GED technologies, with some implementing product-based regulations and others process-based (Entine et al., 2021).

For example, the United States, similarly to Argentina, does not have specific GMO or GED laws and instead uses current existing laws to promulgate GMOs regulations, focusing on the product rather than the method used to produce it (EPA website, 2017). On the other hand, the Court of Justice of the European Union (EU) determined in 2018 that organisms obtained through new mutagenesis techniques (including GED) are GMOs. As a result, GED products are currently still subject to the GMO-specific sets of regulations in the EU, which focus more on the process rather than the product (Van Der Meer et al., 2020). Concerning this issue, a recent article by industry authors Jenkins et al. argues that "process-based differential regulatory systems will also have a negative effect on the democratization of the technology" and that "regulation based on process will not advance common goals of nutrition, sustainability or consumer preference" (Jenkins et al., 2023). These authors focus on biotech companies' growth, access to technology and regulatory burdens rather than food sovereignty challenges related to family farming which is common in some LAC countries.

In addition to these technical struggles, some contingents of advocacy groups and segments of the public continue to raise concerns about potential hazards of biotechnology products, adding to political and economic pressures that have shaped the design of regulatory regimes in countries such as those included in this article. For example, international environmental NGOs raise questions about potential hazards of GED and GMO products. While academics, regulators, and policymakers tend to regard these concerns as unscientific, concerns about toxicity and hazard potential of these products are still an important part of the landscape of innovation and potential deployment of GED products.

In this paper we focus on nine countries in the Latin America and the Caribbean (LAC) region. Drawing on concepts such as regulatory regimes, policy windows and policy entrepreneurs to describe and analyze the governance of agricultural GED in most of the LAC countries, we focus on the political dimensions that shape agricultural GED regulatory regimes by exploring how the domestic politics of nine LAC countries have created a heterogeneous patchwork of regulatory systems. Finally, we analyze the role of policy entrepreneurs in shaping policy discourse and regulatory regimes in the region.

2 Background: Governance of GED in LAC

2.1 Agriculture, biodiversity and innovation for agriculture in LAC

Accounting for more than 5% of GDP in over twenty countries and generally between 8% and 30% of employment (Morris et al.,

2020; World Bank, 2023), primary agriculture, or cultivation of crops and breeding of livestock, remains a fundamental economic activity throughout the LAC region. This importance compounds further when 'backward' linkages to input sectors and 'forward' linkages to processing, transport, and retail sectors are considered. For example, while primary agriculture may compose only 3.8% of GDP in Chile, the compounded value-added share of GDP within the agri-food sector is estimated to reach 6.4% (Foster and Valdés, 2015). While experiencing sometimes volatile year-over-year fluctuations, the growth of agriculture (including fisheries) in the LAC region averages about 2.7% over the past 2 decades (OECD, 2019). Commodity trade is a particularly key export sector and source of foreign currency. Export products such as soybeans, pork, beef, maize, poultry, animal feed, sugar, coffee, fruits, and vegetables are drivers of LAC's agricultural sector (OECD, 2019). The leading food exporter is Brazil (USD 79.3 billion in 2017), followed by Argentina (USD 35.0 billion), Mexico (USD 32.5 billion), Chile (USD 17 billion), Ecuador (USD 10.4 billion), and Peru (USD 8.8 billion) (OECD, 2019). During the past 2 decades, LAC's agricultural trade surplus has increased, reaching USD 104.3 billion in 2017 (OECD, 2019).

As suggested by Roca et al., 2004, the LAC region is an important center of origin and diversity for different organisms that contribute to the world's food security. However, LAC's biodiversity is under pressure, since an estimated 12% of known wild plant and animal species are under threat of extinction (Brooks et al., 2016). The LAC region is the source of around 60% of the global terrestrial, freshwater and marine biodiversity (UNEP-WCMC, 2016). Another important dimension of LAC's biodiversity is agrobiodiversity, which is defined as the genetic diversity of crop and non crop species (Morris et al., 2020), and is the result of the interactions between natural and human systems (Bioversity International, 2017).

Because of the abundance of its biodiversity, the LAC region has become a hub for innovation and technology development for agrifood systems. For instance, plant biotechnology has become relevant for increasing LAC's production, economic and social growth (Gatica Arias, 2020). The same is true for GED in animals, where de Almeida Camargo and Pereira, (2022) argue that through gene editing local dairy cattle breeds, milk production can be increased, contributing to food security. Farmers in countries that allow GMOs may be able to produce more food per unit of land with fewer inputs, cultivate areas considered not suitable for agriculture and agrobiodiversity (Gatica Arias, 2020). However, as mentioned above, LAC's NGOs and environmental groups are very likely to remain hesitant about these technologies.

Another critical dimension of the landscape of GED in LAC is how intellectual property rights (IPRs) intersect with agrobiodiversity conservation, the privatization of seeds and food security. According to Lokhandwala 2022, a relevant amount of the agrobiodiversity legal framework "lies within the intellectual property space". Some authors have described how countries such as Argentina, Brazil and Paraguay have created favorable environments for biotechnology and IPR regimes (Newell, 2009; Filomeno, 2014). Intellectual property protections generally go hand in hand with maturity of biosafety regulations and are generally critical for private sector entrance into this space. The strength of IPR regimes have been used to categorize the maturity of biotechnology infrastructure in Latin American countries (Trigo et al., 2010). During the 1990s, most LAC countries adopted a neoliberal approach to agriculture due to severe financial problems that those countries were facing between the 1980s and the 1990s (Filomeno, 2014). Agriculture was seen as a strategic economic sector to continue paying foreign debt and achieve monetary stabilization. Intellectual property surrounding seeds and plant varietal development is quite controversial to some authors, while absolutely essential to others.

In a policy brief for the Inter-American Development Bank focused on Latin American biotechnology and the patent and licensing environment, Bagley (2021) notes the rapid growth of CRISPR patent families in the region and the importance of licensing structures to facilitate access to GED technologies. Foundational CRISPR-Cas9 patent holders are US-based, though (at the time of writing) the firm Corteva offers a bundle licensing approach for plant agriculture, namely,: 1) an internal only R&D license; 2) a commercial seeds and crop trait products license; 3) a commercial license for other (non-livestock) agricultural products (such as using a plant as a factory to produce therapeutic proteins); 4) a license to provide CRISPR-Cas9 services; and 5) a no-cost academic research license. The manner in which Latin American public and private sector entities are able to effectively access licenses and translate innovations to their populations will be extremely important in determining to what extent small scale producers will ultimately benefit from novel GED technologies.

2.2 Differences in LAC regulations for GED and GMOs

Since the emergence of the first generation of GMOs, the various countries of the region have taken different stances towards the applications of these technologies in agriculture, for multiple reasons illustrated in recent scholarship (for example, Roca et al., 2023). Some of these key differences are illustrated in Figure 1 below[1].

Based on a recent study (Kuiken & Kuzma, 2021), it seems that countries with a longer history of biotechnology regulation such as Argentina, Colombia and Brazil are more open to innovation in general. On the other hand, countries such as Bolivia and Peru, that have a complicated history with biotechnology, seem to have more active and influential anti GMOs groups engaged in domestic politics. Countries such as Brazil and Argentina, which have a stronger culture of industries and startups, have more training opportunities compared to countries in which the private sector is not actively exploring opportunities to invest such as Paraguay (Zarate et al., 2023). Additionally, another important difference between countries' regulations is the way in which they regulate cisgenic and transgenic organisms. In the next paragraph we explain more in detail these differences.

1 It is important to note that Mexico's current administration has a different attitude towards GMO and GED regulation. Additionally, it is only since 2016 that Guatemala and Honduras have a harmonized regulation driven by the Customs Union Agreement. In our results section we explain more in detail the development of GED regulations in these countries.

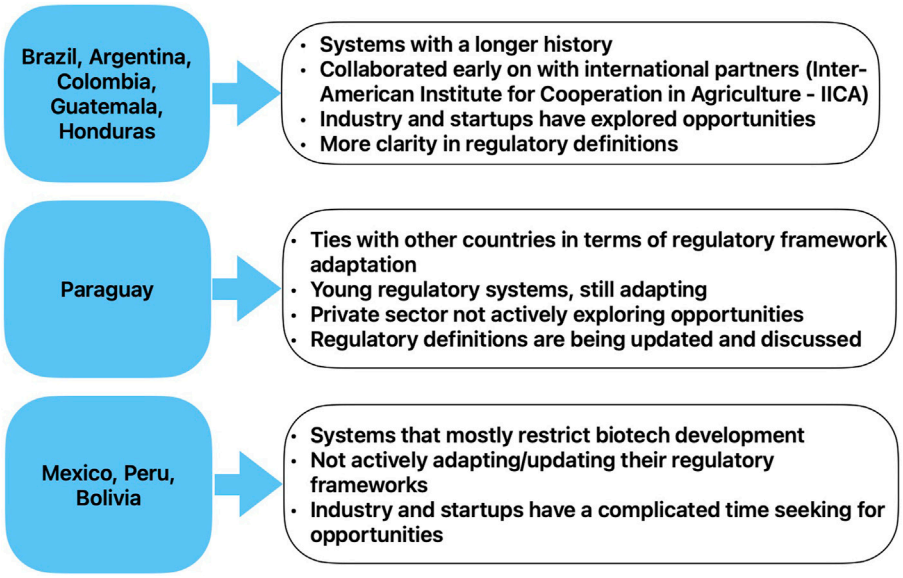

FIGURE 1
Broad illustration of some differences in LAC countries' regulation and key policy aspects.

Due to its advanced regulatory approach to GED products, Argentina is perceived as being a leader in the region, at least according to most regulators and decision makers that we interviewed. Argentina is among the world's top producers of GM crops, having approved 48 varieties for commercial use (Whelan and Lema, 2019) and has one of the oldest regulatory systems for biotechnology in LAC. In Argentina, the decision of whether GED products are subject to GMO regulations is taken by the National Advisory Commission for Agricultural Biotechnology (CONABIA, Comisión Nacional Asesora de Biotecnologia Agropecuaria) on a case-by-case basis according to the criteria of "novel combination of genetic material" (Kuiken & Kuzma, 2021). In particular, some varieties of GED crops most likely will not be considered GMOs in Argentina if the final product submitted to the authorities does not contain any transgenic DNA (Kuiken & Kuzma, 2021). GMO regulations were not altered and no exemptions were established for GED crops. It is important to mention that Argentina, like the United States, uses pre existing laws for the protection of the environment, food, animal health and plants to regulate GMOs and biotechnology in general. Argentinian regulators are considered global experts in this field. Based on our interviews, regulators and researchers in favor of promoting the use of GED in varietal development seem to share a desire for harmonizing regulations in the region based on the Argentinian model. Argentina is also one of the few countries that did not ratify the Cartagena Protocol on Biosafety (CPB) which is part of the Convention on Biological Diversity (Convention on Biological Diversity website, 2014). The CPB, which regulates the transboundary transfer of GMOs, was negotiated from 1996 to 2000 and entered in force in 2003 (Gupta and Falkner, 2006). This is important because other countries included in this study (Bolivia, Brazil, Colombia, Peru, Guatemala, Honduras, Mexico and Paraguay) have ratified it, therefore they need to implement new regulations to comply with these commitments (ECLAC website, 2003).

Brazil is another top country in the region for biotechnology crop production. It is actually the second in the world, with more that 100 GM events approved for consumption (Kuiken & Kuzma, 2021). Brazil is considered to have a robust regulatory capacity (Roca et al., 2023), with specific GMO regulations, and also ratified the CPB. The National Technical Commission on Biosafety (Comissão Técnica Nacional de Biossegurança - CTNBio) is in charge of determining whether a GED product is considered GMO or not on a case-by-case basis. Similarly to Argentina, if the GED product does not contain transgenes, it will most likely not be considered a GMO (Kuiken & Kuzma, 2021). On the other hand, in recent times, Mexico appears to have changed its regulatory stance towards GMOs despite being the 16th country in the world for biotechnology crops planted (Kuiken & Kuzma, 2021). In February 2023, the Mexican president issued a decree (President of the United Mexican States, 2023; Swanson and Qiu, 2023) which replaced the 2020 decree (President of the United Mexican States, 2020) that proposed a phased ban on all imports and approvals of GMO corn[2]. The new decree still requires a phased ban of glyphosate applications and GMO corn imports while at the same time requiring regulatory bodies to provide sustainable and culturally appropriate alternatives.

It is important to note that Mexico has not yet decided whether GED products will be considered GMOs or not under the Biosafety law, which currently regulates biotechnology related products (Kuiken & Kuzma, 2021). Similarly, Venezuela imposed a ban on

2 The current government has not approved any new GMOs since May 2018, rejecting additional permits to plant GMO cotton in 2019 although previously approved (Kuiken & Kuzma, 2021; Roca et al., 2023).

GMO cultivation, as did Ecuador (constitutional prohibition) and Peru (GMO moratorium extended to 2030). Other countries in the region, such as Bolivia, do have regulations that govern the use, importation, and trade of GMOs as part of the CPB implementation process (Kuiken & Kuzma, 2021). However, there is the need to clarify and align the definitions contained in those laws in order to determine whether GED applications will be subject to the GMO legislation in Bolivia (Kuiken & Kuzma, 2021). Honduras also ratified the CPB and has regulated biotechnology products since 1998. Honduras is ranked 20th in the world for biotech crop planted area.

Although some countries took a very different stance on GED, the majority of the LAC region appears to share similar approaches to GED governance (Kuiken & Kuzma, 2021), with some GED products not being regulated as GMOs. However, Kuiken & Kuzma point to the uncertainty of how those differences will impact further negotiations at the global level, particularly within the CPB, where the EU and other reticent countries may hold strong influence. As it will be illustrated later in this article, the ratification of the CPB appears to have motivated policymakers in some LAC countries to expedite decision making on biotechnology and GED normatives.

This paper seeks to better understand the complexities of LAC's regulatory landscape by focusing on the political and social dimensions of agricultural GED regulation across nine countries. LAC's GED or GMOs legislation and policy often represents the outcome of multiple negotiations between parties such as governments, regulators, scientists and activists. The next section will explain the theoretical framework designed to understand how domestic and international politics have shaped agricultural GED and GMOs regulation in the region.

3 Theoretical framework: regulatory regimes, policy windows and policy entrepreneurs

3.1 Regulatory regimes

The framework of regulatory regimes will demonstrate how political standpoints and values around GED technology have shaped agricultural biotechnology regulation across different countries of the LAC region. Using a regulatory regime's lens, we can explore "a range of risk-assessment techniques and policy-making approaches to distinguish the different scientific and bureaucratic practices, techniques, and cultures embodied in different fields of risk regulation" (Hood, 2001). This concept is useful to analyze the interests and motivations behind the integration or fragmentation of regulation, unwritten rules or statutory codes, inputs, processes and products, penalties or incentives, professional or cultural biases, rigor and preferred policy instruments, and biases towards market type incentives (Hood, 2001). We are interested in understanding how economic and political interests have shaped regulatory regimes in the LAC region. We focus on the mobilization of those interests across regulatory regimes rather than reducing decision making to maximizing actors' own interests (Hayden, 2003).

Additionally, we are interested in investigating how regulatory regimes are shaped by policy processes. We follow the Weible Christopher, (2014) characterization of policy process research, defined as the "study of the interactions over time between public policy and its surrounding actors, events, and contexts, as well as the policy or policies' outcomes". In this framework, individuals and collectives can be considered actors that make decisions in the context of ambiguity. Events are defined as anticipated or unanticipated incidents, such as elections or crises. Contexts are considered to be shaped by socioeconomic, cultural, infrastructural and biophysical conditions, as well as institutions. According to Feldman, ambiguity is defined as "a state of having many ways of thinking about the same circumstances or phenomena" (Feldman, 1989). Ambiguity is understood as opposed to uncertainty, since the latter refers to the inability to predict an event and the former may be thought of as ambivalence (Zahariadis, 2014).

3.2 Policy entrepreneurs and policy windows

To understand the concept of policy windows as defined by Kingdon, it is necessary to explain the three streams of policy processes: problems, policies and politics. Problems are considered the issues that policymakers and citizens want addressed (Zahariadis, 2014), such as the COVID-19 pandemic. Policies are the ideas and plans developed by experts that compete to gain acceptance in policy networks (Zahariadis, 2014). Politics include the national mood (thinking along common lines and mood swings), pressure groups, and administrative or legislative turnover (Zahariadis, 2014). According to Kingdon, policy windows are "opportunities for advocates of proposals to push their best pet solutions, or to push attention to their special problems" (Kingdon, 2003). Those could include, for example, new elections, a negative event concerning a problem or the ratification of an international agreement.

Policy entrepreneurs are defined as those individuals or corporate actors that have the skill to identify and take advantage of policy windows to push for policies. As Zahariadis mentions, particular organizations can be considered policy entrepreneurs, not just their individual representatives. According to Zahariadis, policy entrepreneurs are more than mere advocates of solutions. Instead, they can be considered power brokers or coalition enablers. If the windows close, opportunities are lost and policy entrepreneurs must wait for the next opportunity to come along (Zahariadis, 2014). Additionally, they must be able to "attach problems to their solutions" and find those willing to be receptive to their ideas (Zahariadis, 2014).

3.3 Assessing regulatory regimes through an analysis of policy windows and policy entrepreneurs

We also seek to understand the policy processes that have positioned biotechnology and GED technologies as key drivers of LAC agriculture. We argue that agricultural GED regulatory regimes were shaped by multiple policy entrepreneurs who took advantage of key policy windows that facilitated or blocked the implementation of regulations in the LAC region (see Figure 2 for conceptual framework). These regulations and policies were often negotiated by most of the interviewees who participated in this study. Some relied on networks that facilitated agreements between governments,

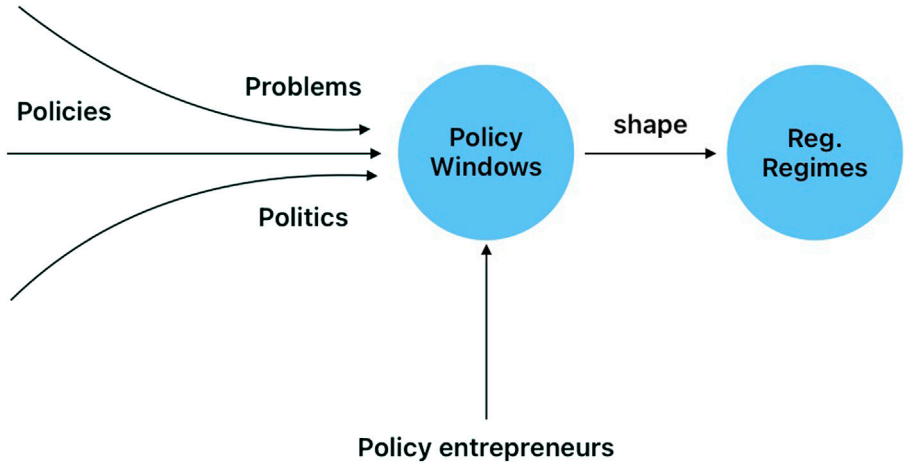

FIGURE 2
Policy entrepreneurs seize policy windows to shape regulatory regimes. Policy windows, according to Zahariadis, are the choices made when "the three streams are coupled or joined together at critical moments".

firms, and universities. Examples will be included in the results section of this paper.

Based on our theoretical framework, we examine how access to markets, legal definitions, formal and informal interactions[3] (Atkinson, 1982; Diefenbach & Sillince, 2011) shape agricultural GED regulation in LAC. Identifying key differences and similarities across LAC's regulatory regimes can contribute to the design and implementation of robust regulatory policies able to tackle key challenges such as increasing capacity, public engagement and public-private partnerships. Finally, this article also addresses a gap in the LAC literature about the governance of emerging technologies since we include the agency of the stakeholders as well as the societal system in which those actions take place.

4 Methods

We conducted 41 semi-structured interviews with experts and other stakeholders on the topic of GED for agriculture. The interviews were conducted over Zoom due to the ongoing COVID-19 pandemic. The data gathered to develop this paper was collected during a broader project carried out through a collaboration between the Inter-American Development Bank (IDB) and the North Carolina State University's Genetic Engineering and Society (GES) Center, which main goals were to evaluate the current state of policies in the LAC region, analyze case studies to understand potential effects of policies directions, and identifying Bank investment priorities[4]. However, while carrying out the interviews analysis, we noticed the emergence of additional relevant information that sparked the idea for this paper and the subsequent analysis with the chosen theoretical frameworks.

The goal was to obtain a clear picture of the situation in the region, particularly concerning the regulatory frameworks in the different countries. The different criteria used to choose the interviewees are the following:

- **Country of origin**
- **Occupation.** The goal was that of interviewing individuals from different sectors, which include regulators, policymakers, researchers in public as well as private institutions and representatives of environmental groups and farming communities. Due to the scope of the original project, our research only included a small group of environmental activists and NGOs.
- **Position toward GED.** with the attempt to capture and reflect the different points of view in the region. As a result, the interviewees were either neutral, leaning pro or doubtful about the application of biotechnology and more specifically GED.

We performed multiple rounds of coding on the interview scripts and notes. First, we focused on the revision of the notes and scripts to identify adequate keywords that would capture the different topics that emerged from the interviews and that we deemed to be relevant for our study. Secondly, we checked that the keywords were used consistently and potentially expanded on additional complementary information. This phase was fundamental to identify some patterns and commonalities in the region concerning GED and biotechnology more broadly and helped us have a better understanding of the situation in the region. Afterwards, based on our understanding of the situation and the observed emerging patterns across the various interviews, we chose relevant existing theoretical frameworks through which to analyze the quotes, which are the ones introduced in the above sections. We therefore focused on some of those keywords that we thought were particularly important to our analysis and some of the corresponding quotes are going to be illustrated in the following

3 Formal interactions are interactions in formal spaces such as those defined by internal policies and norms, or legislation. For instance, Congress is a formal space for passing regulation. Informal spaces are the personal interactions that individual actors or organizations have to negotiate between them without necessarily moving to a formal space.

4 For further information, please visit the project's website at the following link: https://research.ncsu.edu/ges/research/idb-crispr/#top

section. Lastly, an audio and video revision has been carried out to confirm the accuracy of the selected quotes. The more we analyzed the interviews, the more our initial ideas evolved, and therefore some keywords' original meaning was updated to reflect our new interpretation.

The software Taguette was used to perform the above mentioned coding, through which was possible to work collaboratively during this fundamental step of the analysis.

5 Results

5.1 Regulatory regimes: Politics create a landscape of heterogeneous regulatory systems

In Latin America there is a set of diverse regimes, with a tendency from countries with "developing" regulatory regimes to learn and harmonize with countries with more "developed" regimes. However, there is still a desire to maintain a certain degree of autonomy between LAC regulatory regimes. There are multiple reasons why those that are developing their regulatory regimes feel the need to harmonize and improve their regulations to "catch up" with those considered more advanced. The main one is the influence that regulations exercise on the ability to develop products and commercialize them, giving more options to develop ties between product developers, corporations and research institutions like universities. There are multiple interviewees that mention, for example, complications that include expensive processes for approval, problems at the border, problems at acquiring equipment and a worrying tendency of students to go abroad for both graduate education and employment.

As explained above, some LAC countries (e.g., Argentina) interpret existing legislation to promulgate regulations for biotechnology and GMOs, while others (e.g., Brazil and Honduras) have specific legislation for GMOs. In both those cases, often a case-by-case approach is chosen to determine whether a GED organism is subject to GMOs regulations or not. Other countries such as Peru have opted to ban the technology due to its perceived resemblance to transgenic GMOs. The Peruvian Congress decided to ban Living Modified Organisms (LMOs) through Law 29811 and Law 31111 (Peruvian Congress, 2011; Peruvian Congress, 2021). However, Peruvian legislation does not differentiate between transgenic GMO, LMOs or GED bans. At the same time, LAC's regulatory regimes that established more relaxed pathways for non-transgenic GED include countries such as Honduras, Guatemala, Colombia, Brazil and Argentina.

Interviewees affiliated with NGOs tend to argue that there are similarities between those technologies, representing a "GMO 2.0" in terms of social and environmental impacts. In this case, GED is rejected due to its perceived similarity to GMOs. Even though some regulators and policymakers may raise concerns about this transition, we argue that this debate and its regulatory and societal implications can be transferred to GED governance when LAC countries adopt GED technologies and enforce robust regulations. If (non-transgenic) GED products are regulated the same way as transgenic products, then it becomes important to revisit the way in which perception, legal definitions and access to markets change or remain the same.

5.1.1 Guatemala and Honduras: regulation shaped by Free Trade Agreements and customs unions

Both countries' regulations were primarily shaped by the Free Trade Agreement that facilitated the development of biotech regulations and expedited product development, and have since evolved with the adoption of Customs Union agreements. The Dominican Republic-Central America-United States Free Trade Agreement (CAFTA-DR) was signed by the United States of America, the Dominican Republic, Costa Rica, El Salvador, Guatemala, Honduras, and Nicaragua in 2004 and went into effect in Guatemala in 2006 (International Trade Administration website, 2022). Since the late 1990s, Honduras has been a country considered an ideal destination for GMOs supporters. At that time, Guatemala was not motivated to approve GMOs because they considered themselves the center of origin of maize:

> Since we began with the regulations in the late 90s, people used to say, "if you'd like to import GMOs, go to Honduras". That was the gate to get into Central America". The position of other countries, specifically Guatemala, was that they considered themselves the center of origin of maize. They were not eager to approve GMOs, at least for corn. For a long time, we were the only ones. In 2016/17 came this commercial agreement, called something like a customs agreement between Guatemala and Honduras. In 2017, I went there to advise their officials and academics. With our advice and training, they designed their legislation in the same terms as ours.

According to one interviewee, the USDA wanted the customs union agreement to become a reality and pushed for it: "I think the people in Guatemala, internally, did not agree. Some said we must sustain our claim to be a center of diversity and stuff like that, but others said that we need to catch up with the rest of the world. USDA put a policy in place, paid our trip to Guatemala, and promoted the meeting there. USDA wanted the agreement to come into place".

In May 2016, the Guatemalan Congress approved the customs union with Honduras which allowed the "free movement of people and goods between the two countries" (International Trade Administration website, 2022). A year later, both countries carried out the first stage of the customs union process right after addressing regulatory, technical and administrative procedures. In 2019, these countries approved a "harmonized biotechnology and biosafety regulation" for GED plants, which is considered the first in Central America (USDA, 2020). It is important to note that Guatemala had a moratorium in place with the previous regulation that "did not allow for the commercial production of GED plants" (USDA, 2020). According to one interviewee, the new regulation offer simplified procedures:

> In Guatemala, we are open to edited products. We handle them as conventional. A form is filled out, and in a week the user is informed, and an authorization is given without a time limit. We have an agricultural biosafety committee, but it is only for genetically modified organisms and commercial authorizations. We have a simplified procedure, which is common in Central America.

5.1.2 Argentina and Colombia: learning from neighbors

Argentina is considered to have one of the most developed regulatory frameworks in Latin America. While Argentina did not ratify the CPB, its regulations include definitions that are compatible with the CPB (Kuiken & Kuzma, 2021). As one interviewee argues, the problem was its *non-technical* considerations:

> Our regulations are completely in line with the technical part of Cartagena protocol. The safety assessment and the definition of a GMO. All of that has been in the regulation from the start. On the technical side, Argentina has always been in compliance with the protocol. The problem that the country had was with the non-technical part like liability and redress, socioeconomic considerations.

Another feature of Argentina's regulatory regime is the way in which neighbor countries "mimic" its regulation. This happens with the way in which the definition of GMOs is shared: "We came to this strange situation in which our approach can be mimicked by other countries in Latin America or in other regions because they use the same definition [LMO definition]". The LMO (living modified organism) definition is used in the CPB (Whelan and Lema, 2015). Definitions are important for regulators and risk analysts. According to an interviewee, there is a desire to harmonize regulations and achieve a synchronization of approvals. Ideally, harmonized regulations could reduce costs and spread benefits easily and effectively.

One of the LAC countries that has learned from Argentina is Colombia. Interviewees recognize the differences across both regulatory regimes. However, they consider that there is a need to learn from others. In particular, an interviewee expressed the importance of "catching up" to others and that regulation in Colombia should not inhibit research:

> The pressure [to "catch up"] came from Brazil and Argentina, which also had regulatory frameworks. Colombia decided that if they did it already, we must do it. For plants, the institution in charge is ICA, the equivalent of USDA. It was good to have ICA doing the regulatory framework for plants, as they have a very long experience with plants.

The Instituto Colombiano Agropecuario (ICA) was created in 1962 as an agency of the Colombian Ministry of Agriculture and Rural Development (ICA website, 2022). ICA participated in the negotiations of bilateral or multilateral sanitary and phytosanitary agreements (ICA website, 2022). Other research centers such as the International Center for Tropical Agriculture (CIAT), have a longstanding reputation in Colombia. According to one interviewee, there is a need to think in advance about the technology and its relationship with the regulatory landscape. Researchers should be proactive and "bring regulators to the lab".

5.1.3 Brazil: overcoming an embroiled regulation

Brazil has a regulatory regime that is slightly different from other countries in Latin America. Much like the discourse in Argentina, there was a debate around the CPB and the CBD. Nevertheless, in terms of its regulatory framework, interviewees considered that it was embroiled until 2005:

> There were a lot of missed opportunities because the regulatory scenario was really embroiled, completely embroiled in the beginning until 2005 [. . .] And then it changed for the better. But still there was a long way to go. Now I think regulation is mature, and is ready to receive applications from universities, from small companies, from startups. Now with gene editing, we can exclude some products from assessment. An easier and cheaper assessment. Now the field is open for biotechnology, but it was not like that 10 or 12 years ago.

In 2016, Brazil approved what is known as the Normative 16. Before this normative, it was not clear from a regulatory standpoint if products would be considered GM or not. One interviewee mentioned that this normative was created to facilitate companies to invest:

> In 2015, CTNBio [Brazilian National Technical Commission on Biosafety] decided to create a working group to start thinking about creating a normative to regulate gene editing in Brazil. At that time Argentina, the United States, Canada, other countries like Chile, Colombia were thinking about creating something related to gene editing. The process became clear to us, even for the government. We should follow the same pathway, the same standards to create this process to give chances to not just big companies to be on the market. At least in our view, regulation, the whole process of regulation of GM products is so expensive, and so complicated and so different in different countries that create a difficult environment.

Similar to Argentina, precision with definitions is important in Brazil. Some interviewees mentioned that the wording of Normative 16 is in accordance with the 2005 Law and the CPB, and that this measure does not intend to modify Brazilian Law. As with the regulations designed for Guatemala and Honduras, the goal of Normative 16 was to save time and reduce costs. CTNBio regulates technologies on a case-by-case process and interviewees claim that, under this normative, it takes approximately between two to 3 years to commercialize a product in the market. Compared to the transgenic products, regulatory compliance costs are considered much lower for GED products. Lastly, Normative 16 was also developed to reduce bureaucracy that, as interviewees mentioned, causes 'asynchronous approvals' in which there is a lag between the timing of cultivation approval and import approval.

5.1.4 Mexico, Peru and Bolivia: regulation shaped by politics

This group of countries' regimes seem to be influenced by domestic politics and environmental activist networks. The governments of Mexico, Bolivia and Peru, as well as civil society and indigenous groups, have opposed the development of transgenics and biotechnology. In the case of Mexico, regulations seem contradictory and confusing to some interviewees:

> There are contradictions, inexplicable moratoriums, there are irreconcilable points, of the agencies that protect the environment and those that promote the agricultural sector. There is little understanding between what the law says and what officials do. The law had many heated debates, which reconciled biosafety from gradual, experimental liberations, pilot tests, and commercial evidence, which obeys international principles.

Additionally, interviewees mentioned that the regulation has not been modified since 2009 except for corn. To this date, this means that Mexican Law cannot be applied to products developed with GED. According to one interviewee, the regulation in place for agricultural developments is the National Biosafety Law from 2005:

> The legal framework in place for any agricultural development in GMO, follows the 2005 national law of biosafety. This law gives the general framework. It has several considerations, [such as] the regulatory aspects of biotechnology, health, forests, etc. What the law regulates is the product and the process in Mexico. Despite its known or unknown implications, you must go to several stages of development.

Interviewees mentioned that before moving towards a commercial release, researchers first need to design an experimental research and a pilot stage. If these products are for human consumption, the Ministry of Health is involved. Additionally, some products are allowed to be released while others are not:

> The policy in terms of GMOs, commercial, research, field release for cotton is in place. Maize is not released in Mexico [. . .]. We used to have a permit for soybeans, but currently there are no permits. Transgenics are not well received, it is a big debate.

In the case of Peru, an ongoing GMO moratorium affects the development of biotechnology. The original moratorium, approved in 2011, was established with considerable support from civil society organizations opposed to agricultural biotechnology. Law 29811 stipulated a 10 years moratorium that sought to prevent the entry and production of LMOs for "cultivation or breeding purposes, including aquatic ones, to be released into the environment" (Peruvian Congress, 2011). Through Law 31111, in 2021, the Peruvian Congress approved the extension of the moratorium until 2035 (Peruvian Congress, 2021). According to an interviewee, the Executive instead aimed for a new Biosafety Law and a shorter moratorium. Additionally, according to the same interviewee, previous regulations approved in 2002 for LMOs were not implemented efficiently.

The position of the Peruvian Ministry of Environment on this matter was not seriously considered by the Peruvian Congress in 2021. Interviewees argue that this was a political decision that had both positive and negative impacts:

> The Ministry of Environment issued its opinions, its pros and cons, which were not taken into account in this commission. Finally, they approved it for 15 years even though other projects proposed 10 years. It was a political decision. The positive impacts of it were the availability of greater resources, fostering agrobiodiversity, and family agriculture. The negative impact was the restriction of a technology, to improve the productivity of small farmers.

A common problem in Peru and Bolivia is getting reagents and other key laboratory materials through customs. Interviewees from both countries mentioned different challenges such as substantial paperwork (Peru) and an association with illegal activities such as drug trafficking (Bolivia):

> There are restrictions regarding the production of drugs. The law pursues you. They will come to your laboratory to see that you used your reagent, even if it is pedagogically. Importation (of reagents) is very bureaucratic. They must make a report on the health and food implications. Import is expensive, and many legal processes are involved.

GMOs were banned in Bolivia from 1997 to 2005 due to the pressure from the environmental groups. According to one interviewee, even with this pressure it was possible to publish a supreme decree in which a shorter procedure was included for risk assessment:

> There was a moratorium on all GMO events from 1997 to 2005 due to this pressure from the environmental groups that persists. But there are scientists and academics that talk about benefits. Even while developing regulations, we talk about the importance of science over the economic, social, and cultural fears. Bolivia is trying this, starting from the producers, and I think that a positive sign in the country's politics came in 2018. The government of Morales was very close to regulating and using biotech, but then it published a supreme decree where a shorter procedure was introduced to evaluate the risks.

5.1.5 Paraguay: an evolving regulatory regime

Between 2005 and 2012 there was an official restriction from the Paraguayan government to release new transgenic crops. This changed in 2012, when the Paraguayan government was open to the release of new events and transgenic crops such as cotton:

> It started with the acceleration of commercialization, first with transgenics. Then between 2005 and 2012 there was an official restriction with the release of new transgenic crops when I had to go in that direction. There was political pressure to avoid the release of new events; there were not many transgenics at that time. In 2012, new government policies started to focus on the analysis and to be open to the release of new events and transgenic crops. Cotton was released, others in the same way because we had issues with Argentina that had releases in 2016.

In 2019, the Paraguayan government published a resolution for crops developed using GED and other new breeding techniques (Genetic Literacy Project, 2020). Additionally, Paraguay issued a joint statement alongside twelve other countries including Argentina, Brazil, Australia and the United States to the World Trade Organization supporting relaxed regulations for GED (WTO, 2020). According to an interviewee, the pandemic slowed down this process:

> Then we had the pandemic that has slowed and stopped what occurs in regulatory systems [. . .] Requests for microorganisms have increased, but I do not know if they have been regulated by GED regulation.

5.2 Policy entrepreneurs and policy windows in the LAC region

We consider environmental activist groups and grassroots organizations as policy entrepreneurs. Those groups are defined as public interest groups, which may be understood as counterpoints to the self-interest groups such as industry (Kingdon, 2003). In most cases, these groups are mobilized internationally through advocacy networks in a process called "transnational advocacy" (Keck and Kathryn, 1998). This term refers to the situation in which states are unresponsive to the demands of their citizens, and therefore activists may seek the support of international allies. Their main goal is to push public attention to issues such as food sovereignty, indigenous rights and agroecology. We also consider regulators, risk analysts, developers and scientists as policy entrepreneurs if they have actively influenced the adoption or rejection of new GED regulations.

In this study we aim to understand how these different policy entrepreneurs took advantage of policy windows to reconfigure agricultural GED regimes. We are paying attention to how domestic affairs (national legislation, elections, agriculture and environmental policies) and internationally-driven events (trade agreements, ratification of international agreements, partnerships) constitute policy windows.

Public interest groups often aim to establish transnational advocacy networks to increase their relevance and the resources available to them, primarily blocking the development of regulations that allow applications of GED in agriculture while pushing for alternatives. On the other hand, the other set of policy entrepreneurs (regulators, policymakers, risk assessment experts) primarily focus on pushing for the development of regulations that would allow the use of GED in agriculture with an eye on harmonization around the LAC region. For example, policy entrepreneurs have taken advantage of international agreements to steer legislation in favor of GED technologies, such as in the case of Guatemala and Honduras.

5.2.1 Domestic policy windows

An example of a negative domestic event that opened a policy window that was seized by NGOs and civil society representatives in Peru is the finding of GM corn in the environment. According to a Peruvian interviewee, in 2008 a report identified the presence of GM yellow corn in the Barranca valley (Gutierrez-Rosati, 2008). This interviewee mentioned that this report triggered the establishment of the current moratorium:

> In 2008 due to a report of transgenics in the environment, civil society organizations, farmers and social movements declared the country free of transgenics. In 2011, after a few years, Ollanta Humala [former Peruvian president], promulgated [the GMO moratorium] in December 2011, valid for 10 years.

In Bolivia, small farmers, concerned with GM crop imports from Argentina, triggered a shift inside the government that opened the possibility to discuss the matter:

> So these small farmers said "why did our government import corn from Argentina when we can produce our own corn in Bolivia, with our techniques, our tastes". This caused some shift inside the government and this allowed an opening towards this discussion.

Therefore, some small producers that are in favor of GED and GMOs crops, particularly from the Santa Cruz region in Bolivia, are trying to act as policy entrepreneurs and seize the policy window to push to have clear regulations about GED:

> It is important to say that even the smaller producer is convinced of the benefits of the biotech and is open to the new tech like CRISPR because they know they can get more benefits. So, they are trying to influence the current government to make sure that these technologies have clear regulations and that can help the producers to produce more, and more sustainable agriculture. Small producers are very important in Bolivia.

As explained before, national elections are usually perceived as a policy window. In fact, an interviewee from Honduras mentioned it was an awaited event to push for new regulations:

> It is a matter of time. The regulation that comes with the law. It is a matter of time [. . .] We have a lot of pressure to do it [. . .] Congressional elections coming in November, there is a chance to publish this regulation.

In Honduras, it seems that those that acted as policy entrepreneurs belong to the private sector, who apparently influenced the Ministry of Agriculture:

> The Ministry of Agriculture and private entrepreneurs pushed for inclusion of regulatory updates for GED. When they wanted to import new technology they would not have any problem with it. The Ministry of Agriculture has focused on this issue, but on suggestions from the private sector.

5.2.2 Internationally-driven policy windows

An example of policy windows that opened thanks to internationally-driven events is described by an interviewee from Guatemala, who explained that the Customs Union Agreement helped to pass regulations for biotechnologies, which was supported by the private sector and academia:

> The regulations [for biotechnology] would not have passed without the Free Trade [i.e., Customs Union] Agreement [. . .] It was fundamental, it would not have been possible if it had not been done under that premise, it allows the regulation to be maintained [. . .] The Ministry of Economy negotiates the treaties. The Ministry of Agriculture carries out the technical proposals. There was support from businessmen, the private sector, and the academic sector.

Interviewees mentioned that the ratification of the CPB and the subsequent requirement to pass national regulations to comply with it pressured the different governments to act further in the biotechnology sector. The CPB's influence is tied to international trade imperatives mediated by domestic politics (Gupta and Falkner, 2006). However, as Gupta and Falkner suggest, the flexible interpretation of the CPB has motivated countries to choose their own paths in biosafety policies. For example, one interviewee mentioned that due to the ratification of the CPB, the decision making moved faster:

> When the Cartagena Protocol was ratified, the law established that the entity in charge and the focal point was not the Ministry of Agriculture but the Ministry of the Environment. This made things move very quickly for 2 years because not everything had to go to the national biotechnology commission.

Interviewees from both Honduras and Guatemala also mention the Inter-American Institute for Cooperation in Agriculture (IICA) and its role in supporting the development of regulations and policies in both those countries. IICA is an international agency based in Costa Rica specialized in agriculture of the Inter-American System; their goal is to support the Member States in agricultural development and rural wellbeing (IICA website, 2022). One interviewee explained that IICA respects local systems and regulations, working both with stakeholders in favor and against GMOs, as they aim at providing them with information about the regulations that exist elsewhere to make informed decisions.

Based on our interviews, it seems that IICA also acted as a policy entrepreneur in the LAC region. In a sense, it acts as a mediator between governments and other economic and social actors involved in LAC agriculture and rural living (IICA website, 2022). Most of our interviews highlight IICA's role in fostering ties between academia and regulators. For example, an interviewee from Guatemala mentions that IICA supported academics and authorities in advancing and harmonizing regulations. The same interviewee mentioned that some individual consultants were particularly active during this process.

5.2.3 Pressure from international environmentalists: the perceived European influence over LAC countries

We note an interesting pattern in our data: the repeated references to the influence of international environmental organizations in LAC, primarily from Europe. Most of the interviewees are concerned with the perceived European influence. One interviewee even described the European Union as "the worst enemy". An example that shows these widespread concerns is the quote below from a Bolivian interviewee:

> In Bolivia, as well as in other Latin American countries, we suffer from interference from European environmental organizations with strong investments and [. . .] (they) introduce a lot of fear over not only the production of transgenic crops but also on the consumption of these products. They also introduced fear [. . .] these new technologies, like CRISPR, can potentially change the genome of humans that consume products obtained with CRISPR.

This interviewee continued by mentioning the perceived presence of a heavily financed environmentalism in Bolivia, with influence in the civil society but also in the State. The interviewee frames the interference as a problem that affects not only the current government but also previous ones. The interviewee argues that the trend is observable since the 2000s, mentioning a moratorium on all the GMO events from 1997 to 2005 due to this pressure from the environmental groups.

Multiple interviewees also articulated that those environmental groups do not have strong local roots, primarily being foreign organizations. This concept is represented in the following quote from an interviewee from Brazil:

> Activist groups are always international [. . .] Very rarely we saw small farmers, or agriculture, or students connected with those movements. It was not a spontaneous presence, it was organized internationally. The same issues were brought back, same questions were brought to other countries.

One aspect that caught our attention was the discrepancy between how those European organizations are perceived by those interviewees, that primarily come from a policy or academic background, and the activists themselves. The former, who tend to be in favor of GED, appear to support the idea that the European organizations are behind the anti-GMO network in the LAC region, particularly in some countries (for example, Mexico, Bolivia and Peru). Interviewees suggested that local opinions are in fact influenced by anti-GMO organizations' agendas.

However, the activists themselves described a strong local presence, referring to specific events that motivated the formation of local organizations. Additionally, from the interviews it emerged that while they do actively seek international support, both from Europe and from other countries in the LAC region, international support is a strategy to gain additional strength and help in advocating for their domestic issues. As this interviewee from Brazil mentions:

> People from outside, global vision, internationalize the fight and the hope, to have another dimension. Without the external pressure, the situation would be worse. It is an additional help for our internal fights.

The same interviewee adds that their organization has activities in the macroregion, collaborating with individuals from other countries in the region including Peru, Chile, Argentina, Paraguay and Nicaragua.

As explained above, the need to network with international organizations (i.e., transnational advocacy), appears to be triggered by the fact that the interviewees feel that their concerns are often not properly addressed in the decision making process concerning GED/GMO applications in agriculture. Some of those concerns include potential health hazards, accessibility to the technology and ultimately for whom GED is going to be more beneficial. For example, a shared concern is the possibility that the technology will primarily favor the big corporations rather than smallholders, as explained by an interviewee from Paraguay:

> Our question is, how will this benefit us? There is incredible technological development, but how can this development benefit these poor people? How can we protect our seeds? And how can we access this? Technology needs to develop us as much as it develops the big companies. This is our fear: develop technology, but in the hands of big companies, and not in favor of small indigenous farmers.

Similarly, an interviewee from Brazil argued that big corporations are supported by the government, having a considerable amount of food exported while at the same time "local people continue to starve". This interviewee felt that the public should benefit from these technologies:

> The government does nothing for the farmers, but supports the big agribusiness. There is not even a single incentive for the farmers' production. There is nothing positive for women farmers in the Bolsonaro's government. [. . .] There are a lot of people that starve. [. . .] If people from Brazil would benefit then yes, but it is all exported.

As mentioned at the beginning of the paper, although the LAC region is quite resource rich, there still persist food insecurity and poverty. GED could be used to increase agricultural production. However, the interviewed members of local environmental and farmers organizations fear that this increased crop production would primarily be exported, rather than used to address domestic food insecurity.

> [In Paraguay], big agrobusiness produce to export. At the roots of poverty and the death of our people. We are not against development, but we are against the exploitation of nature [. . .] privatization of seeds that are a heritage of our people. Today it is more and more in the hands of companies.

As highlighted in the above quote, it is important to note that activists are not necessarily against the use of technologies such as GED. They consider them to provide opportunities for development if used in a transparent and fair manner.

6 Discussion

The development of agricultural GED regulation requires the involvement of stakeholders familiar with science, legislation, policy and public engagement, as well as keeping pace with evolving domestic and international trade agreements and other treaties. While fostering deliberation around these technologies may appear to hinder technology adoption (Kuiken et al., 2021), it reflects the negotiations undertaken by regulators, product developers, and social movements around food sovereignty. Often, these negotiations are not known or explored, and thus deliberation may turn into tension or conflict. As Kuiken and Kuzma (2021) suggest, it is important to consider where GED is going to be implemented, whether there are markets for biotechnology products, and whether the public approves, trusts, and has equitable access. Kuiken et al. (2021) consider that the international governance of GED, in particular CRISPR, will play a crucial role in food and agricultural markets.

This paper showed that the differences between agricultural GED regulatory regimes across the LAC region can be explained partially by the variety of ways in which policy entrepreneurs (the different interest groups represented by academics, industry, ministries, congress, regulators, NGOs, scientists) have influenced agricultural GED regulation through policy windows.

The complexity of the stakeholder landscape and the dynamic political cultures we have studied contribute to heterogeneous agricultural GED regulatory regimes. We have shown this through the analysis of our interviews, where we particularly focused on domestically and internationally driven policy windows, as well as on the political pressure exerted by networks of activists and NGOs. These stakeholders are able to shape regulation through political interactions in formal and informal spaces. The role of policy entrepreneurs, especially in our definition that includes transnational advocacy networks, is a critical and potentially overlooked causal factor in the complexity of the regulatory landscape. In other words, without careful consideration of a wider range of policy entrepreneurs, we may be missing important context for what gives rise to different policy regimes.

How might lessons from LAC transfer to other geographies? What ideas might translate? Which are specific to the LAC region?

Additionally, little is known about the perspectives of growers and potential end users of GED technologies in the region. In regulatory cultures that emphasize being scientific or evidence-based, we would like to highlight that systematic social science data is indeed evidence and there are clear gaps where social science data is needed, particularly in the context of growers and historically marginalized groups. Our research only included a small group of environmental activists and NGOs. While these preliminary conclusions suggest compelling complexity, our research would be bolstered by additional research particularly in local communities.

In our case, public interest groups constituted by the NGOs primarily work to block the development of regulations that would allow the use of GED/GMOs in agriculture, while pushing for alternatives (e.g., agroecology). On the other hand, industry and academia tend to advocate for harmonious, comprehensible, and permissible regulations that would allow the application and diffusion of GED/GMO products which it is believed would foster R&D and overall economic growth. The identified groups, or groups that we call policy entrepreneurs, invest a considerable amount of resources like time, energy, reputation and money in the attempt to see their solutions transformed into regulations. It is important to note that this influence was undertaken also through formal and informal negotiations. In Guatemala and Honduras, informal negotiations (between the country's ministries and external organizations like IICA, with particularly active consultants) led to the current regulatory framework. In countries such as Peru, negotiations in formal spaces such as the Congress led to the current GMO moratorium.

As mentioned by Hood, (2001), an analysis of regulatory regimes may explain the integration or fragmentation of regulatory frameworks, as well as the policy instruments and potential biases towards market type incentives. For instance, the regimes of countries like Argentina, Honduras, Guatemala, Colombia and Brazil are designed to support a stronger

relationship with external markets and are generally more open about applications of the GED technology in agriculture to increase production. These regimes tend to foster public engagement as well as harmonization of regulatory frameworks across LAC. On the other hand, regimes of countries such as Peru, Bolivia, and Mexico generally restrict agricultural GED applications primarily due to its perceived resemblance to GMOs. Stakeholders that oppose agricultural GED have pressured their governments with a stated purpose to protect family agriculture and food sovereignty.

At the same time, there are common issues across the LAC region in terms of the definitions used in regulation. Since legislation changes across countries, similar definitions of what is and what is not a GED product may impact product development and regulation. In order to move towards a more harmonized regulatory framework across the region, the concerns of public interest groups (environmental NGOs, farmers and indigenous communities organizations) need to be taken into account. This could be achieved by providing clear and transparent information about the differences with GMOs, how GED works, the safety of these technologies and how these will benefit them.

Although we support the need for science (particularly molecular biology and risk evaluations) to inform regulations, we also believe that dismissing social science data may be detrimental to achieving the goal of regulation development and harmonization in LAC countries. The general belief that the anti-GMO movement in the region was born due to international influence, particularly from the European Union, appears to conflict with local organizations' self-description of their origins and scope. The interviewed activists explained the local roots of their organizations, adding that they are not necessarily against GED and GMOs, but they are concerned with the health impacts of products obtained through these technologies, and question the benefits for small producers. Therefore, we believe that there is the need to collect sound social science data on those local groups to be included in the body of knowledge considered to formulate regulations. The conversation on those technologies should be broadened to potentially positively or negatively impacted, marginalized communities to obtain a complete picture of the political landscape in the region.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by North Carolina State University's Institutional Review Board. The patients/participants provided their written informed consent to participate in this study, IRB protocol number 23647.

Author contributions

IC and SZ conducted the analysis, developed the framework, and wrote most of the manuscript. MR participated in data collection and manuscript editings. MJ conducted the interviews and contributed to the manuscript drafting process. SB-D conducted the interviews, oversaw the analysis, and edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

Funding for this project, "Assessment of the Regulatory and Institutional Framework for Gene-editing via CRISPR-based Technologies in Latin America and the Caribbean" was provided by the Inter-American Development Bank, project number RG-T3431. IDB works to improve lives in Latin America and the Caribbean. Through financial and technical support for countries working to reduce poverty and inequality, IDB helps improve health and education, and advance infrastructure to achieve development in a sustainable, climate-friendly way.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Atkinson, J. M. (1982). Understanding formality: The categorization and production of "formal" interaction. *Br. J. Sociol.* 33 (1), 86–117. doi:10.2307/589338

Bagley, M. (2021). Genome editing in Latin America: CRISPR patent and licensing policy. Environment, rural development and disaster risk management division. Available at: https://publications.iadb.org/publications/english/viewer/Genome-Editing-in-Latin-America-CRISPR-Patent-and-Licensing-Policy.pdf.

Bioversity International (2017). *Mainstreaming agrobiodiversity in sustainable food systems: Scientific foundations for an agrobiodiversity index*. Rome (Italy): Bioversity International.

Brooks, T. M., Resit, H., Burgess, N. D., Butchart, S. H. M., Hilton-Taylor, C., Hoffmann, M., et al. (2016). Analysing biodiversity and conservation knowledge products to support regional environmental assessments. *Sci. Data* 3, 160007. doi:10.1038/sdata.2016.7

Convention on Biological Diversity website (2014). Nagoya Protocol on access and benefit-sharing. Available at: https://www.cbd.int/abs/.

de Almeida Camargo, L. S., and Pereira, J. F. (2022). Genome-editing opportunities to enhance cattle productivity in the tropics. *CABI Agric. Biosci.* 3, 8. doi:10.1186/s43170-022-00075-w

Diefenbach, T., and Sillince, J. A. A. (2011). Formal and informal hierarchy in different types of organization. *Organ. Stud.* 32 (11), 1515–1537. doi:10.1177/0170840611421254

ECLAC website (2003). The Cartagena Protocol on biosafety to the convention on biological diversity. Observatory on principle 10 in Latin America and the caribbean. United Nations. Available at: https://observatoriop10.cepal.org/en/treaties/cartagena-protocol-biosafety-convention-biological-diversity.

Entine, J., Felipe, M. S. S., Groenewald, J. H., Kershen, D. L., Lema, M., McHughen, A., et al. (2021). Regulatory approaches for genome edited agricultural plants in select countries and jurisdictions around the world. *Transgenic Res.* 30, 551–584. doi:10.1007/s11248-021-00257-8

EPA website (2017). Update to the coordinated framework for the regulation of biotechnology. Availabe at: https://www.epa.gov/regulation-biotechnology-under-tsca-and-fifra/update-coordinated-framework-regulation-biotechnology.

Feldman, M. S. (1989). *Order without design: Information production and policy making*. Stanford, CA: Stanford University Press.

Filomeno, F. (2014). "Monsanto and intellectual property in south America," in *International political Economy series* (London, UK: Palgrave Macmillan London). doi:10.1057/9781137356697

Foster, W., and Valdés, A. (2015). Measuring the size of the renewable resource sector: The case of Chile. *World Food Policy* 2, 19–35. doi:10.18278/wfp.2.1.2

Gatica-Arias, A. (2020). The regulatory current status of plant breeding technologies in some Latin American and the Caribbean countries. *Plant Cell, Tissue Organ Cult. (PCTOC)* 141 (2), 229–242. doi:10.1007/s11240-020-01799-1

Genetic Literacy Project (2020). Paraguay crops/food. Global gene editing regulation tracker. Available at: https://crispr-gene-editing-regs-tracker.geneticliteracyproject.org/paraguay-crops-food/.

Gupta, A., and Falkner, R. (2006). The influence of the Cartagena Protocol on biosafety: Comparing Mexico, China and south Africa. *Glob. Environ. Polit.* 6 (4), 23–55. doi:10.1162/glep.2006.6.4.23

Gutierrez-Rosati, A. (2008). Investigaciones sobre la Presencia de Transgenes en Perú: caso Maíz (Zea mays L) 13o Congreso Latino Americano de Genética, 9o Congreso Peruano de Genética. *Rev. Latinoam. Genética. Segunda Épo*-1 (1), 89–108.

Hayden, C. (2003). *When nature goes public: The making and unmaking of bioprospecting in Mexico*. Princeton, NJ: Princeton University Press.

Hood, C. (2001). *The government of risk: Understanding risk regulation regimes*. Oxford and New York: Oxford University Press.

ICA website (2022). Instituto Colombiano Agropecuario. El ICA. Available at: https://www.ica.gov.co/el-ica.

IICA website (2022). Inter-American Institute for cooperation on agriculture. Available at: https://www.iica.int/en.

Innovative Genomic Institute website (2022). What is Crispr? Available at: https://innovativegenomics.org/education/digital-resources/what-is-crispr/.

International Trade Administration website (2022). Guatemala country commercial guide. US department of commerce. Available at: https://www.trade.gov/country-commercial-guides/guatemala-trade-agreements.

Jenkins, D., Juba, N., CrawfordWorthington, B. M., and Hummel, A. (2023). Regulation of plants developed through new breeding techniques must ensure societal benefits. *Nat. Plants* 9, 679–684. doi:10.1038/s41477-023-01403-2

Keck, M. E., and Kathryn, S. (1998). *Activists beyond borders: Advocacy networks in international politics*. Ithaca, NY: Cornell University Press.

Kingdon, J. W. (2003). *Agendas, alternatives, and public policies*. 2nd ed. New York: Longman.

Kuiken, T., Barrangou, R., and Grieger, K. (2021). (Broken) promises of sustainable food and agriculture through new biotechnologies: The CRISPR case. *CRISPR J. Febr.* 4, 25–31. doi:10.1089/crispr.2020.0098

Kuiken, T., and Kuzma, J. (2021). *Genome editing in Latin America: Regional regulatory overview*. Washington, DC, USA: Inter-American Development Bank. doi:10.18235/0003410

Kuzma, J. (2018). Regulating gene-edited crops. *Issues Sci. Technol.* 35 (1), 80–85.

Lokhandwala, Z. (2022). Peasants' rights as new human rights: Promises and concerns for agrobiodiversity conservation. *Asian J. Int. Law* 12 (1), 105–120. doi:10.1017/S2044251322000042

Morris, M., Sebastian, A. R., and Perego, V. M. E. (2020). *Future foodscapes: Re-Imagining agriculture in Latin America and the caribbean (English)*. Washington, D.C.: World Bank Group.

Newell, P. (2009). Bio-Hegemony: The political Economy of agricultural biotechnology in Argentina. *J. Lat. Am. Stud.* 41 (1), 27–57. doi:10.1017/S0022216X08005105

OECD/Food and Agriculture Organization of the United Nations (2019). "Latin American agriculture: Prospects and challenges," in *OECD-FAO agricultural outlook 2019-2028* (Rome: OECD Publishing, Paris/Food and Agriculture Organization of the United Nations).

Peruvian Congress (2011). Law 29811. Available at: https://leyes.congreso.gob.pe/Documentos/ExpVirPal/Normas_Legales/29811-LEY.pdf.

Peruvian Congress (2021). Law 31111. Available at: https://busquedas.elperuano.pe/download/url/ley-que-modifica-la-ley-29811-ley-que-establece-la-moratori-ley-n-31111-1917468-1.

Pixley, K. V., Falck-Zepeda, J. B., Paarlberg, R. L., Phillips, P. W. B., Slamet-Loedin, I. H., Dhugga, K. S., et al. (2022). Genome-edited crops for improved food security of smallholder farmers. *Nat. Genet.* 54 (4), 364–367. doi:10.1038/s41588-022-01046-7

President of the United Mexican States (2020). Mexico decree banning glyphosate and GE corn. Available at: https://www.dof.gob.mx/nota_detalle.php?codigo=5609365&fecha=31/12/2020 (Accessed May, 2023).

President of the United Mexican States (2023). Mexico decree banning glyphosate and GE corn. Available at: https://www.dof.gob.mx/nota_detalle.php?codigo=5679405&fecha=13/02/2023#gsc.tab=0 (Accessed May, 2023).

Roca, M. M., Falck-Zepeda, J., and Paes-Andrade, P. (2023). "Cisgenics and genome editing or second generation biotechnologies: A Latin America and caribbean perspective," in *Cisgenic crops: Safety, legal and social issues. Concepts and strategies in plant sciences*. Editors A. Chaurasia and C. Kole (Cham: Springer). doi:10.1007/978-3-031-10721-4_9

Roca, W., Espinoza, C., and Panta, A. (2004). Agricultural applications of biotechnology and the potential for biodiversity valorization in Latin America and the caribbean. *AgBioForum* 7 (1and2), 13–22.

Shukla-Jones, A., Friedrichs, S., and Winickoff, D. (2018). "Gene editing in an international context: Scientific, economic and social issues across sectors," in *OECD science, technology and industry working papers* (Paris: OECD Publishing). No 2018/04. doi:10.1787/38a54acb-en

Swanson, A., and Qiu, L. (2023). U.S. To challenge Mexican ban on genetically modified corn. New york times. March 6th, 2023. Available at: https://www.nytimes.com/2023/03/06/us/politics/mexico-ban-us-corn.html#:~:text=Mexico%20has%20planned%20to%20phase,United%20States%20is%20genetically%20modified.

Trigo, E., Falck-Zepeda, J. B., and Falconi, C. (2010). Biotecnología agropecuaria para el desarrollo en América Latina: Oportunidades y Retos. Available at: https://publications.iadb.org/publications/spanish/viewer/Biotecnolog%C3%ADa-agropecuaria-para-el-desarrollo-en-Am%C3%A9rica-Latina-Oportunidades-y-retos.pdf.

UNEP-WCMC (2016). *The state of biodiversity in Latin America and the caribbean: A mid-term review of progress towards the aichi biodiversity targets*. Cambridge, UK: UNEP-WCMC.

USDA (2020). Guatemala: Agricultural biotechnology annual. Biotechnology and other new production technologies. Global Agricultural Information Network. Available at: https://apps.fas.usda.gov/newgainapi/api/Report/DownloadReportByFileName?fileName=Agricultural%20Biotechnology%20Annual_Guatemala%20City_Guatemala_10-20-2019.

Van der Meer, M., Angenon, G., Bergmans, H., Buhk, H. J., Callebaut, S., Chamon, M., et al. (2020). The status under EU law of organisms developed through novel genomic techniques. *Eur. J. Risk Regul.* 14 (1), 93–112. doi:10.1017/err.2020.105

Weible Christopher, M. (2014). "Introducing the scope and focus of policy process research and theory," in *Theories of the policy process*. Editors A Sabatier Paul and M Weible Christopher. 3rd (Boulder, CO, USA: Westview Press).

Whelan, A. I., and Lema, M. A. (2019). *Regulation of genome editing in plant biotechnology: Argentina. In regulation of genome editing in plant biotechnology*. Berlin, Germany: Springer International Publishing. doi:10.1007/978-3-030-17119-3_2

Whelan, A. I., and Lema, M. A. (2015). Regulatory framework for gene editing and other new breeding techniques (NBTs) in Argentina. *Gm. Crops Food* 4, 253–265. doi:10.1080/21645698.2015.1114698

World Bank (2023). Employment in agriculture (% of total employment) (modeled ILO estimate) - Latin America and Caribbean. Available at: https://data.worldbank.org/indicator/SL.AGR.EMPL.ZS?locations=ZJ (Accessed May, 2023).

WTO (2020). Committee on sanitary and phytosanitary measures. G/SPS/GEN/1658/Rev.4 - international statement on agricultural applications of precision biotechnology. Available at: https://docs.wto.org/dol2fe/Pages/SS/directdoc.aspx?filename=q:/G/SPS/GEN1658R4.pdf&Open=True.

Zahariadis, N. (2014). "Ambiguity and multiple streams," in *Theories of the policy process*. Editors A Sabatier Paul and M Weible Christopher. 3rd ed. (Boulder, CO: Westview Press), 25–58.

Zarate, S., Cimadori, I., Roca, M. M., Jones, M., and Barnhill-Dilling, K. (2023). *Stakeholder interviews. Assessment of the regulatory and institutional framework for agricultural gene editing via CRISPR-based technologies in Latin America and the caribbean*. Washington, DC, USA: Inter-American Development Bank.

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Mini Review
PUBLISHED 06 July 2023
DOI 10.3389/fbioe.2023.1224992

OPEN ACCESS

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RECEIVED 18 May 2023
ACCEPTED 27 June 2023
PUBLISHED 06 July 2023

CITATION
Mungeyi P, Chimwamurombe PM and Kangueehi GN (2023), Assessing the adoption and application of the Namibian biosafety labelling regulations and determining their impact on Namibian food and feed importers.
Front. Bioeng. Biotechnol. 11:1224992.
doi: 10.3389/fbioe.2023.1224992

Assessing the adoption and application of the Namibian biosafety labelling regulations and determining their impact on Namibian food and feed importers

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The study was carried out to investigate the implications of the Namibian biosafety regulations on Namibian food and feed importers. After the Biosafety Act, 2006 (Act No. 7 of 2006), the biosafety regulation was gazetted in 2016, which saw the implementation of the national framework, the impact of food and feed importers was not known. The objective of the study was to assess the adoption and application of the national biosafety labelling regulations by food and feed importers. In addition, the impacts of these regulations on Namibian food and feed importers were assessed. The study used a structured online and hard copy survey questionnaire based on responses from 340 Namibian importers of food and feed products from eight identified Namibian regions: Khomas, Erongo, Kavango West, Kavango East, Omusati, Oshana, Ohangwena, Oshikoto, and Zambezi who have the knowledge required for the adoption and application of the Namibian biosafety labelling regulations. Using the Mann-Whitney test, the study confirmed that individuals who are aware of the biosafety Act, 2006 (Act No. 7 of 2006) are less likely to agree with statements such as experiencing problems in fulfilling requirements under the biosafety regulations. It was further concluded that there is a need to reduce the current administrative burdens for handling applications and improve dialogue between regulators and the food and feed importing industry while increasing the competence of regulators and creating more labelling regulation awareness for food and feed importers. The study further suggests that public awareness is required beyond food and feed importers.

KEYWORDS

biosafety, GMOs, GM products, importers, labelling

1 Introduction

Food and feed consisting of, containing, or derived from GMOs have been circulating the globe since the beginning of the 21st century (Aguilera et al., 2013).

Many countries that are using GMOs and derived products have put in place biosafety regulations to ensure the safe handling, transport, and use of GMOs. During the last 15 years, more than 40 countries have approved labelling laws. However, the level of implementation

of these labelling laws differs greatly from one country to another based on their characteristics, (Phillips and McNeill, 2000; Carter and Gruère, 2003; Haigh, 2004). Twardowski and Małyska (2015) stated that in countries that have biosafety regulatory frameworks in place, such as in the European Union (EU), the regulations are rather complicated and that leads to the slow approval process of genetically modified crops and products. In many African countries, these regulations are still lacking and making it impossible to approve the use of GMOs (Adenle et al., 2017). Kaur et al. (2018), have indicated that, out of 54 countries, the use of GMOs has only been approved in a few African countries like South Africa, Eswatini, Malawi, Kenya, Ethiopia, Sudan, Nigeria, and Burkina Faso. One of the requirements for regulation is the labeling of GM food products and processed products with the aim to give consumers choices (Twardowski and Małyska, 2015). General rules on food labelling can be divided into two categories, namely, rules on nutritional information and rules on labelling. The rules on labelling are associated with obligatory information for all food stuffs (Borges et al., 2018). Aarts et al. (2002) have further highlighted that GM products should be labelled to ensure traceability of these products at all stages of the food supply chain. The primary goals of these standards are to safeguard consumers' health and safety, as well as to guarantee that food traders implement fair international and regional trading procedures. (Borges et al., 2018). One such standard is guided by Codex General Standard for The Labelling of Prepackaged Foods (Codex) which recommends applying a fair labelling rule including GM products considering risk assessment of such products (Borges et al., 2018). Over time, extensive literature has been developed on the importance of labelling food products. One emphasis was put on consumer health on which Twardowski and Małyska (2015) argued that labelling help customers in understanding the ingredients in terms of specific doses and/or spotting ingredients that may cause allergies. Kedisso et al. (2022) argue that labelling is important since it is a tool that regulators employ to guarantee that traders disclose information to consumers in hopes of decreasing scientific uncertainty and consumer arguments over the safety of GMOs.

As a result of the high degree of competition in the global food market, customer choice can have a considerable influence on the sort of product selected. Hence, consumer choice also involves distinguishing between GMO and non-GM food products (Albert, 2010). Hence, this is an indication that the buying behaviours of consumers' are driven by health concerns associated with processing aids used in the production and manufacturing of the food products. Hu et al. (2021) further argued that consumers will choose GM, but only when it is significantly less expensive than non-GM. These findings are supported by Azila-Gbettor et al. (2013) who added that consumer choice becomes difficult to achieve in some regions of the world due to the diversity of products in the market and the ability of consumers to read a particular language due to low literacy levels. Therefore, when it comes to labelling of food products, Choi (2010) stressed that labelling based on the percentage threshold level of GM content may also be a barrier to trade. Thus, there is a lot of uncertainty in the implications of mandatory labelling regulations in terms of what requirements must be satisfied for food to be considered a GM product (MacFadden, 2017). Asioli et al. (2017) contend that, unlike in poor nations, consumers in advanced nations are more interested in knowledge about food production methods than in the ingredients of the food products they eat. Other barrier to trade in terms of consumer choice is associated by pricing. Thus, if looking at the angle of the cost implication of labelling, some proponents of mandatory labelling are of the opinion that food companies change labelling to reduce the cost associated with this effect McFadden (2017). Thus, researcher have therefore argued that when it comes to the labelling of food products, consideration should not only be based on national laws such as the biosafety laws, but great consideration should be put on the interpretation and align laws in line with the Word Trade Orgaisation agreements (Borges et al. (2018; Komen, 2012; Van der Walt, 2001).

Namibia now depends on imports to fulfill domestic demand since it cannot produce enough maize as most (over 70%) of the white maize grain imported now comes from South Africa (NAB, 2021). As outlined in the Biosafety Act of 2006, Namibia, like many other nations, has implemented legislation requiring the labelling of genetically modified food and feed items (Biosafety Act No. 7, 2006). All registered Namibian firms dealing with genetically modified foods or feed must mark them in accordance with the Act's three 3) categories (separately or individually packaged raw agricultural commodity, Raw agricultural commodity, which is not separately or individually packaged and processed genetically modified food or feed). The regulation that allows for the labelling of GMOs and products under the Biosafety Act was gazetted on 1 November 2016. The labelling criteria for GM food and feed in Namibia is 0.9 percent, while in South Africa is 5%. According to Jacobs (2018), the Namibian labelling Biosafety regulations have made it more stringent and costly for South African products exporters to modified and label their packaging of products in line with Namibia's, labelling regulation requirements. Other criticisms included the fact that Namibia's GMO labelling threshold was impracticable and had a major negative impact on South African local grain exporters. This is an indication of regional trade barriers between Namibia and South Africa caused by the Biosafety Labelling Regulations. Trade-related regulations in developing countries in terms of the trend toward harmonizing regulations with regards to trade-related regulations of GM food have been advocated by Gruère (2006). Although the GM labelling requirements exist in some countries, they are often challenging to implement in others due to a variety of circumstances, including a lack of understanding among consumers and those who should follow the regulations, such as companies who import or export GMO-related products (Bain and Dandachi, 2014; Adalja et al., 2022). Several empirical studies have focused on investigating consumer perceptions of food security, genetically modified crops, and food safety (Albert, 2010; Aerni et al., 2011; Valente and Chaves, 2018). Unfortunately, the existing research has been limited in assessing the overall implications of GMO labelling on producers, processors, and importers. No study was conducted in Namibia to assess the impact of the national biosafety labelling regulation on producers, processors, and importers after enactment. Hence the necessity to critically investigate the implications of the Biosafety Act, 2006, considering the trading partners.

2 Materials and methods

Assessing the adoption and application of the Namibian Biosafety labelling regulations and determining their impact on Namibian food and feed importers was undertaken using a

TABLE 1 Information on the respondents' context.

Sample	N	%
Gender		
Female	90	66.7
Male	45	33.3
No of years in the food and feed industry		
0–5 Years	30	22.2
6–10 Years	29	21.5
>10 Years	76	56.3
Position of the respondent		
Business Owner	43	31.9
Executive Management	20	14.8
Middle Management	59	43.7
Junior Management	8	5.9
Junior Staff	5	3.7
Full-time employees in the company		
0–20	49	36.3
21–40	24	17.8
41–60	22	16.3
61–80	9	6.7
81–100	5	3.7
>100	26	19.3

quantitative research approach. The response to the assessment were based on data collected through a structured questionnaire survey. The questionnaire survey was sent to 180 Namibian importers (importing food and feed products) based in most regions identified, namely Khomas, Erongo, Kavango West, Kavango East, Omusati, Oshana, Ohangwena, Oshikoto, and Zambezi who answered the survey from 28 April 2020 to 12 January 2021. A purposive sampling method was used to identify the respondents who were required to have basic knowledge on Namibian Biosafety labelling regulations adoption and application. A sample of experts comprised of the following groups: Business Owner, Executive Management, Middle Management, Junior Management and Junior Staff were surveyed. Contextual information regarding the background of the respondents is shown in table 1.

Questions in the survey were technical and specific to the Namibian biosafety labelling regulations, therefore only those who have knowledge or have been involved in the adoption and application of the Namibian biosafety labelling regulations and their impact on the food and feed importers in Namibia were included, hence the public was excluded. Survey questions were answered based on a five-point Likert scale. For instance, Robayo-Avendaño et al. (2018) used a 3-point scale, Coşkun and Olhan, (2022) as well as Nowamukama (2022) used a 5-point scale while Shooshtari et al. (2022) used a 6-point scale to assess the awareness of GMOs in terms of the Iran Biosafety Act: case study of Tehran city. The five-point scale used in this study provided the required detail for the evaluation while reducing potential over-complication caused by a higher number of alternatives (e.g., a 6-point scale (Leung, 2011). The survey responses in this study were rated as follows: 1—Strongly Disagree (SD); 2—Disagree(D); 3—Neither Agree nor Disagree (NAD); 4—Agree A); 5—Strongly Agree (SA).

A total of 137 were responded, making it 76.1% of the response rate and only 135 were responded correctly making up 98.5% usefulness in the analysis. Thirteen (13) of the non-response were no more in business and 30 were either not interested or non-responding at all. Survey results were analysed using the Statistical Package for the Social Sciences (SPSS 26) and Microsoft Excel (2020). Descriptive statistic statistics were employed to tabulate data, cross tabulations, and Chi Square. According to Pimentel (2010), the five-point Likert scale is an interval scale with a highly significant mean of 1–1.8, which means strongly disagree, 1.81 to 2.60, disagree, 2.61 to 3.40, neutral, 3.41 to 4.20, agree, and 4.21 to 5, which means strongly agree. When Likert scale, items are considered to have an interval measurement, and the information for all respondents is frequently summarized in the form of a weighted mean. Following this technique, the resultant weighted mean was interpreted using an interval with a matching verbal explanation.

3 Results

This section explores the perceptions of various actors on substantive effectiveness of the Namibia Biosafety framework as presented under three elements: a) Biosafety Act awareness, b) The BSL adoption and application, c) Impact of labelling regulations and d) Eliminating trade barriers posed by the Biosafety labelling regulations.

3.1 Biosafety Act awareness

3.1.1 Awareness of the existence of the Biosafety Act, 2006

Table 2 shows the perceptions of the survey respondents on the awareness of the existence of the Biosafety Act, 2006. The z value that was based on the Mann-Whitney Test ranks on the awareness of the existence of the Biosafety Act, 2006 is -2.328, with a significance level of $p = 0.020$. This result shows that since the p-value calculated is significant at 0.02, individuals who are aware of the existence of the Biosafety Act, 2006 are less likely to agree with the labeling of Genetically Modified Food and Feed (processed) as required by the Biosafety labelling regulations as compared to individuals who are not aware of the existence of the Biosafety Act, 2006.

3.1.2 Awareness of the labelling requirement under the biosafety Act, 2006

The Mann-Whitney Test was further run to establish whether individuals who are aware of the labelling requirements under the Biosafety Act, 2006 agree with the labeling of Genetically Modified Food and Feed (processed) as required by the Biosafety labelling regulations as compared to those whose companies are unaware. The z value is -1.101 with a significance level of $p = 0.271$. The

TABLE 2 Mann-Whitney Test ranks on the awareness of the existence of the Biosafety Act, 2006.

Are you aware of the existence of the biosafety Act, 2006?	n	Mean rank	Sum of ranks
Yes	125	65.80	8,225.00
No	10	95.50	955.00
Total	135		
Test Statistics			
Mann-Whitney U	350.000		
Wilcoxon W	8.225E3		
Z	−2.328		
Asymp. Sig. (2-tailed)	0.020		

TABLE 3 Mann-Whitney Test ranks on the awareness of the labelling requirement under the Biosafety Act, 2006.

Are you aware of the labelling requirement under the biosafety Act, 2006	n	Mean rank	Sum of ranks
Yes	98	65.74	6,442.50
No	37	73.99	2,737.50
Total	135		
Test Statistics			
Mann-Whitney U	1.592E3		
Wilcoxon W	6.442E3		
Z	-1.101		
Asymp. Sig. (2-tailed)	0.271		

results shows that there is sufficient evidence to conclude that there is no significant difference in the agreeance of individuals who are aware of the labelling requirement under the Biosafety Act, 2006 with the labeling of Genetically Modified Food and Feed as required by the Biosafety labelling regulations in Table3.

3.2 The BSL adoption and application

The study sought to determine how biosafety labelling regulations are adopted and applied in Namibia. To achieve this objective, the study looked at general Biosafety Act, 2006 awareness and how it related to adoption before assessing the acceptance of biosafety labelling regulations. Twardowski and Małyska (2015) have indicated that stagnation in the implementation of biosafety frameworks is due to a lack of awareness.

By running the Mann-Whitney Test, the study confirmed the findings that individuals who are aware of the existence of the Biosafety Act, 2006, including those who are experiencing problems in fulfilling the requirements of the Biosafety Act, 2006, are less likely to agree with the labeling of Genetically Modified Food and Feed (processed) as required by the Biosafety labelling regulations. Responses of individuals from companies that have applied to fulfil the requirements of the Biosafety (Secretariat of the Convention on Biological Biodiversity, 2000) indicated that they are more likely to agree with the labeling of Genetically Modified Food and Feed (processed) as required by the Biosafety labelling regulationsin Table4.

3.2.1 Companies applying to fulfil the requirements of the Biosafety Act, 2006

A Mann-Whitney test was run to assess whether individuals whose companies applied to fulfil the requirements of the Biosafety Act, 2006 agree with the labeling of Genetically Modified Food and Feed (processed) as required by the Biosafety labelling regulations compared to those whose companies are unaware. The z value is -2.502 with a significance level of $p = 0.012$. Based on the mean rank values observed in the ranks table, it can be concluded that individuals whose companies have applied to fulfil the requirements of the Biosafety Act, 2006 are more likely to agree with the labeling of Genetically Modified Food and Feed as required by the Biosafety labelling regulations as compared to individuals whose companies have not applied to fulfill the requirements of the Biosafety Act, 2006.

TABLE 4 Mann-Whitney Test ranks companies applying to fulfill the requirements of the Biosafety Act, 2006.

Has your company applied to fulfil the requirements of the biosafety Act, 2006	N	Mean rank	Sum of ranks
Yes	73	75.71	5,526.50
No	62	58.93	3,653.50
Total	135		
Test Statistics			
Mann-Whitney U	1.700E3		
Wilcoxon W	3.654E3		
Z	-2.502		
Asymp. Sig. (2-tailed)	0.012		

TABLE 5 Mann-Whitney Test ranks companies experiencing problems in fulfilling requirements of the Biosafety Act, 2006.

Is your company experiencing problems in fulfilling the requirements of the biosafety Act, 2006?	N	Mean rank	Sum of ranks
Yes	54	85.28	4,605.00
No	81	56.48	4,575.00
Total	135		
Test Statistics			
Mann-Whitney U	1.254E3		
Wilcoxon W	4.575E3		
Z	-4.222		
Asymp. Sig. (2-tailed)	0.000		

3.2.2 Companies experiencing problems in fulfilling requirements of the Biosafety Act, 2006

Individuals whose companies are experiencing problems in fulfilling requirements of the Biosafety Act, 2006 are less likely to agree with labeling of Genetically Modified Food and Feed as required by the Biosafety labelling regulations in comparison to individuals whose companies are not experiencing problems in fulfilling requirements of the Biosafety Act, 2006 Table5.

3.3 Impact of labelling regulations

3.3.1 Threats posed by the biosafety labelling regulations

3.3.1.1 The most significant threats posed by labelling regulations to a company

As seen from the Likert scale mean in Table 6, the response for all the questions regarding the biggest threats posed on companies surveyed regarding labelling regulations falls in the range of (2.01–3.00). Thus, it can be concluded based on the range used that the average response recorded for all the questions were neutral. The responses were slightly leaning towards monopoly in the supply chain.

3.3.1.2 Long-term consequences for businesses as a result of labelling regulations

Results show that out of the six long-term effects for businesses caused by labelling regulations, the threats posed by the Biosafety labelling regulations causes long term consequences such as reduction in production, revenues, increase in production costs, loss of markets, retrenchment of employees and closing of the company. As seen from the Likert scale mean in Table 7, the response for all the questions regarding the long-term consequences faced by companies if the biggest threats posed to their companies regarding labelling regulations are not resolved falls in the range of (2.01–3.00). Thus, it can be concluded based on the range used that the average response recorded for all the questions were neutral with responses leaning toward 'Lead to loss of markets'.

3.3.1.3 Policy recommendations for eliminating trade threats imposed by labelling regulations

The policy recommendations towards the elimination of trade threats imposed by labelling regulations looked at weather the policy should reduce regulatory administrative burden, increase the competence of regulators, improve regulatory monitoring processes, improve dialogue between regulators and industry or

TABLE 6 The most significant threats posed by labelling regulations to a company.

Statements concerning the biggest threats posed to your company regarding labelling regulations	N	Minimum	Maximum	Mean	Std. Deviation
Increases Business costs	135	1	5	2.50	1.021
Increases foreign competition	135	1	5	2.70	1.093
Lengthy administration process	135	1	5	2.40	1.001
Increase in taxes and permit fees	135	1	5	2.61	1.100
Monopoly in the supply chain	135	1	5	2.79	1.153
Too many import laws	135	1	5	2.42	1.143
Lack of technical capacity of regulators	135	1	5	2.50	1.085
Limited understanding of the law by importers	135	1	5	2.50	1.139
Lack of uniformity in the Namibia biosafety regulations	135	1	5	2.63	1.028
Inadequate capacity to export products to markets	135	1	5	2.63	1.028
c Note. 5 strongly agree (4.01–5.00), 4 agree (3.01–4.00), 3 neutral (2.01–3.00), 2 disagree (1.01–2.00), 1 strongly disagree (0.01–1.00)					

Reliability Coefficient	Cronbach's Alpha	N of Items
	0.912	10

TABLE 7 Long-term consequences for businesses as a result of labelling regulations.

Long-term consequences faced by companies if the biggest threats posed to your company regarding labelling regulations are not resolved	N	Minimum	Maximum	Mean	Std. Deviation
Reduction in production	135	1	5	2.47	0.853
Reduction in revenues	135	1	5	2.53	1.028
Increase in production costs	135	1	5	2.38	0.969
Lead to loss of markets	135	1	5	2.59	1.095
Lead to retrenchment of employees	135	1	5	2.46	1.091
Lead to closing of the company	135	1	5	2.41	0.988
c Note. 5 strongly agree (4.01–5.00), 4 agree (3.01–4.00), 3 neutral (2.01–3.00), 2 disagree (1.01–2.00), 1 strongly disagree (0.01–1.00)					

Reliability Coefficient	Cronbach's Alpha	N of Items
	0.882	6

TABLE 8 Policy recommendations for eliminating trade threats imposed by labelling regulations.

What policymakers should do to remove the threats in question caused by labelling regulations to allow importing companies to remove trade barriers	N	Minimum	Maximum	Mean	Std. Deviation
Reduce regulatory administrative burden	135	1	5	2.52	1.028
Increase the competence of regulators	135	1	5	2.25	1.020
Improve regulatory monitoring processes	135	1	5	2.46	1.131
Improve dialogue between regulators and industry	135	1	5	2.16	1.052
Create more awareness of the regulation	135	1	5	2.26	1.044
c Note. 5 strongly agree (4.01–5.00), 4 agree (3.01–4.00), 3 neutral (2.01–3.00), 2 disagree (1.01–2.00), 1 strongly disagree (0.01–1.00)					

Reliability Coefficient	Cronbach's Alpha	N of Items
	0.909	5

create more awareness of the regulation. As seen from the Likert scale mean in Table 8, the response for all the questions regarding what policymakers should do to remove the threats in question caused by labelling regulations to allow importing companies to remove trade barriers falls in the range of (2.01–3.00). Thus, it can be concluded based on the range used that the average response recorded for all the questions were neutral, however, respondents were more on 'improve regulatory monitoring processes'.

3.4 Trade barriers posed by the biosafety labelling regulations

As seen from the Likert scale mean, the response for all the questions (*Statements concerning trade barriers posed by the Biosafety labelling regulations*) falls in the range of (2.01–3.00), therefore it can be concluded based on the range used, the average response recorded for all those questions were neutral. Respondents were more leaning towards 'different labelling regulations between trading partners'. This is a clear illustration of the respondents' neutral position on the biggest threats to companies as a result of biosafety labelling regulations in Table 9.

3.5 Eliminating trade barriers posed by the biosafety labelling regulations

Table 10 shows that with the reliability test of Cronbach's Alpha coefficient ($\alpha = 0.847$), means that the scale has good internal consistency. As seen from the Likert scale mean, the response for all the questions (Statements concerning trade barriers posed by the Biosafety labelling regulations) falls in the range of (2.01–3.00), therefore it can be concluded based on the range used, the average response recorded for all those questions were neutral. However, respondents have highlighted that integrating regional labeling regulations could eliminate trade barriers. The respondents have a neutral position on what needs to be done to eliminate trade barriers posed by the Biosafety labelling regulations.

4 Discussions

4.1 Biosafety Act awareness

This result shows that individuals who are aware of the existence of the Biosafety Act, 2006 are less likely to agree with the labeling of Genetically Modified Food and Feed (processed) as required by the Biosafety labelling regulations as compared to individuals who are not aware of the existence of the Biosafety Act, 2006. The results might suggest that those that are aware of the existence of the Biosafety Act, 2006 do not agree with the labelling of GMO processed food and feed. Based on the findings of similar studies, a more plausible explanation is that there is an ongoing debate about whether genetically modified foods should be labelled, with some arguing that consumers should have the right to know everything about what's in their food and others arguing that there is no evidence that such foods harm health and that labelling is not necessary (Yang and Chen, 2016). While past studies have concentrated on consumer protection with regard to GMOs (Monien and Cai, 2018), there have been less investigations on Food and Feed (processed) importers and exporters who are required to label Food and Feed (processed). According to studies conducted on importers and exporters, the economic implications of labelling GMOs are the stumbling block

TABLE 9 Trade barriers posed by the Biosafety labelling regulations.

Attributes	N	Minimum	Maximum	Mean	Std. Deviation
Different labelling regulations between trading partners	135	1	5	2.60	1.154
Lengthy and different timing approval processes between trading partners	135	1	5	2.45	1.124
Difficult in accessing food or feed risk assessment information from other countries	135	1	5	2.47	1.071
Unintentionally and unauthorized movement of unlabeled products	135	1	5	2.56	0.944
Inadequate segregation of unlabeled food and feed products (during production, storage, and transportation)	135	1	5	2.33	0.864
*c Note. 5 strongly agree (4.01–5.00), 4 agree (3.01–4.00), 3 neutral (2.01–3.00), 2 disagree (1.01–2.00), 1 strongly disagree (0.**01**–1.00)*					

Reliability Coefficient	Cronbach's Alpha	N of Items
	0.733	5

TABLE 10 Eliminating trade barriers posed by the Biosafety labelling regulations.

Attributes	N	Minimum	Maximum	Mean	Std. Deviation
Integrate regional labeling regulations	135	1	5	2.74	1.178
Ban unlawful importing companies	135	1	5	2.12	1.127
Harmonize administration and enforcement of Namibian laws required for import	135	1	5	2.41	1.135
Improve public engagement for the newly introduced laws	135	1	5	2.24	1.272
c Note. 5 strongly agree (4.01–5.00), 4 agree (3.01–4.00), 3 neutral (2.01–3.00), 2 disagree (1.01–2.00), 1 strongly disagree (0.01–1.00)					

preventing them from those wishing to label them even when they are aware of the labelling legislation (Grebitus et al., 2018). As a result, the Food and Feed importers and exporters who participated in the study and are required to label the GMO Food and Feed are aware of the Biosafety Labelling regulation, and they are less likely to label GMOs as they might be concerned with the economic implications of labelling. These findings support Oh and Ezezika's (2014) contention that such labelling may raise food costs, impeding attempts to meet food security requirements. Companies that develop GMO products are more likely to charge higher prices for their products such as GM seeds, with the intention of recouping their investments on research and development which can have a repel effect on the price of GMO product prices (Rutivi and Mugwagwa, 2009). The expenses of GMO review are determined not only by the criteria, but also by the length of time required to get a permit and the type of label on the products. These findings are supported by a study conducted in the United States as it indicated that when consumers were presented with conventional, organic, and non-GMO food options, the additional amount consumers were willing to pay for organic food was insignificant, indicating that producers would benefit economically by using a non-GMO label rather than organic certification (Grebitus et al., 2018). This suggests that being GMO-free may have a benefit, since it offers makers of GMO-free products with a significant motivation to market their products in this manner, putting less economic advantages on GMO-branded products. Due to the lack of data on the economic value of labeling in terms of cost and time of GMs due to the existence of the Biosafety Act, 2006 in Namibia, the results cannot confirm why those who are aware of the labelling regulations are less likely to agree with labelling of labeling of GMO Food and Feed because of economic value. Thus, the need for further studies in this regard.

The results shows that there is sufficient evidence to conclude that there is no significant difference in the agreeance of individuals who are aware of the labelling requirement under the Biosafety Act, 2006 with the labeling of Genetically Modified Food and Feed (processed) as required by the Biosafety labelling regulations. Twardowski and Małyska (2015) have indicated that stagnation in the implementation of biosafety frameworks is due to a lack of awareness.

4.2 The BSL adoption and application

Studies examining the companies applying to fulfil the requirements of the Biosafety Act are part of BSL adoption and application review. Many countries have several mechanism and requirements for BSL adoption and application, but few evaluate the companies applying to fulfil the requirements of the Biosafety Act with the purpose of improving the BSL adoption and application. This study assessed weather individuals whose companies applied to fulfil the requirements of the Biosafety Act, 2006 agreed with the statements regarding the labeling of genetically modified food and feed as required by the Biosafety labelling regulations in comparison to the companies who are unaware of the Biosafety labelling regulations. The Mann-Whitney test done indicates that individuals whose companies have applied to fulfil the requirements of the Biosafety Act, 2006 are more likely to agree with the statements regarding the labeling of genetically modified food and feed as required by the biosafety labelling regulations as compared to individuals whose companies have not applied to fulfill the requirements of the Biosafety Act, 2006.

The legal framework for labelling GMO products before placing the products on the market was necessary and thus the need for companies to apply to fulfil the requirements of regulations such as the Biosafety Act, 2006. The Mann-Whitney test agreeing that companies that have applied to fulfil the requirements of the Biosafety Act, 2006 are more likely to label GMO feed and food as required by the Biosafety labelling regulations is an indication of the agreement of BSL adoption and application by companies. The BSL adoption and application by companies is very important because the Biosafety Act was enacted so that all activities such as importation, production, release, and distribution are regulated to limit possible harmful consequences to the environment. According to the Government Gazette that deals with the Biosafety Act that regulates genetically altered products, non-compliance will lead to a fine not exceeding N$8,000 or imprisonment for a period not exceeding a 2 years or both.

The study found that individuals whose companies are experiencing problems in fulfilling requirements of the Biosafety Act, 2006 are less likely to agree with labeling of GM Food and Feed as required by the Biosafety labelling regulations in comparison to individuals whose companies are not experiencing problems in fulfilling requirements of the Biosafety Act, 2006. Existing regulations mandate labelling for both imported and domestically produced end goods (food and feed) to ensure that products are labelled in such a way that buyers are aware of the presence of GMOs in the products. But non-etheless, the Biosafety Act now requires permits to import, process, and transport such things, which were previously not necessarily due to the country's long history of importing GMO food. Additionally, the law mandated that the permission application process be made public, and the public was allowed to express themselves whether such GMO permit should be given or denied. The Biosafety Council's goal was to collect public inputs about GMO permits before determining whether to approve or reject such licenses. However, there is little to no research on the overall time it takes to fulfilling requirements of the Biosafety Act, 2006. However, the overall approval process for fulfilling requirements of the Biosafety Act, 2006 is longer than established 90 days to make a decision. The Cartagena protocol (article 11, 6b)) gives a maximum of 270 days) which is considering as the whole application to come to a decision.

4.3 Impact of labelling regulations

Results show that out of the ten statements concerning the biggest threats posed to companies regarding labelling regulations, the most significant threats posed by the Biosafety labelling regulations to companies are the increases in foreign competition and monopoly in the supply chain. A well-thought-out Biosafety labelling regulation should include many components that do not threaten companies, resulting in more good competition and less monopoly within the supply chain (Van der Walt, 2001). In the GMO labeling context, Kim et al. (2022) argue that a GMO label mandated by a regulatory body may send a negative signal that consumers should avoid a product with GM. Seeing that despite the presence of commercial farms in Namibia, the country has struggled to maintain the essence of food security thus striving through food imports, companies in Namibia would see the Biosafety labelling regulations as a threat towards foreign competition. A Biosafety labelling regulation that does not threaten companies in terms of ensuring that there are no impediments sch as foreign competition and monopoly in the supply chain towards companies can mitigate the impact of labelling regulations. However, Albert ed (2010) contends that, given the high level of competition in the global food market, consumer`s attitudes towards GM foods can heavily influence decisions made by farmers, commodity dealers, food manufacturers, and food retailers about whether to produce and market GM foods or use conventional varieties. Therefore, an understanding of the perceptions of, and likely reactions toward, genetically modified (GM) foods is crucial for decision making by both policymakers and biotechnology companies developers (Spence and Townsend, 2006). Institutions entrusted with enforcing the Biosafety labelling regulations should be strengthened to ensure that they understand the perceptions of, and likely reactions toward, genetically modified (GM) foods when making decisions to lessen the impacts of labelling regulations.

Results show that out of the six long-term effects for businesses caused by labelling regulations, the views on the threats posed by the Biosafety labelling regulations in terms of long-term consequences such as reduction in production, revenues, increase in production costs, loss of markets, retrenchment of employees and closing of the company are neutral. A neutral choice, according to DeMars and Erwin (2005), rewards responders who tilt slightly towards a favourable or unfavourable judgement. Thus, these findings can mean that the respondents were leaning slightly towards a favorable or unfavorable long-term effects for businesses because of labelling regulations stating that the labelling regulations are more neutral, but they are more geared towards leading to loss of markets with a mean of 2.53 and reduction in revenues with a mean of 2.59. In an ideal world, a well-thought-out mandatory labelling regulation would not jeopardize a company's economic sustainability through market loss and revenue decrease (Van der Walt, 2001; Oh and Ezezika, 2014). Therefore, the long-term effects for business due to labelling regulations can have an impact on the Namibian economy.

In terms of revenue, food companies believe that the loss is due to the significantly high costs involved with mandatory labelling of

GM products, as well as customer reaction towards GMOs. For example, research done in Kenya found that, while their food sector regarded it as vital to track GM products, many do not support labelling of GM products because of the additional costs and the likelihood of negative customer reactions (Bett et al., 2010). While studies (James, 2011; Oh and Ezezika, 2014; MacFarland and Yates, 2016; Strauss and Sax, 2016; Huffman and McCluskey, 2017) have been conducted, all former, demonstrating that mandatory labelling will incur additional costs that will eventually be passed on to the consumer, actual increases in food prices because of mandatory GM labelling have yet to be reported. Nevertheless, a 2001 research based on current EU legislation discovered that obligatory GM labelling adds an additional *per capita* yearly cost of around US$0.23 (Jones, 2001). A similar study done in Canada have shown that mandatory labelling in Canada would increase retail prices by 9%–10% (KPMG, 2000). Therefore, mandatory GM labelling law makes it important to fuel discussions about the costs of implementation in terms of the whole value chain as this have a significant impact on the companies dealing the GM products. According to Oh and Ezezika (2014), African governments including Namibia can benefit from reliable studies that allow them to examine the economic sustainability of regulating the mandate of GM labelling inside their individual nations before enacting a labelling law, as well as whose stakeholders may be impacted. In South Africa, Reddy (2017) showed that the direct cost increase of mandatory labelling to the consumer depends on many factors, but the average is calculated to be between 9% and 12%. This means that most of the market will suffer the expenses of GM labelling. More studies assessing the potential economic costs of mandatory labelling in Africa, however, are required, as most cost experiments pertaining to mandatory GM labelling are based on the experiences of countries other than Africa. Therefore, further case-by-case analyses of the economic consequences of mandatory GM labelling on Namibian food industry companies are required**.** Moreover, Namibia has developed and gazetted of genetically modified product list of GM transformed events that should first be approved for safe use even before consignments entering Namibian territory.

The policy recommendations towards the elimination of trade threats imposed by labelling regulations looked at wether the policy should reduce regulatory administrative burden, increase the competence of regulators, improve regulatory monitoring processes, improve dialogue between regulators and the industry or create more awareness of the regulation. The study findings were neutral on all five 5) statements even though the response was more geared towards the policy reducing regulatory administrative burdens and improving regulatory monitoring processes. This finding is consistent with studies that show the weaknesses within Namibia's own Biosafety legal system (Geingos, 2018). Parker and Kirkpatrick (2012) highlighted that weakness within a legal system can be caused by regulatory administrative burdens and improving regulatory monitoring processes. However, administrative burdens in regulating GMO`s in Namibia are also rooted in the fact that even though Namibian regulatory framework made provision for a national GMO testing facility, this facility is still in the development phase and is not yet accredited. A GMO testing facility is essential for GM tracking, monitoring, and surveillance, as well as full compliance with national requirements (Kaiser et al., 2015). From the result, it can be inferred that limited regulatory monitoring processes such as that of the GMO testing laboratory require policy intervention in strengthening and reducing regulatory administrative burdens. However, Grechkina et al. (2019) concluded that the implementation of customs control over cross border movement of GMO foods improves efficiency and ensures the protection of the rights of citizens of the EAEU member States. Therefore, Namibia have developed and gazetted a genetically modified product list even though there is still a need to implement border control to sample GMO product consignments entering Namibia to ensure that permits granted are adhered to.

4.4 Trade barriers posed by the biosafety labelling regulations

The overall rating of the respondents shows that their response towards the trade barriers posed by the Biosafety labelling regulations is neutral. From the results, it can be inferred that the removal of trade barriers posed by the Biosafety labelling regulations in Namibia are more towards the different labelling regulations between trading partners and the unintentionally and unauthorised movement of unlabeled products. Trading of GMO products in Namibia as regulated by the Biosafety labelling regulations is affected by various contextual and administrative challenges such as poor regulatory frameworks. GMOs are widely available across the world, but they are also contentious and susceptible to regulatory scrutiny in terms of mandatory *versus* voluntary GMO labelling (Bullock and Desquilbet, 2002; Bhalerao and Kadam, 2010; Buah et al., 2021). Thus, different countries have developed and are implementing different GMO labeling policies to inform consumer choice if imported from countries with different percentage threshold levels of GMOs and labelling requirements. A good example is a difference in labelling requirements between the Biosafety Act, 2006 of Namibia and the South African Consumer Protection Act, 68 of 2008. When the GM content of a product exceeds 0.9%, it must be labelled as opposed to that which requires labelling when the GM content exceeds 5.0% (Charnovitz, 2000). Jacobs (2018) added that this GMO labelling regulations caused a severe negative impact on South Africans grain exporters to Namibia, it is expected to have very little impact on the South African grain industry as we export fairly small quantities to Namibia. This overlap between different country labelling may be an interesting area for future work as this may help to narrow the focus on finding a solution to the trade impacts of Biosafety labelling regulations. Hence the necessity to conduct further studies to critically review the way to ensure that labelling regulations between trading partners are married.

4.5 Eliminating trade barriers posed by the biosafety labelling regulations

The study looked at how trade barriers posed by the Biosafety labelling regulations can be eliminated based on six variables mainly in terms of integrating the regional labeling regulations, banning unlawful importing companies, harmonising administration and enforcement of Namibian laws required for import and improving

public engagement for the newly introduced laws. The study responses were neutral but was more leaning towards favoring the integration of the regional labeling regulations. These findings are supported by Jacobs (2018) who found that South Africa's labelling regulations is not in harmony with those of Namibia, with Namibia's being more explicit than South Africa. However, the need to integrate labeling regulations has not only been a regional issue but it has been advocated at the international levels. There are also large differences in import-approval and marketing policies for GM food worldwide that can impact trade. Gruère (2006) noted that at the international level, harmonization efforts are led by the Codex Alimentarius Commission, the Cartagena Protocol on Biosafety (CPB), and the World Trade Organization (WTO). While internationally harmonized guidelines for safety approval have been finalized at the Codex Alimentarius, there is no clear consensus on labelling regulations for GM food, some of which could be found inconsistent with the WTO, and there is an increasing risk of conflicts between the CPB and the WTO. Even though there have been harmonization efforts at the international level, their consensus is more on safety approval, not on labeling. From a policy perspective, Gruère (2006) argues that there are three main spillover effects of national and international regulations on developing countries' according to policymaking: 1) compliance with international agreements that do not necessarily correspond to domestic objectives, 2) the fear of export loss due to trade-related regulations implemented by the large importing countries, and 3) the trend toward harmonizing domestic regulations with those of the large importers. As a result, Namibia from a policy perspective must ensure that the Biosafety labelling requirements for GMOs are based on international standards while considering regional constraints and national preferences that give considerable productivity benefits to local businesses.

5 Recommendation

- Considering these conflicting viewpoints, the study suggests more research into how various GMO labelling legislation regimes affect the goods Namibian consumers choices. This will provide valuable input towards the Biosafety Act in terms of whether the sort of labelling being used affects customer choice.
- In practice, the research further suggests that the government raise public knowledge of GM foods through advertising, as well as boosting media coverage of GM safety and consumer choice through GM food labelling.
- To improve the adoption and application of the BSL, Namibia might learn from other countries e.g., South Africa and the EU that have already established processes.
- From a policy perspective, Namibia must ensure that the Biosafety labelling requirements for GMOs are based on best practices while taking into account regional constraints and national preferences that give considerable productivity benefits to local businesses
- Due to the lack of data on the economic value of labeling in terms of cost and time of GMOs due to the existence of the Biosafety Act, 2006 in Namibia, the results cannot confirm why those who are aware of the labelling regulations are less likely to agree with labelling of labeling of GMO products because of economic value. Thus, the need for further studies in this regard.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Acknowledgments

We are grateful to the department of Biology, Chemistry, and Physics and the Department of Agricultural Sciences and Agribusiness for the provision of resources to produce this work. This work is part of the Ph.D. study for the first author, the two authors are supervisors of the Namibian University of Science and Technology. The authors have drafted and revised the manuscript for submission.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Aarts, H. J., van Rie, J.-P. P., and Kok, E. J. (2002). Traceability of genetically modified organisms. *Expert Rev. Mol. Diagnostics* 2 (1), 69–77. doi:10.1586/14737159.2.1.69

Adalja, A., Liaukonytė, J., Wang, E., and Zhu, X. (2022). GMO and non-GMO labeling effects: Evidence from a quasi-natural experiment. *Mark. Sci.* 42, 233–250. doi:10.1287/mksc.2022.1375

Adenle, A. A., Ford, J. D., Morton, J., Twomlow, S., Alverson, K., Cattaneo, A., et al. (2017). Managing climate change risks in Africa - a global perspective. *Ecol. Econ.* 141, 190–201. doi:10.1016/j.ecolecon.2017.06.004

Aerni, P., Scholderer, J., and Ermen, D. (2011). How would Swiss consumers decide if they had freedom of choice? Evidence from a field study with organic, conventional and GM corn bread. *Food Policy* 36 (6), 830–838. doi:10.1016/j.foodpol.2011.08.002

Aguilera, J., Gomes, A. R., and Olaru, I. (2013). Principles for the risk assessment of genetically modified microorganisms and their food products in the European Union. *Int. J. Food Microbiol.* 167 (1), 2–7. doi:10.1016/j.ijfoodmicro.2013.03.013

Albert, J. (Editor) (2010). *Innovations in food labelling* (Rome, Cambridge, UK, Boca Raton, FL: Food and Agriculture Organization of the United Nations).

Asioli, D., Aschemann-Witzel, J., Caputo, V., Vecchio, R., Annunziata, A., Næs, T., et al. (2017). Making sense of the "clean label" trends: A review of consumer food choice behavior and discussion of industry implications. *Food Res. Int.* 99, 58–71. doi:10.1016/j.foodres.2017.07.022

Azila-Gbettor, E. M., Avorgah, S. M. K., and Adigbo, E. D. (2013). Exploring consumer knowledge and usage of label information in Ho Municipality of Ghana. *Eur. Sci. J.* 9 (28), 1–10. doi:10.19044/esj.2013.v9n28p%25p

Bain, C., and Dandachi, T. (2014). Governing GMOs: The (counter) movement for mandatory and voluntary non-GMO labels. *Sustainability* 6 (12), 9456–9476. doi:10.3390/su6129456

Bett, C., Ouma, J. O., and De Groote, H. (2010). Perspectives of gatekeepers in the Kenyan food industry towards genetically modified food. *Food Policy* 35 (4), 332–340. doi:10.1016/j.foodpol.2010.01.003

Bhalerao, S., and Kadam, P. (2010). Sample size calculation. *Int. J. Ayurveda Res.* 1 (1), 55. doi:10.4103/0974-7788.59946

Borges, B. J. P., Arantes, O. M. N., Fernandes, A. A. R., Broach, J. R., and Fernandes, P. M. B. (2018). Genetically modified labeling policies: Moving forward or backward? *Front. Bioeng. Biotechnol.* 6, 181. doi:10.3389/fbioe.2018.00181

Buah, E., Linnanen, L., and Wu, H. (2021). Augmenting the communication and engagement toolkit for CO2 capture and storage projects. *Int. J. Greenh. Gas Control* 107, 103269. doi:10.1016/j.ijggc.2021.103269

Bullock, D. S., and Desquilbet, M. (2002). The economics of non-GMO segregation and identity preservation. *Food Policy* 27 (1), 81–99. doi:10.1016/s0306-9192(02)00004-0

Carter, C. A., and Gruère, G. P., 2003. *Mandatory labeling of genetically modified foods: Does it really provide consumer choice?*. Germany: Springer.

Charnovitz, S. (2000). The supervision of health and biosafety regulation by world trade rules. *Tulane Environ. Law J.* 13 (2), 271–302.

Choi, E. K. (2010). International trade in genetically modified products. *Int. Rev. Econ. Finance* 19 (3), 383–391. doi:10.1016/j.iref.2009.04.002

Coşkun, S. T., and Olhan, E. (2022). Genetically modified organisms and biosafety as perceived by professionals according to their sociodemographic characteristics. *Int. J. Agric. Environ. Food Sci.* 6 (1), 121–129. doi:10.31015/jaefs.2022.1.16

DeMars, C. E., and Erwin, T. D. (2005). *Neutral or unsure: Is there a difference*. Washington, DC: American Psychological Association.

Geingos, I. L. (2018). "The role of law in the development transition to a knowledge-based economy–A study of Namibia's drive towards a sustainable biotechnology regulation," Doctoral dissertation. (Sheffield: University of Sheffield).

Grebitus, C., Peschel, A. O., and Hughner, R. S. (2018). Voluntary food labeling: The additive effect of "free from" labels and region of origin. *Agribusiness* 34 (4), 714–727. doi:10.1002/agr.21558

Grechkina, O. V., Agamagomedova, S. A., Zyryanov, S. M., and Spector, L. A. (2019). *Customs' control on genetically modified food products across the border of the*. United States: Eurasian Economic Union.

Gruère, G. P. (2006). *An analysis of trade related international regulations of genetically modified food and their effects on developing countries*. Germany: Springer.

Haigh, M. (Editor) (2004). *Handbook on the labelling of genetically modified foods, ingredients and additives* (United States: Leatherhead Food International).

Hu, Y., House, L. A., McFadden, B. R., and Gao, Z. (2021). The influence of choice context on consumers' preference for GM orange juice. *J. Agric. Econ.* 72 (2), 547–563. doi:10.1111/1477-9552.12416

Huffman, W., and McCluskey, J. (2017). Food labels, information, and trade in GMOs. *J. Agric. Food Industrial Organ.* 15 (1), 38. doi:10.1515/jafio-2016-0038

Jacobs, N. (2018). Namibian labelling regs drive up hassle factor for SA exporters. Available at: https://www.freightnews.co.za/article/namibian-labelling-regs-drive-hassle-factor-sa-exporters.

James, C. (2011). *Global status of commercialized biotech/GM crops*. Ithaca, NY: isaaa.

Jones, S. (2001). *Economic appraisal of options for extension of legislation on GM labelling: A final report for the food standards agency*. United States: National Economic Research Associates.

Kaiser, A. D., Assenmacher, M., Schröder, B., Meyer, M., Orentas, R., Bethke, U., et al. (2015). Towards a commercial process for the manufacture of genetically modified T cells for therapy. *Cancer gene Ther.* 22 (2), 72–78. doi:10.1038/cgt.2014.78

Kaur, P., Stoltzfus, J., and Yellapu, V. (2018). Descriptive statistics. *Int. J. Acad. Med.* 4 (1), 60. doi:10.4103/ijam.ijam_7_18

Kedisso, E. G., Maredia, K., Guenthner, J., and Koch, M. (2022). Commercialization of genetically modified crops in Africa: Opportunities and challenges. *Afr. J. Biotechnol.* 21 (5), 188–197. doi:10.5897/ajb2021.17434

Kim, Y., Kim, S., and Arora, N. (2022). GMO labeling policy and consumer choice. *J. Mark.* 86 (3), 21–39. doi:10.1177/00222429211064901

Komen, J. (2012). The emerging international regulatory framework for biotechnology. *Medicine* 4 (1), 78–84. doi:10.4161/gmcr.19363

KPMG (2000). *Phase 1 report: Economic impact study: Potential costs of mandatory labeling of food products derived from biotechnology in Canada*. Germany: Springer.

Leung, S. O. (2011). A comparison of psychometric properties and normality in 4-5-6-and 11-point Likert scales. *J. Soc. Serv. Res.* 37 (4), 412–421. doi:10.1080/01488376.2011.580697

MacFarland, T. W., and Yates, J. M., 2016. *Introduction to nonparametric statistics for the biological Sciences using R*. 1st ed. Cham: Springer International Publishing.

McFadden, B. R. (2017). The unknowns and possible implications of mandatory labeling. *Trends Biotechnol.* 35 (1), 1–3. doi:10.1016/j.tibtech.2016.09.009

Monien, J., and Cai, Y. (2018). *Genetically modified organisms law, handbook of agri-food law in China, Germany, European union: Food security, food safety, sustainable use of resources in agriculture*. Germany: Springer, 517–549.

NAB (2021). Rules pertaining to the processing of yellow maize grain for human consumption. Available at: https://www.nab.com.na/wp-content/uploads/2022/05/RULES-PERTAINING-TO-THE-PROCESSING-OF-YELLOW-MAIZE-GRAIN-FOR-HUMAN_-ONSUMPTION.pdf.

Nowamukama, M. (2022). *Public perceptions, knowledge and factors associated with the acceptability of genetically modified foods in Kampala city*. Uganda: Springer, 2022–2111.

Oh, J., and Ezezika, O. C. (2014). To label or not to label: Balancing the risks, benefits and costs of mandatory labelling of GM food in Africa. *Agric. Food Secur.* 3 (1), 8. doi:10.1186/2048-7010-3-8

Parker, D., and Kirkpatrick, C. (2012). *Measuring regulatory performance*. Germany: Springer.

Phillips, P. W., and McNeill, H. (2000). *A survey of national labeling policies for GM foods*. Germany: Springer.

Pimentel, J. (2010). A note on the usage of Likert scaling for research data analysis. *Science* 18, 109–112.

Reddy, S. (2017). *Determining awareness, attitudes, and behaviors towards genetically modified food among consumers in Durban, South Africa*. Doctoral dissertation. (South Africa: University of South Africa).

Robayo-Avendaño, A., Galindo-Mendoza, M. G., Yáñez-Estrada, L., and Aldama-Aguilera, C. (2018). Measurement of public perception of GMOs with a likert-type scale. *Agrociencia* 52 (5), 767–781.

Rutivi, C., and Mugwagwa, J. (2009). *Socio-economic impact of GMOs on african consumers*. United States: Socio-economic impact of GMOs on African consumers, 55.

Secretariat of the Convention on Biological Biodiversity (2000). *Cartagena protocol on biosafety to the convention on biological diversity*. Montreal: Springer, 11–12.

Shooshtari, A., Hajatpour, Z., Ghaffari, M. R., Seraji, F., and Loni, F.,(2022). Awareness of GMOs in terms of the Iran biosafety Act: Case study of tehran city. *Available at SSRN 4298126*.

Spence, A., and Townsend, E. (2006). Examining consumer behavior toward genetically modified (GM) food in Britain. *Risk Anal.* 26 (3), 657–670. doi:10.1111/j.1539-6924.2006.00777.x

Strauss, S. H., and Sax, J. K. (2016). Ending event-based regulation of GMO crops. *Nat. Biotechnol.* 34 (5), 474–477. doi:10.1038/nbt.3541

Twardowski, T., and Małyska, A. (2015). Uninformed and disinformed society and the GMO market. *Trends Biotechnol.* 33 (1), 1–3. doi:10.1016/j.tibtech.2014.11.006

Valente, M., and Chaves, C. (2018). Perceptions and valuation of GM food: A study on the impact and importance of information provision. *J. Clean. Prod.* 172, 4110–4118. doi:10.1016/j.jclepro.2017.02.042

Van der Walt, W. J. (2001). Trade barrier issues related to modern biotechnology. *J. New Seeds* 3 (1), 71–82. doi:10.1300/j153v03n01_05

Yang, Y. T., and Chen, B. (2016). Governing GMOs in the USA: Science, law and public health. *J. Sci. Food Agric.* 96 (6), 1851–1855.

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Hypothesis and Theory
PUBLISHED 04 August 2023
DOI 10.3389/fbioe.2023.1209054

OPEN ACCESS

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RECEIVED 20 April 2023
ACCEPTED 12 July 2023
PUBLISHED 04 August 2023

CITATION
Mueller S (2023), Recombination between coronaviruses and synthetic RNAs and biorisk implications motivated by a SARS-CoV-2 FCS origin controversy. *Front. Bioeng. Biotechnol.* 11:1209054. doi: 10.3389/fbioe.2023.1209054

Recombination between coronaviruses and synthetic RNAs and biorisk implications motivated by a SARS-CoV-2 FCS origin controversy

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The urgent need for improved policy, regulation, and oversight of research with potential pandemic pathogens (PPPs) has been widely acknowledged. A 2022 article in Frontiers in Virology raises questions, reporting on a 100% sequence homology between the SARS-CoV-2 furin cleavage site (FCS) and the negative strand of a 2017 patented sequence. Even though Ambati and collaborators suspect a possible inadvertent or intentional cause leading to the FCS insert, the related underpinnings have not been studied from the perspective of potential biorisk policy gaps. A commentary on their article contests the low coincidence likelihood that was calculated by Ambati et al., arguing that the sequence match could have been a chance occurrence alone. Additionally, it has been suggested that the odds of the recombination event may be low. These considerations seem to have put many speculations related to any implied viral beginnings, notably from a research setting likely outside the Wuhan Institute of Virology, to rest. However, potential implications for future disasters in terms of biosafety and biosecurity have not been addressed. To demonstrate the *feasibility* of the Ambati et al. postulate, a theoretical framework is developed that substantially extends the research orientations implicated by these authors and the related patent. It is argued that specific experimental conditions, in combination, could significantly increase the implied recombination profile between coronaviruses and synthetic RNAs. Consequently, this article scrutinizes these largely unrecognized vulnerabilities to discuss implications across the spectrum of the biological risk landscape, with special attention to a potential "crime harvest." Focusing on insufficiently understood features of interaction between the natural and man-made world, vulnerabilities related to contaminants, camouflaging, and various misuse potentials fostered by the digitization and computerization of synthetic biology, it highlights novel biorisk gaps not covered by existing PPP policy. Even though this work does not aim to provide proof of the viral origin, it will make the point that, in theory, a convergence of under-appreciated lab experiments and technologies could have led to the SARS-CoV-2 FCS insert, which analogously could be exploited by various threat actors for the clandestine genesis of similar or even worse pathogens.

Abbreviations: CoV, coronavirus; DDR, DNA damage response; DU, Dual-Use; DURC, dual-use-of-concern; ENISA, The European Union Agency for Cybersecurity; FCS, furin cleavage site; GoF, gain of function; MSH3, The DNA mismatch repair protein MutS Homolog 3; NLS, nuclear localization signal; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2.

KEYWORDS

DURC, recombinability, interactions between the natural and man-made world, coronavirus, biorisk spectrum, deliberate attacks, crime harvest, Trojan horse

1 Introduction

The realization that an analysis such as the one below was necessary arose over a 2-year discussion with The European Union Agency for Cybersecurity which had been tasked with evaluating cybersecurity gaps in the life sciences (Mueller and Barros Lourenco, 2023) as fostered by the digitization of biology, computerized applications, and web-interfaces. This led to the question of applications, particularly in synthetic biology, which, when compromised, could have systemic implications and endanger critical infrastructure. These technologies may fall under the new European NIS2 Directive (The European Commission, 2023) which makes organizations engaged in such type of work compliant with the requirements necessary for the protection of critical infrastructures (Mueller and Barros Lourenco, 2023).

In this light, this article investigates specific regulatory and assessment gaps that arise from a controversy surrounding a postulated origin of the furin cleavage site (FCS) insertion into SARS-CoV-2. This particular topic is not analyzed to prove the beginnings of the virus, but rather, to highlight the feasibility of these controversial issues and the ensuing potentials for future malicious exploitation.

Background: The Covid pandemic has been one of the most destructive events in modern human history. Over 3 years since the first emergence of the new virus, there is still no consensus about its origin. While initially, a natural spillover event was essentially taken for granted (Andersen et al., 2020), studies and investigations by the Lancet Commission, the FBI, and an assessment by the Energy Department concluded with varying degrees of confidence that its likely source was an accidental release from the Wuhan Institute of Virology (WIV)[1]. In an April 18 bombshell release[2], a report by the U.S. Senate Committee on Health, Education, Labor and Pensions asserts that SARS-CoV-2 likely resulted from an accidental leak at a laboratory in Wuhan.

In recent years, gain-of-function (GoF) work has been heavily criticized, and many leading experts, including former Centers for Disease Control and Prevention (CDC) Director Dr. Redfield, argue that even while such a type of work aims to prevent or prepare for a pandemic by artificially improving "the ability of a pathogen to cause disease"[3], that, on the contrary, it "caused the greatest pandemic we've ever seen[4]."

With certain coronaviruses (CoVs), it has long been known that the presence of a furin cleavage site (FCS) plays a key role in cell tropism and pathogenesis of these viruses. Over the years, this observation has triggered many research projects to better assess how the insertion of such cleavage sites into specific viruses could possibly enhance their transmissibility, expand their tropism, and increase their pathogenicity (reviewed in (Chan and Zhan, 2022)). A common observation has been that the insertion of an FCS has always made these viruses more dangerous. Significantly, while several CoVs do have an FCS, SARS-CoV-2 is the only member of the subgenus *sarbecovirus* with this characteristic. The finding of this unique FCS, absent among all SARS-related CoVs but inserted into SARS-CoV-2, has already rather early during the pandemic been seen as a significant piece of evidence to suggest that SARS-CoV-2 may be the product of laboratory manipulation (Chan and Zhan, 2022; Harrison and Sachs, 2022). Albeit, by just looking at the sequence, there is no way to determine whether humans or nature inserted this novel site into the virus (Cyranoski, 2020), which highlights but one difficulty of how "risky" research could be attributed, identified, and regulated.

The question as to what type of research should be regarded as "too dangerous" has triggered hefty discussions. For example, a recent policy analysis by a U.S. biosecurity panel found numerous loopholes and weaknesses in current regulation and oversight. Even though the panel agreed on a long-awaited set of recommendations, they are concerned about the vagueness of some of these, and in practice, the U.S. rules for risky pathogen research remain unclear (Reardon, 2023).

Specific research, such as experiments with an FCS, has previously been recognized as "too risky," as evidenced by past expert advice. Notably, the insertion of FCS sequences into SARS-like viruses, a stated goal of the "DEFUSE" project, was in 2018 rejected by DARPA because the risks were deemed too high (Harrison and Sachs, 2022). While this does not exclude the possibility that such work was carried out using different funding sources, the research community is largely aware of potential perils associated with FCS research.

The key point raised here is that in addition to the debates surrounding the tradeoffs between the risks/benefits of inserting an FCS, or stated manipulations on the spike protein overall (Jocelyn, 2023), there may be yet another possibility for the emergence of the FCS altogether that has fallen outside regulatory oversight.

Specific research that may escape regulation: At the core of the ongoing controversy of what type of research should be restricted is that lab work is inherently dual-use (DU): the same research can be used for both benevolent and harmful purposes. Some DU work could potentially lead to disasters with far-reaching potential, known as DU research "of concern (DURC)," which, in a synthetic biology context essentially means that pathogens are being made even more dangerous[5].

It will be suggested below that it may be possible that even research that does not have stated DURC/GoF risks (such as recognized for the FCS) may cause significant damage and even

1 https://www.washingtonpost.com/investigations/interactive/2023/virus-research-risk-outbreak/?itid=hp-top-table-main_p001_f001&mc_cid=1aed65cce5&mc_eid=6cb23747ff

2 https://www.marshall.senate.gov/newsroom/press-releases/sen-marshall-releases-bombshell-covid-19-origins-report/

3 https://www.phe.gov/s3/dualuse/Pages/GainOfFunction.aspx

4 https://www.youtube.com/watch?v=g4rF91BeSJU

5 A detailed list of relevant DURC research criteria can be found in (Godbold et al., 2023).

TABLE 1 Poorly recognized biorisks which can be inferred from the scenarios suggested by Ambati et al. (2022): The finding of a purportedly proprietary sequence in SARS-CoV-2 encompassing the FCS, in the context of cancer research, raises many questions related to unrecognized biosafety and biosecurity dangers.

Key points made by Ambati et al. (2022)	Alternative views, main questions, and comments
Ambati et al. report on the presence in SARS-CoV-2 of a 19-nucleotide RNA sequence • The novel insert encompasses and encodes the novel FCS of its spike protein • It has 100% identity to the reverse complement of a proprietary MSH3 mRNA sequence (identified as SEQ ID11652, nt 2751-2733, see below) • The insert could have happened during laboratory research via some recombination event	Although the beginnings of COVID have not been unambiguously established, the main hypotheses include either a natural genesis (zoonosis (Andersen et al., 2020; Calisher et al., 2020)) of the virus, or a laboratory origin (in the context of viral and GoF research). The hypothesis by Ambati et al. (2022) raises the potentiality for a radically different laboratory origin, even if of the SARS-CoV-2 FCS alone, that is outside the scope of viral GoF/DURC policy and regulation
• Copy-choice recombination could have been realized during cancer research via infection of SEQ ID11652-MSH3-transduced human cells by a SARS-like virus • This proprietary sequence (SEQ ID11652) is found in a US patent filed by Moderna on Feb. 4, 2016 (Bancel et al., 2017) • Specifically, the sequence listing in US9587003B2 revealed an artificial sequence fragment comprising 5′-CTACGTGCCCGCCGAGGAG-3' (nt 2733-2751 of SEQ ID11652). The corresponding mRNA would have 3′- GAUGCACGGGCGGCUCCU C - 5′, or equivalently, 5′- CU CCU CGG CGG GCA CGU AG - 3,' which is a 100% match to the original SARS-CoV-2 strain from Wuhan (ntds 23547-23565 in the SARS-CoV-2 genome), in which the four codons CCU CGG CGG GCA exactly yield the PRRA furin cleavage site	The explicit goal of the Moderna patent (Bancel et al., 2017) is to enhance cancer research. However, • *A priori*, the motivation for combining human cancer research with SARS-based viral research is not clear • Even though Ambati and collaborators suspect an inadvertent or intentional act during the course of viral research, the odds of the implied recombination event could be low
According to Ambati et al. (2022), the reason for using MSH3 may have been that • Overexpression of MSH3 is known to interfere with mismatch repair • Mismatch repair deficiency could have been important during the research with SARS-like viruses	The context of such viral experiments is not clear at the outset. MSH3 is a human DNA repair gene
Accordingly, Ambati et al. propose the following mechanism leading to the integration of the novel sequence surrounding the SARS-CoV-2 FCS • Human cell lines may have been transfected with MSH3 • This could inadvertently or intentionally have induced mismatch repair deficiency • Such cells co-transfected with a SARS-like virus (expressing appropriate enzymes such as RdRp) could have led to copy-choice recombination between the MSH3 and the virus	This specific research context raises several immediate questions • Is there a rationale for conducting research that combines a) DNA repair pathways, b) induction of DNA repair deficiency, c) CoV research involving SARS-like viruses, and d) cancer research (the goal of the patent)? • What is the potentiality of CoV evolution/escape via recombination in such a research context (deliberate or accidental)? • Could a specific experimental framework increase the odds of the postulated recombination event?
The actual proprietary sequence does not represent the corresponding sequence surrounding the FCS in SARS-CoV-2, but its reverse complement. Hoverer, according to Ambati et al. (2022) • Single stranded RNA viruses such as SARS-CoV-2 utilize negative strand RNA templates in infected cells • Copy choice recombination with a negative sense SARS-like RNA could have led to the integration of the MSH3 negative strand • That is, recombination of the sequence may have happened despite being on the opposite strand of the open reading frame	From a biorisk perspective, this is critical • Dangerous sequences could effectively be camouflaged by their harmless-looking reverse complement • Via this concealment, the dangerous sequence itself would not be detected during screening • Such camouflaging is a substantial vulnerability to both unintended mishaps and deliberate forms of misuse • Specifically, the integration of short fragments from antisense strands has been observed in experimental models (see (Ambati et al. (2022) for references)

lead to a pandemic. Seemingly taboo topics in the context of biorisk assessment and management have been 1) that of well-intended research to engender adverse outcomes, and 2) that in a criminal context, benign R&D could be hijacked and have catastrophic consequences. Clearly, allegations that research or applications in synthetic biology that are widely regarded as beneficial could result in disasters nonetheless, would have significant disruptive effects across academia and industry, even if unsubstantiated. This makes it even more important that careful attention be placed on research efforts that fall outside of regulation, to help minimize the accidental or deliberate exploitation of any previously unrecognized gaps.

Concretely, this work scrutinizes specific aspects of CoV recombination in a laboratory setting. This is not done from the perspective of targeted viral mutations *per se*, for example, via direct mutagenesis or stated DURC/GoF work, but in the context of benign experiments such as in cancer research. More specifically, the starting point will be the discovery of (the reverse complement of) a proprietary sequence encompassing the SARS-CoV-2 FCS (Ambati et al., 2022), which will serve as a key example to investigate largely unrecognized gaps (for a summary of (Ambati et al., 2022), see Table 1 and Sect. 2).

Explicitly, Ambati et al. (2022) identify an unexpected relationship between this new sequence insert in SARS-CoV-2 and a previously patented sequence. More precisely, they obtain the coincidence probability for the occurrence of the sequence homology between the new FCS insert in the SARS-CoV-2 genome and (the negative strand of) the patented sequence as 3.21×10^{-11}.

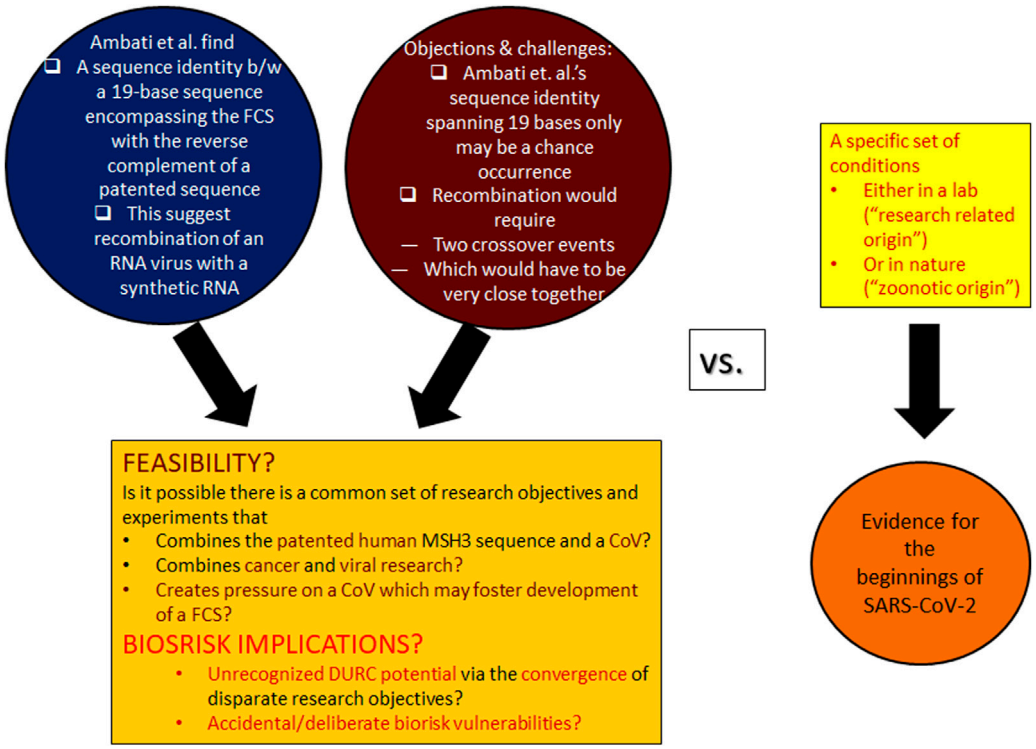

FIGURE 1
Postulated presence of the negative strand of a patented sequence in SARS-CoV-2, the feasibility of chance homologies, and implications to inform biorisk assessment. Left: The main focus of the article by Ambati et al. (2022) was to draw attention to the critical sequence insert surrounding the SARS-CoV-2 FCS, which, according to their analysis, is 100% complementary to the negative strand of a previously patented sequence. In Ref. Ambati et al. (2022), the authors also calculated, and obtained, a very small probability for this unexpected sequence homology. They also gave a very basic framework of how the purportedly patented sequence could have been integrated into a SARS-like virus, but they leave several questions about the rationale of such a research entertainment unanswered. Additionally, it has been suggested that the specific recombination event may happen very rarely, and, furthermore, the calculated coincidence probability has been contested and published as a Commentary (Dubuy and Lachuer, 2022) to Ref. (Ambati et al., 2022). Against this backdrop, the analysis undertaken here investigates the feasibility of the implied viral recombination event and consequential biorisk dangers with special attention to deliberate misuse potentials for future exploitation. It is important to contrast the current analysis, which asks if/how specific laboratory experiments could have led to the special insertion in SARS-CoV-2 or other recombination events, from the actual investigations of the beginnings of SARS-CoV-2 (right).

This extremely low number is the basis of their conclusion that this represents a "highly unusual" phenomenon, asking for potential explanations for this correlation which should be further investigated.

Their extremely low coincidence probability has been contested. A commentary (Dubuy and Lachuer, 2022) to Ref. (Ambati et al., 2022) questions the BLAST search conducted by Ambati et al. as well as how the probabilities were calculated. Furthermore, the recombination between a CoV and a synthetic RNA as suggested in Ref. (Ambati et al., 2022), requiring two crossover events that would have to be very close together, may be regarded as practically unlikely[6]. These constraints and the counterargument offered in (Dubuy and Lachuer, 2022) that the observed sequence homologies may just be a chance occurrence seem to have put the question raised in (Ambati et al., 2022) to rest. However, these developments and controversies have not been sufficiently scrutinized. The genetic recombination envisioned by Ambati et al., if proven feasible, has grave biorisk implications for future events (Figure 1).

It is important to note that biological risk covers a spectrum encompassing naturally occurring, unintended, and deliberate risks (The Royal Society and the International Council for the Life Sciences, 2009). Furthermore, adding to the difficulty of distinguishing natural from man-made risks, as seen in the ongoing Covid origin debate, the analogous problem applies to parsing out unintended from deliberate events. In the context of increased reliance of synthetic biology on technology, separating safety (which focuses on vulnerabilities fostered by unintentional issues) from security (which targets deliberately induced vulnerabilities) may not be easy (Mueller, 2020). Now, as there is no sound rationale that supports the notion that SARS-CoV-2 was intentionally released from a lab, past origin discussions have not paid special attention to factors that could be deliberately misused. However, looking ahead, it is important to additionally scrutinize security aspects as well. As with all emerging technologies, a failure to do so may lead to a scale of exploits that previously has been called a "crime harvest" (Pease, 1997; Elgabry et al., 2022).

6 I thank one of the reviewers of an earlier version of this article for this important observation.

Key open questions to be addressed: This article first investigates the very feasibility of the emergence of the FCS as suggested by Ambati et al. or via related experiments. It then scrutinizes the resulting vulnerabilities, across the full range of the biorisk landscape, ranging from unintended accidents to deliberate malicious exploitation.

Just as the actual beginnings of SARS-CoV-2, due to a lack of early data, are to a large part limited to a rational investigation, the analysis conducted here builds on logical deduction. The main questions analyzed below are the following.

- Even though Ambati et al. suggest that the novel insert in SARS-CoV-2 could have come about inadvertently or intentionally, the odds of the implied recombination event may be low. Notwithstanding this, is there a logical rationale for some rather feasible experimental underpinnings that could substantially increase these odds?
- Is it possible that the disparate research goals implicated in Ref. (Ambati et al., 2022) (research with SARS-like viruses) and the patent (Bancel et al., 2017) (cancer research) can be reasonably extended so that whilst neither includes the integration of an FCS into a CoV, they could have converged into one joint set of laboratory experiments enabling its integration nonetheless?
- If the likelihood for the implied sequence homologies is as high as argued in (Dubuy and Lachuer, 2022), what does this mean in terms of biorisk potentials, especially regarding deliberate exploitation?
- What do these factors *combined* mean from an existing biorisk management perspective, especially related to crime risks and deliberate attacks?

Outline: The article begins with a description of the postulated genesis of the FCS in SARS-CoV-2 from a laboratory context as suggested in (Ambati et al., 2022). It continues with a review of the feasibility and orientation of the implicated research objectives, as well as those extended below, and argues that these could have aggregated in a unifying research project which may have favored viral evolution and escape and, consequently, recombination with synthetic RNAs as envisioned by Ambati et al. It then highlights challenges with existing biorisk policy, placing special focus on deliberate attack potentials. Finally, it concludes with a summary and recommendations.

2 Viral research in the context of cancer research: an analysis of the feasibility of the Ambati et al. postulate

This section gives a brief introduction to the postulated route of how the SARS-CoV-2 FCS could have evolved as first suggested in (Ambati et al., 2022). Given that the implied research setting, along with their apparently vastly disconnected features, may not have favored the particular viral recombination in question, additional rational research aims and contexts are identified that could provide the necessary framework and substantially increase the chance of such events.

2.1 Synopsis of the hypothesis by Ambati et al. concerning the proprietary sequence in SARS-CoV-2 and main open questions

As mentioned, in early 2022, a publication in Frontiers in Virology (Ambati et al., 2022) described an intriguing finding. First, Ambati and collaborators note that among numerous point mutation differences between SARS-CoV-2 and the bat RaTG13 CoV, only the 12-nucleotide FCS exceeds 3 nucleotides. During the pandemic years, the FCS has been regarded as one of the most, if not *the* most important novel characteristics of SARS-CoV-2. CoVs, just as RNA viruses in general, are subject to numerous random point mutations. Given the high error rate of the RNA replicase and the very structure of the virus genome itself, it is unclear how a random insertion mutation could explain the emergence of the FCS without substantial additional changes throughout the genome (Romeu and Ollé, 2021).

Intriguingly, specifically related to the SARS-CoV-2 FCS, Ambati et al. (2022) report on a BLAST search for the 12-nucleotide insertion which, surprisingly, revealed a 100% reverse match in a Moderna patented sequence listing (SEQ ID11652) in US patent 9,587,003 filed on Feb. 4, 2016 (Bancel et al., 2017). Furthermore, according to Ambati et al., an examination of SEQ ID11652 showed that the match extended beyond the 12-nucleotide insertion to a 19-nucleotide sequence that encompasses the FCS (Table 1).

The reverse complement sequence present in SARS-CoV-2 may occur randomly. However, Ambati and collaborators report that the artificial 19-nucleotide sequence fragment is without precedence in any mammalian or viral genome in the BLAST database except in SARS-CoV-2.

Ambati et al. also point out that the unprecedented sequence encompassing the FCS not only is a 100% complementary match to the Moderna proprietary sequence in (Bancel et al., 2017); furthermore, SEQ ID11652 is transcribed to the human mutS homolog (MSH3), which they think is codon optimized for humans.

The aim of the Moderna patent in question, titled "Modified Polynucleotides For The Production Of Oncology Related Proteins And Peptides," is cancer treatment. Specifically, it "relates to compositions and methods for the preparation, manufacture and therapeutic use of oncology-related polynucleotides, oncology-related primary transcripts and oncology-related mmRNA [modified mRNA] molecules." In line with this, Ambati and collaborators suggest that MSH3 replacement with a codon-optimized mRNA sequence for human expression likely has applications in cancers with mismatch repair deficiencies. More specifically, this leads to their far-reaching hypothesis: they postulate that a specific recombination event during cancer research may have led to the integration of the MSH3 negative strand, including the FCS, into the genome of a precursor of SARS-CoV-2, leading to the novel FCS.

Concerning the postulated occurrence of the patented sequence in the SARS-CoV-2 genome, the identified sequence is on the opposite strand of the open reading frame in SEQ ID11652. Nonetheless, Ambati et al. provide a mechanistic explanation that captures the molecular underpinnings to resolve this apparent limitation. Single-stranded RNA viruses such as SARS-CoV-2 utilize negative-strand RNA templates in infected cells. Ambati

et al. suggest that the artificial 19-nucleotide sequence present in the human MSH3 gene might have been introduced into the SARS-CoV-2 genome through copy choice recombination with a negative sense SARS-CoV-2 progenitor RNA in infected human cells. If so, this would imply that from a biorisk perspective, homologies and recombinations between pertinent strands as well as those involving their complements need to be taken into consideration.

The above raises numerous questions which will be further analyzed below.

- Feasibility: Is the scenario hypothesized by Ambati et al. theoretically feasible?
- The reasons why Moderna could have engaged in particular experiments: specifically, is there a legitimate reason why/how to combine cancer research, viral research, and synthetically modified mRNAs?
- Genetic recombination: The postulated recombination would have required two crossover events. As the novel insert comprises only 19 nucleotides, these crossovers would have to be very close together, which some may think makes the frequency of such events very low. Nonetheless, is it possible that specific laboratory settings that exploit the natural recombinability of CoVs and other unrecognized properties, increase the odds of such a genetic recombination event?
- Misuse: What is the practical feasibility to maliciously exploit the implicated and related gaps?
- What do sequence homologies, especially if they are rather likely as argued in (Dubuy and Lachuer, 2022), mean for laboratory safety and security, and in particular, those where both the original as well as the reverse complement may be of biological relevance (e.g., with MSH3 and FCS, see below)?

2.2 CoV recombination - insights from decades-old research

CoV recombination has been increasingly investigated since the Covid pandemic. However, the focus of past studies has mainly been that of inferring natural evolution and relationships between SARS-CoV-2 and its potential progenitors. For instance, as done in (Yang et al., 2021) via a detailed automated analysis, such type of investigation relies on the number and diversity of representative CoV genomes available, and therefore cannot directly predict recombination characteristics between CoVs and synthetic RNAs, and even less so, fostered by enhanced laboratory evolutionary pressure. In the following, therefore, the analysis places special focus on well-established recombination characteristics that lend themselves to the situation postulated by Ambati et al.

RNA recombination was first identified in the early 1960s as an exchange of genetic material between closely related RNA viruses. CoVs, in particular, have long been known to utilize RNA recombination, possibly because of their large genome size and the large number of errors during RNA replication. Indeed, already in 1996, Lai discovered that for mouse hepatitis virus (MHV) not only was recombination frequency high. Many of the recombinants had even multiple cross-overs. Furthermore, the recombinant viruses grew at non-permissive temperatures and became the predominant virus population after only two tissue culture passages. The only explanation for this was that recombinant viruses had evolutionary advantages over parental viruses under experimental conditions (Lai, 1996).

For at least 20 years now, precise details for a variety of RNA recombination events have been clearly established (Chetverin, 1999). In the context of the possible genesis of the SARS-CoV-2 FCS, as postulated in (Ambati et al., 2022), several features of RNA recombination are worth mentioning.

- Already in 1996, it was suggested that CoVs in particular may utilize RNA recombination to counter the possibly deleterious effects of their high mutation rate. In fact, at that time it was already well-established that CoVs undergo recombination at a very high frequency of nearly 25% of the entire genome.
- Already in the 1970s, it was known that special “defective interfering (DI) particles” (previously known as “inactive viruses” - so called because they lacked some viral genes) were able to propagate following some non-homologous RNA recombination events. Importantly, the components could be identified that made it possible to provide the missing proteins in *trans*: they then could be detected as particles contaminating the virus preparation (Chetverin, 1999).
- Ref. (Rowe et al., 1997) established 25 years ago that for CoVs, recombination can also happen during passage in tissue culture. This establishes the very basis that the proposed recombination event postulated by Ambati et al. (2022) was not the result of slow adaptations among naturally occurring viruses but indeed could have been realized in a laboratory context.
- Additionally, in 1997, Rowe et al. (1997), specifically analyzing the spike protein of MHV to study spike deletion variants, found that RNA recombination can occur during either positive or negative strand synthesis - thereby supporting the suggestion offered by Ambati and collaborators of the recombination event involving negative sense RNA intermediates.

In general, the generation of a recombinant sequence can mechanistically be conceived in two different ways (Chetverin, 1999): 1), via breaking the parental sequences and joining the resulting fragments, or 2) via *de novo* synthesis by the viral replicase which switches to another template after it has copied a portion of the first template. Notably, already more than two decades ago, details and refinements of these were known.

- Back in 1999, one of the most surprising discoveries was that of RNA *self*-recombination. This means that RNA molecules can recombine without any DNA intermediates. Precisely, self-combination is a general property of RNA, requiring nothing but RNA itself and Mg2+. Chetverin (1999) concluded that it must be ubiquitous in nature and involve both viral and cellular RNAs. (By extension then, the same could also apply to lab experiments and also synthetic RNAs.)
- Apart from RNA recombination performed by, and as an inherent feature of, RNA itself, recombination is also known to be promoted by some proteins. These include replicase- (more below) and ribozyme-assisted recombination (Chetverin, 1999).

- The template-switch mechanism for RNA recombination has been demonstrated in two other interesting contexts. Both were initially believed to not be available for (natural) RNA viruses but may be especially relevant in the context of lab experiments.
  (i) The first involves retroviral reverse transcriptases. Notably, Negroni et al. (1995) showed that Moloney murine leukemia virus reverse transcriptase (RT) alone promotes homologous recombination efficiently. The important point they are making, for lab experimentation in particular, is that while RNA concentration itself has little effect on recombination frequency, there is a clear correlation between the amount of RT used in the assay and the extent of recombination observed.
  (ii) Furthermore, for RNA recombination, the 'switching between template' mechanism has also been directly demonstrated for DNA polymerases during PCR (Chetverin, 1999; Innis et al., 2012).
  (iii) Chetverin (1999) believes that both the mechanism via the retroviral RT activity and PCR assist the template-switch mechanism in that they enable dissociation of the nascent strand base-paired to its template. In the former case, the RT has an inherent RNA template degradation mechanism, and in the latter, this is realized during the heat-induced melting of the DNA duplexes. As pointed out by Chetverin, RNA viruses may have evolved analogous mechanisms to overcome the duplex problem. It seems feasible that the repeated melting during either PCR or RT-PCR under laboratory conditions may help dissociate the nascent RNA strand from their template. In addition, while not a focus of ref. (Chetverin, 1999), with CoVs, the mechanism of the viral polymerase itself is now known to facilitate that step very efficiently (more below).

2.3 Recombination in CoVs and basic links between cancer and viral research that could support the Ambati et al. hypothesis

The fact that CoVs are very amendable to recombination has been known for decades. Pivotal work in this regard was first obtained by Lai (1996) who argued that for CoVs, due to their large genome size, recombination is a valuable tool for virus evolution, to counter the large number of errors made during replication, but also to provide diversity in genomic structure and hence, offer evolutionary advantages for recombinants under specific conditions (including experimental).

The important point is that CoV evolution may thereby not happen by a slow accumulation of adaptive mutations in a piecemeal fashion, as has been the basis of substantial pandemic research on the origin of SARS-CoV-2—which has often centered on individual ntd changes and sequence-based measures and determinants but which may not be the most optimal (Piplani et al., 2021). A notable exception is a paper by Gallaher (2020) who proposed that RaTG13, a relatively recent ancestor of SARS-CoV-2, likely experienced a number of sudden changes which can be explained, he argues, by several recombination events.

The usefulness and potential of recombination in the lab were already known in 1999 when Lai described how RNA recombination could be utilized to achieve desirable consequences for CoV studies. Notably, the setup is exactly the same as postulated in (Ambati et al., 2022): the basic step consists of manipulating certain mRNA constructs which are then transfected into virus-infected cells. Based on several success stories in the lab, Lai concludes that this approach is very useful for introducing certain sequences into viral RNA. He concludes that, given the limitations of CoV research at that time (owing to their size), such a "recombination strategy provides an alternative method for introducing site-specific mutations into the viral genome."

Research has significantly advanced during the last few decades. It is not clear to what extent Moderna was, or was not, attempting to use viral recombination for research purposes. From a theoretical perspective, a key question is why/how cancer research could have been linked to viral evolution. It seems feasible that in a laboratory context, Moderna (Bancel et al., 2017) attempted to do a combination of approaches. These are discussed in greater detail below for their underappreciated potentials related to biorisk assessment and mitigation and not to imply any culpability of Moderna related to the origin of SARS-CoV-2.

- *mmRNAs as therapeutics against viruses implicated with cancer*: Central to Moderna's patent is the use of novel (modified) mRNAs that Moderna aimed to deploy as therapeutic agents. Since viruses can compromise human health at many levels, and have been implied with the development of various cancers as well (Zapatka et al., 2020), it seems feasible that Moderna may have tested various synthetic mRNAs as therapeutic modalities in the context of various viral infections and viral variants.
- *Accidental (unintended) viral modification*: The development of therapeutics against certain cancer-implicated viruses would likely have involved viral mutagenesis. During the last few decades of viral research, prominent methods have emerged such as synthetic genomics techniques which have even enabled the rapid reconstruction of SARS-CoV-2 from synthetic DNA (Thao et al., 2020), and the focus may have shifted away from targeted RNA recombination. Thus, Moderna may not have been sufficiently aware that their research setup (Bancel et al., 2017) mimicked the very experimental conditions of Ref. (Lai, 1996) to influence viral evolution in the lab. Given that the mechanism of recombination may create new viruses, this may have happened unexpectedly, e.g., via contaminated or accidently switched cell lines infected with some SARS-CoV-2 precursors.
- *Targeted viral mutagenesis to study cancer-causing viruses and their susceptibility to the therapeutic agents*: As mentioned, in 1999, Lai described some success stories of CoV research of how recombination was able to replace some previously "defective genes" in specific CoVs. That is, when specific RNA fragments were transfected into cells infected with a mutant carrying a defective N gene, recombinant viruses with a functional N gene were obtained. In similar events, RT-PCR

confirmed the presence of the transfected RNA fragment. It seems feasible that Moderna may have used recombination as one of the means to gain new insights regarding CoVs and their cancer-causing properties. Additionally, the aim may have been to assess viral survival and evolution when in the presence of the new therapeutic mmRNAs.

- *Viral mutagenesis for the development of recombinant CoVs as a cancer vaccine and tested in cells transfected with mmRNAs*: Rather than developing certain mmRNAs as therapies against cancer-causing viruses, CoVs themselves may have been analyzed for their potential as a vector to deliver the therapeutics, and tested in susceptible human cells (e.g., those over-expressing MSH3 to induce DNA repair deficiency). CoVs may have been of great interest as they represent an RNA virus that was long believed to be unable to integrate into the host genome (but see below).

2.4 Feasibility of integration of a short stretch of synthetic RNAs into a CoV

As stated, it has been known for decades (Lai, 1996; Rowe et al., 1997; Graham and Baric, 2010; Gallaher, 2020) that the mechanism of recombination in RNA viruses is template switching. In this case, recombination takes place during RNA replication, i.e., when the RNA polymerase pauses at certain sites of the RNA template. As first postulated by Lai (Lai, 1996), the nascent RNA transcripts separate from the original template, and then join themselves to a different RNA template to continue RNA synthesis.

From this perspective, one of the key questions that remain to be addressed when assessing Ambati et al.'s hypothesis is: how is it possible that only a short stretch of 19 ntds was integrated into a SARS-like genome even though during the experiments, cells would have been transfected with the full-length sequence that codes for MSH3? That is, why is it that transfection did not result in the integration of the full sequence, and instead, just included the short 19-ntd part including and surrounding the FCS alone? Interestingly, intrinsic features of CoV transcription itself may explain, theoretically, at least, how this could have happened.

- Notably, for CoVs, mRNA transcription is done in a discontinuous manner (Lai, 1996; Rowe et al., 1997), with the viral polymerase functioning in a piecemeal fashion rather than progressing the entire viral genome at once. This fact is well established, as summarized by a recent publication by the NIAID (Sattar et al., 2023): "These coronaviruses contain a positive-strand RNA genome with a few unique features: two-thirds of the viral RNA is translated into a large polyprotein, and the remainder of the viral genome is transcribed by a discontinuous transcription process into a nested set of subgenomic mRNAs."
- Necessary for the above discontinuous mechanism is that the viral polymerase and nascent RNA transcripts disassociate from the RNA template regularly during RNA transcription, and by necessity then, the CoV polymerase must jump between different RNA molecules during RNA synthesis (Lai, 1996).
- The realization that the CoV polymerase is not acting in a progressive manner is essential also for recombination - which is reminiscent of the disassociation from, and rejoining to RNA templates during mRNA transcription (Lai, 1996). Likewise, then, the RNA polymerase complex may jump to a spatially proximal template, and thus by falling off and rejoining, contribute to RNA recombination.
- Importantly, however, recombination is not a totally random event. Recombinants with chimeric viral proteins derived from different parental viruses are often unstable and have inferior replication ability. Noting that some cross-over sites were hardly detected among mutants of mouse hepatitis virus strains, Lai (1996) suggests that for recombination, certain cross-over sites appear to be restricted. He postulates that some aberrant recombination events would render the recombinants not viable under selection pressure and that for optimal viral growth, recombinants are favored that reflect specific viral RNA or protein structure requirements.
- Rowe et al. (1997) also determined that the functioning of the RNA polymerase, including its fragmented way of operation, is significantly dictated by specific secondary structures. Concerning the copy-choice mechanism of recombination, it was therefore long believed that recombination will occur frequently at RNA sites of strong secondary structure, based on the observation that these structures promote transcriptional pausing (Mills et al., 1978).
- Recent years have shown that recombination is a promiscuous event that is not significantly influenced by any single factor. Notably, in 2020, Alnaji et al. (2022) argued that recombination in positive-sense RNA viruses is not influenced by RNA structure, or even the RNA donor or acceptor sequence. Instead, they posit that genome function and fitness are of greater importance in determining the identity of recombinant progeny. This seems to contradict previous studies that emphasize the role of RNA structure, sequence identity, and the amount of base pairing in the donor and acceptor sequence (Zúniga et al., 2010; Sola et al., 2011), and may reflect variation in the recombination processes involved between different viruses or the involvement of host factors (Wang et al., 2022).
- Consequently, then it seems that both CoV RNA sequence and structural factors as well as selective pressure are responsible for recombination, with the former contributing to bringing particular regions of the RNA molecule in sufficiently close proximity, and the latter enabling the selection of propagation of more advantageous recombinants.

These underlying features enabling recombination seem important for the genesis of the patented sequence in SARS-CoV-2 as proposed by Ambati et al. (2022). They provide the rationale for the RNA polymerase to jump to a spatially proximal template and to come off after a short stretch. As highlighted by more recent research, RNA viruses undergo frequent and continuous recombination events over a prolonged period of time and favor the selection of the fittest recombinant genome (Bentley and Evans, 2018; Wang et al., 2022). This could explain the selection and retention of specific variants, e.g., those with the short insert that constitutes and encompasses the novel FCS. The

hypothesis of lab-imposed selective pressure as a key factor to support such as recombination event will be further analyzed below where this will be linked to particular research experiments that would have made sense in the context under consideration, and which may have resulted in a new nuclear localization signal (NLS) that happens to be an FCS.

3 A potential framework that increases the odds of the genetic recombination as envisioned by Ambati et al.

Even though Ambati and colleagues believe that accidental or deliberate acts may have led to the viral recombination resulting in the new genetic insert in SARS-CoV-2 (Ambati et al., 2022), this section envisions a more detailed framework that could also increase the odds of such recombination events.

3.1 Cancer research, host DNA repair, and potentials for viral recombination

From a logical perspective, the postulated sequence insert in SARS-CoV-2, essentially identical to a patented sequence, may seem difficult to grasp since the patent in question targets cancer research in humans. How could this possibly be linked to viral research so that MSH3-transfected cells, then infected with a SARS-like virus, could have resulted in genetic viral recombination? This section analyzes potential research objectives of how such apparently disconnected issues could converge, and under which settings the odds for such type of recombination could be substantial.

3.1.1 Disruption of DNA repair by DNA and RNA viruses

Viruses are responsible for various human health challenges including serious forms of disease. Some viruses introduce DNA damage and genetic instability in host cells during their lifecycles. Notably, some have been found to manipulate components of the DNA damage response (DDR), a network of complex mechanisms for DNA damage detection and repair to combat DNA damaging agents. Surprisingly, these include RNA viruses as well, even for those species where viral replication takes place exclusively in the cytoplasm. As detailed in (Ryan et al., 2016), by impairing DDR pathways, the resulting DNA damage can be a crucial component of the pathogenicity of RNA viruses, e.g., through the triggering of apoptosis, stimulation of excessive inflammatory immune responses, and the introduction of deleterious mutations in infected cells. The latter, in turn, will likely increase the risk of tumor development.

Since cancer research was one of the main components of the Moderna patent, this relationship between RNA viruses and tumor development may be one common denominator to explain and further refine the apparently disparate research components implicated by Ambati et al. (i.e., CoV research and DNA repair deficiency).

Specifically, Ryan et al. (2016) describe various key mechanisms during the RNA virus lifecycle and how they can induce genetic instability. Even though the exact source of DNA damage and consequences of DDR (de)activation are still unresolved, it is now clear that specific viruses are believed to derive some of their most pathogenic features, including tumorigenesis, through such cellular transformation mechanisms.

In the context of viral-host interactions, numerous questions may have triggered the attention of Moderna[7].

- Mechanisms of how RNA viruses can trigger, influence, or impair DDR pathways: Although a common feature of DNA viruses, it has been known for some years now that also for some RNA viruses, it is frequently the case that viral proteins are often transported to the nucleus (Leon et al., 2012; Ryan et al., 2016). Once in the nucleus, they can obviously perturb various critical cellular functions, including the antiviral response; albeit, details of these mechanisms remain poorly understood.
- Nuclear transport involving CoVs: In 2016, when examining the potential of various RNA viruses, or some of their proteins, to be transported to the nucleus, it became clear that these also include common cold viruses including CoVs. Specifically, Ref. (Ryan et al., 2016) details how the Infectious bronchitis virus (IBV), a highly infectious avian CoV, may impair specific DNA damage signaling pathways and induce DNA replication stress, including via its interaction with DNA polymerase δ and modulation of cell cycle progression. Thus, comprehending key features of CoVs that enable their nuclear transport would be essential from both a scientific and public health perspective.
- Molecular mechanisms involved in the DDR: The DNA Damage Response and DNA repair pathways comprise a highly coordinated network of proteins that are activated in the presence of DNA damage, compromising a host of sophisticated mechanisms to deal with single- and double stranded DNA breaks. One of the most famous involves the cell cycle checkpoint protein p53, the guardian of DNA which promotes cell cycle arrest to prevent the replication of damaged DNA. Repair of single-strand DNA damage is realized via several repair pathways, *inter alia* via various MSH complexes (MutSα or MutSβ). This seems highly relevant, as the sequence implicated in the Moderna patent (MSH3) is part of the MutSβ complex (an MSH2-MSH3 heterodimer) which is involved in tumorigenesis through the maintenance of chromosomal stability. Importantly, both loss of expression and over-expression of MSH3 can lead to tumorigenesis (Marra et al., 1998; van Oers et al., 2014). On the other hand, the involvement of MutSβ has been extensively studied in the context of severe genetic neurological disorders.

The following provides further details on why and how the above is relevant to the Ambati et al. hypothesis.

7 Throughout, Moderna is taken as a proxy for relevant biomedical stakeholders across academia and industry simply because of the related Moderna patent and not to imply their involvement in the genesis of SARS-CoV-2.

3.1.2 Nucleocytoplasmic trafficking of viral proteins - an underappreciated target for antiviral therapy

Classically, it has been recognized that molecules larger than 45–50 kDa generally require specific amino acid sequences known as nuclear localization signals (NLSs) to gain nuclear entry (Leon et al., 2012). More precisely, nuclear protein import requires the recognition of the NLS-signal containing cargo proteins by members of the importin (IMP) superfamily of nuclear import receptors on the cytoplasmic side of the nuclear pore complex (NPC). After the transport complex docks to the NPC, it is translocated to the nucleus through the central pore; consecutively, once it is within the nucleus, the transport complex dissociates to allow the cargo to perform its nuclear function. Nuclear protein export occurs in an analogous fashion, where nuclear export signals are recognized by exportin proteins (Kylie et al., 2011; Leon et al., 2012).

As mentioned, the fact that viruses can facilitate the nuclear import and/or export of viral proteins in infected cells likely benefits viruses to carry out many functions ranging from essential replication activities such as DNA replication (DNA viruses), RNA synthesis (even for some RNA viruses such as Influenza A where synthesis of viral ribonucleoprotein complexes takes place in the nucleus (Ryan et al., 2016)), to the dampening of the host cell immune responses (Leon et al., 2012).

Some 10 years ago, this observation triggered the idea to specifically target the transport of specific viral proteins into the host cell nucleus as a therapeutic strategy (Leon et al., 2012). The inhibition of nuclear trafficking of viral proteins was recognized as an attractive possibility not only for retroviruses but also for many other RNA viruses which, despite their replication occurring in the cytoplasm, nonetheless transport some of their key proteins to the nucleus and thereby impair essential host processes.

The potential of preventing nuclear protein import seemed to be validated by some early studies that showed promises as potential therapies against HIV-1 and dengue by the recognition of a broad-spectrum inhibitor of the nuclear transport receptor importin α/β (Kylie et al., 2011; Leon et al., 2012). Specifically, for the dengue virus (DENV) which replicates in the cytoplasm and with no requirement for its genome to enter the nucleus, the nonstructural protein 5 (NS5), which serves as the viral RNA polymerase, is predominantly found within the nucleus of infected cells. Strikingly, in 2011, Kylie et al. (2011) demonstrated that inhibiting NS5 nuclear import using ivermectin, "a general inhibitor of IMPα/β1-dependent nuclear import," was found to greatly reduce virus production, supporting the potential of targeting nucleocytoplasmic trafficking for therapeutic interventions.

3.1.3 The targeting of nuclear import/export of viral proteins - general objectives.

Conceivably, these consist of the following.

- *CoV research to better comprehend details related to nucleocytoplasmic trafficking of viral proteins, including their consequences in the host.* Studies that have investigated the import of viruses or their proteins have traditionally heavily relied on mutagenesis, to e.g., express specifically mutated viral proteins or even created new viruses altogether. For example, Ozawa et al. (2007) report on the creation of a new influenza-A virus whose nucleoprotein contains amino acid substitutions to abolish its nuclear localization function; doing so helped identify specific viral NLSs that are essential for viral transcription and translation. For HIV-1, it is well established that this virus makes use of multiple import pathways under diverse conditions and in different cell types (Leon et al., 2012). On the other hand, for CoVs less seems to be known in this regard. Trying to comprehend inhibitors of nuclear import or export would likely have involved the transfection of viral proteins or susceptible/mutated viruses into human cells to study the interaction of key human and viral proteins involved in this process.
- *Identification of new drugs able to inhibit the import of viral proteins.* As noted above, in 2011, Wagstaff et al. (Kylie et al., 2011) developed a screening assay for the identification of specific inhibitors of nuclear import. In their case, using the HIV-1 integrase (IN) and importin (IMP) α/β1 interaction as a proof-of-principle, they were able to validate the activity and specificity of mifepristone and ivermectin to inhibit nuclear protein import in HeLa (human cervical adenocarcinoma) cells. The IMP α/β1 pathway is utilized by many RNA viruses, including SARS-CoV-2. Specifically, *in-vitro* studies (Caly et al., 2020) have confirmed that ivermectin is able to bind to and destabilize the IMP α/β1 heterodimer and thereby prevents viral proteins from entering the nucleus. It would have made sense to try to extend this, e.g., to test if analogous inhibitory mechanisms apply to the MutSβ heterodimer viral shuttle (including CoVs, see below).
- *Viral vector vaccines:* As noted, Ambati et al. (2022) suspect that the new gene sequence in SARS-CoV-2 might have arisen in the context of viral research, ostensibly to learn about viruses themselves, as e.g., in the above context. The insights of such an analysis would likely inform the design of novel therapeutics, the main aim of the patent. Thus, in addition to studying CoVs for research purposes in cancer-related pathologies, viruses could have been designed as a vector to deliver specific oncology-related mmRNAs into human cells. The Moderna patent (Bancel et al., 2017) places special emphasis on this step, emphasizing that the novel oncology-related polynucleotide sequence encoding a polypeptide of interest would need to be incorporated into a vector such as plasmids, *viruses,* cosmids, and artificial chromosomes. In this light, certain CoVs may have been engineered as a recombinant vector *vaccine* to express oncology-related genes of interest.

The notion that synthetic mRNAs may help repair the damage done by viral proteins to the host cell immune responses is analogous to that employed for mRNA Covid-19 vaccines: mRNAs specifically designed and introduced into living cells get translated by the host cell machinery which, in turn, is expected to result in the production of the anticipated proteins—with Covid-19, it is the spike antigen of the virus, whereas for therapeutic purposes, it would be key proteins that were compromised by the nuclear viral proteins to support specific immune responses such as DNA repair, or those that inhibit the import of viral proteins, for example. The idea of utilizing synthetic mRNAs as gene-therapy agents to provide

TABLE 2 While the genetic recombination of a CoV with an RNA described by Ambati et al. may happen rarely in a natural context, the above argues it may be realized in a certain laboratory setting as particularly fostered by specific evolutionary pressure during the testing of novel therapeutics.

Type	Arguments objecting to/supporting the recombination event postulated by Ambati et al.
Cons	• The viral recombination of a CoV with a synthetic RNA leading to a certain insert in SARS-CoV-2 as postulated by Ambati et al. requires two template switching events
	• Template switching has been extensively studied. For example, with CoVs, it is known that a major regulator of template switching is the amount of base pairing in the donor and acceptor (Zúniga et al., 2010; Sola et al., 2011)
	• For the new sequence insertion as reported by Ambati et al. (19 nucleotides), it is expected that the frequency of the crossover events would be extremely low because the two crossover events would have to be very close together
Pros	• MSH3 is involved in double-strand break (DSB) repair via homologous recombination (Tseng-Rogenski et al. (2020) and references therein) and it seems to be a shuttling protein itself. Due to its involvement in Huntington's disease (HD) and related human genetic diseases, the control of the subcellular localization of MutSβ (MSH2-MSH3 heterodimer) has been pursued as a novel therapeutic opportunity. Treatments that favors acetylated MutSβ allow it to exit the nucleus but hinder its nuclear reentry.
	• As detailed above, a core pillar of Moderna's cancer research may have been to target the nuclear transport of CoV proteins, a viral feature that is known to disrupt DNA repair. It is reasonable to envision an experimental context wherein MSH3 was tested to a) better elucidate the role of CoV nuclear import and its role in cancer, and b) try to exploit the therapeutic potential of controlling MSH3 localization and mechanisms that inhibit MutSβ nuclear import (notably, NLS acetylation), to impair NLS-enabled viral translocation of SARS-like viruses. By its very nature, such experiments could have created substantial evolutionary pressure on a CoV, fostering the development of escape mutants with improved nuclear transport profiles
	• Recombination between CoVs plays an important role in CoV evolution as it can alter host range, pathogenicity, and transmission patterns. Contrary to previous results that identified RNA structure and sequence identity as the major regulators of recombination in RNA viruses, more recent studies have shown that recombination is a promiscuous event that is significantly influenced by evolutionary mechanisms and selection processes (Alnaji et al., 2022; Wang et al., 2022)
	• For natural genetic viral recombination, their heritability is mediated by the replication fitness of the resulting progeny genome (Graham et al., 2018). However, evolutionary pressure has recently been recognized as a key factor dictating both the selection and maintenance of recombination events in RNA viruses (Bentley and Evans, 2018; Alnaji et al., 2022; Wang et al., 2022)
	• The influence of evolutionary pressure in the lab has not been sufficiently studied to fully understand, let alone eliminate, the potential of RNA viral recombination in such settings
	• In this work, particular experiments are outlined that logically make sense in the context of the Ambati et al. hypothesis. It is suggested that the resulting specific evolutionary pressure on some CoVs may have been in tandem with the development and survival of escape mutants harboring the novel NLS/FCS sequence - which is the core part of the insert indicated by Ambati et al
	• Thus, certain laboratory experiments as explained herein could have favored the genesis and heritability of the recombination events as postulated by Ambati et al

missing or defective proteins is not new and had previously been explored for decades (Malone et al., 1989; Wolff et al., 1990) and is one of the main pillars of the Moderna patent (Bancel et al., 2017).

Interestingly, while Ambati and collaborators suspected that the role of MSH3 was to lead to DNA repair deficiency in human cells, MSH3 is itself a DNA repair protein. It acts to recognize mismatch repair and helps to repair double-stranded breaks (van Oers et al., 2014). Furthermore, MSH3 may be a shuttling protein as well, a feature that is highly relevant in this context, as is the discovery of agents that either promote or prevent nuclear import of MutSβ (MSH2-MSH3 heterodimer) which have been investigated to treat trinuleotide repeat expansions that drive Huntington's disease (HD) and other severe genetic diseases.

3.2 An extended experimental framework that could facilitate the recombination of a CoV with an mmRNA encoding human MSH3

The above extends in a hypothetical manner the experimental underpinnings envisioned by Ambati and colleagues. Doing so not only provides a feasible rationale for a joint research objective that aligns CoVs with cancer research and MSH3. It also outlines in which way the postulated viral recombination event could have materialized.

According to the refined framework envisioned here, a genetic recombination event could have led to the FCS insert in SARS-CoV-2 in several ways.

- *Infection of MSH3-transfected cells with viral vector vaccines*: To test recombinant viral vector vaccines carrying a novel anti-tumorigenic gene, it is likely that cell lines prone to tumorigenesis (e.g., those deficient in DNA repair) would have been injected with different variants of a viral vector vaccine (i.e., different SARS-like viruses encoding different therapeutic mmRNAs). Presence of the optimized MSH3 gene (to evoke DNA deficiency) in the cell culture could have led to recombination with the viral vector vaccine (a SARS-like virus) and resulted in the integration of the novel SARS-CoV-2 FCS insert.
- *Testing the therapeutic potential of (modified) MSH3, the control of its intracellular shuttling/localization, and its potential as a cellular defense (which likewise would have*

TABLE 3 Factors that increase the misuse potential of the type of research indicated by Ambati et al. - and extrapolated herein to highlight the feasibility and danger of these unrecognized vulnerabilities.

Key factor	Comments
Existing policies have cautioned not to over-emphasize hazards and threats, especially downplaying security concerns	The notion that laboratory work in general could be maliciously exploited, has long led to the sentiment to not create unsubstantiated public fear. For example
	• The Royal Society and the International Council for the Life Sciences (2009) cautioned in 2009 that "It is also important not to over emphasise one particular risk, such as terrorism, which can undermine public confidence in risk assessments of the range of hazards and threats."
	• The same sentiment is ongoing, as demonstrated by the fact that there is relatively little published work that analyzes what threat actors could learn from the Covid pandemic
Drug and vaccine R&D has not received adequate scrutiny for their potential to be misused by threat actors	As increasingly seen since the Covid pandemic, suggestions that certain research objectives like those discussed above could be misused, have not been widely appreciated
	• Any such suggestions may quickly be (mis)understood as implying culpability of certain companies related to past events
	• The very notion that vaccines or viruses could be turned into harmful agents has essentially been regarded as 'verboten,' out of fear of political, sociological, or other detrimental consequences to science (Andersen et al., 2020)
	• As before, this very climate and gap in biorisk awareness has created an unprecedented security vulnerability
Zero-day exploits	The feasibility of malign or criminal use of genetic recombination in the context of viral and vaccine research has not been sufficiently recognized
	• This is likely because these applications are inherently seen as being developed with a beneficial objective and with the common goal to save lives and improve the health of humans
	• The underlying biosafety challenges have prompted R&I into preventing accidents and unintended outcomes, albeit at the expense of targeting criminal aspects
	• The convergence of these factors may support a crime harvest and provide substantial advantages to those intending to harm
Challenges with attribution (historical experience)	The ongoing struggles to clearly prove the origin of SARS-CoV-2 can inform future criminals. A lack of attribution has long been recognized as a significant driver for misuse (Murch, 2015)
A general difficulty to distinguish natural from deliberate events	Ironically, whilst synthetic biology strives to mimic nature to enhance the safety and efficacy of bioengineered products, this very same feature may also facilitate misuse
	• Specifically, synthetic genetic material, as it can, and does, play roles similar to its natural counterpart, can therefore become highly attractive for bad actors
	• The very indistinguishability between 'natural' and 'engineered,' may enable threat actors to infiltrate cell lines not only via contaminants but also allow criminal work to be done in secret and additionally fostered by insecure technologies
	• The fact that both positive and negative strand RNAs may play critical roles, would further complicate analysis and detection
The need for tacit knowledge may be minimized	Whilst traditionally, building a bioweapon has relied on intense tacit knowledge and skill, and would have required access to very specific and expensive technology and devices, these constraints are challenged by some of the above
	• Given that recombination does naturally occur between RNAs and CoVs, this may assist bad actors and minimize the skill they need
	• Exploiting the tendency of RNAs to recombine, bad actors may therefore resemble someone with a match in a dry forest
	• When done covertly, this may be able to facilitate (some) recombination events without needing to employ molecular specifics

(Continued on following page)

TABLE 3 (*Continued*) Factors that increase the misuse potential of the type of research indicated by Ambati et al. - and extrapolated herein to highlight the feasibility and danger of these unrecognized vulnerabilities.

Key factor	Comments
Sequence homologies have not been sufficiently scrutinized for their potential for misuse (camouflaging, covert ingression, etc.)	Special features identified above are particularly amendable to malicious exploitation with adverse clinical consequences
	• CoV recombination itself has long been known to play an important clinical role as it can change host/tissue range, increase infectivity and pathogenicity of viruses, and lead to vaccine escape
	• The substantial amino acid sequence matches between CoVs and humans can have profound adverse clinical sequelae. For instance, Harrison and Sachs. (2022) found that SARS-CoV-2's FCS also exists in the α subunit of the human epithelial sodium channel ENaC where it is functional. This "molecular mimicry" between the viral FCS and that of the human ENaC leads to, 1) a detrimental competition for host furin and decreased expression of ENaC related to its ion channel function which is known to compromise airway function, and 2) cross-reactivities of antibodies with human ENaC from SARS-CoV-2 infection, a factor implicated with severe forms of COVID-19
	• In addition to a covert insertion of an FCS, the analogous malicious exploitation of molecular mimicry between other mammalian/human and viral proteins may lead to the disruption of the balance and kinetics of critical host enzymes, auto-antibody development, and other adverse events
Several convergence issues creating knowledge gaps	The traditional biosecurity landscape is substantially increased by a convergence/ blurring of
	• Research objectives, experimental underpinnings, and potential pathways of harm
	• Biotechnology with ICT technology, which creates a vast array of novel cyberbiosecurity gaps (Jean et al., 2018; Murch et al., 2018; Murch and DiEuliis, 2019; Mueller, 2020; Mueller and Barros Lourenco, 2023)

relied on infecting MSH3-transfected human cell lines with SARS-like viruses): Again, this involvement of MSH3 is different than the one envisioned in (Ambati et al., 2022) to evoke DNA deficiency, likely for some research purposes. Alternatively, MSH3 itself may have been examined for its potential to act as a viral protein shuttle/viral defense. This is based on the observation that MSH3 contains Nuclear Localization and Export Signals which in an inflammatory context have been shown to enable nuclear-cytosolic shuttling of proteins (Tseng-Rogenski et al., 2020). A key question that remains is whether the shutting of SARS-like proteins into the nucleus could actually be inhibited. Now, the study of Huntington's disease (HD) and other expansion diseases has revealed potential therapeutic options via the control of MutSβ localization that seems to be relevant in this regard. Intriguingly, Williams et al. (2020) discovered that the acetylation status of lysine residues in the MSH3 NLS effectively controls the subcellular localization of MutSβ. Of note, this gives rise to specific treatment options that either favor deacetylated MutSβ—which can translocate in and out of the nucleus—or the acetylated form—which prevents nuclear reentry. Given that NLS-driven viral protein nuclear translocation is common in SARS-like infections (Sattar et al., 2023), it would have been reasonably to test whether treatments favoring acetylated MutSβ, or others, could likewise impair nuclear import characteristics of CoVs present in the same cell culture.

- *Testing of viral evolution/escape:* In this hypothesized framework, CoVs could have played three roles: a) For the analysis of genetic features which allow SARS-like viruses to translocate some of their proteins into the host cell's nucleus; b) The design of CoVs that express novel anti-tumorigenic genes such that attenuated forms thereof could be used as a cancer vaccine; c) SARS-like viruses as the targets of novel drugs which prevent the nuclear localization of their proteins. Even though a recombination event between those viruses and synthetic mRNAs seems feasible in all these scenarios, their odds may be different and significantly increased by specific evolutionary pressure, e.g., when targeting the ability of CoVs as a carrier of therapeutics or when trying to prevent nucleocytoplasmic transport of viral proteins.

As described in Sect. 2.4, evolutionary pressure may be one of the key factors to foster CoV escape mutants via recombination events. In which way this seems relevant to the Ambati et al. hypothesis is further detailed next.

3.3 Selective pressure in the lab may have created both a novel FCS and an unintended NLS

The above described several hypothetical ways in which viruses, either during viral or vaccine research, could have unwittingly or intentionally been modified via recombination to acquire the purported patented sequence in the SARS-CoV-2 genome. In some of these cases, this could have been fostered by lab-induced pressure leading to viral evolution.

TABLE 4 The motives, mechanisms, and potential outcomes of the possible pathways of harm discussed herein have not previously been analyzed from a biosecurity perspective and are not covered by existing policy and regulation.

Motives	Description and potential outcome
Criminal/for profit	Bad actors could ingress synthetic RNA contamination, which under certain laboratory conditions may enable genetic recombination of RNA viruses. Specific aims of such criminal acts may be to
	• Derail competitor's research programs (e.g., involving viral or oncology-related research) via unrecognized genetic recombination events
	• Create new viruses and blame a competitor for the conducting of forbidden GoF work
	• Corrupt competitor's manufacturing of vaccines or therapeutics
Bioterrorism	Covert/disguised genetic recombination events may be employed for the design of harmful viruses for their actual, staged, or threatened employment as a bioweapon
	• Traditionally, biosafety policy has placed great emphasis on preventing the design and manipulation of pathogens with pandemic potential (PPPs)
	• Focus has been on specific adversaries that are believed to be interested in creating bioweapons
	• Due to a) a lack of significant historical events related to state actors and b) limited dangers seen from non-state actors (i.e., extremists with apocalyptic ideology or sociopathic tendencies or rare mentally ill insiders (The Royal Society and the International Council for the Life Sciences, 2009)), biosecurity has not been regarded as the most imminent threat related to the emergence of PPPs
	• Traditionally, the view has been that the dangers of PPP are mostly caused by zoonosis
	• All the above ignores new technological developments, which could a) enable threat actors to utilize automated processes/AI to optimize laboratory settings which increase the odds of viral recombination, b) exploit the lack of existing security-by-design and by-default of underlying technologies, and c) realize their intrusions at various points of the largely unsecured threat landscape of modern biotechnologies (Mueller and Barros Lourenco, 2023)
Insider attacks	• Insider threats have always played an important role in various security contexts, comprising a range of nuances and motivations including accidental and malicious (Mueller and Barros Lourenco, 2023)
	• Insiders often have direct access to relevant (biological) material, devices, and processes, which may allow the covert infiltration of genetic contaminants, particularly as these are difficult to spot
Circumventing GoF policy	• Given that life-science researchers have always been conscientious, albeit overall lacking a security mindset, dangerous research projects camouflaged as benign might not readily be detected
	• The ongoing controversy as to what type of pathogen research is necessary ("good") vs what is too risky ("bad") has created a gap in clear and uniform biorisk assessment and policy
	• Disparate views, interpretations, and different policies in distinct jurisdictions may increase the likelihood that certain work be hijacked
DU controversies and a new type of ethics-based hacking	By their very nature, DU issues comprise two sides and it may not always be easy to distinguish "good" from "bad." Ironically, this inherent dilemma, which has the potential to significantly polarize scientists and policymakers, may also create a new type of (ICT-based) attackers who feel their views are not adequately appreciated

An interesting aspect related to MSH3 highlighted above is that it recently was recognized as a shuttling protein containing special nuclear localization signals (NLSs) (Tseng-Rogenski et al., 2020). The critical role of novel NLSs in SARS-CoV-2 has only recently become known when it was discovered that this virus has unexpectedly improved nucleocytoplasmic trafficking potentials. Specifically, a recent study by the National Institute of Allergy and Infectious Diseases (Sattar et al., 2023) analyzed novel characteristics of SARS-CoV-2 related to its potential for its proteins to be transported into the nucleus. Notably, Sattar and collaborators found that unexpectedly both the spike (S) protein and mRNA translocate into the nucleus in SARS-CoV-2-infected cells. Even though NLS-driven translocation of some SARS-like proteins is well established, neither of these is as effective as for SARS-CoV-2's S protein.

The critical observation that SARS-CoV-2 proteins, most notably the spike, can translocate to the nucleus was first shown in (Jiang and Mei, 2021) which, however after its first publication appeared as too controversial since it raised the potential of the same mechanisms to also apply to the spike produced by Covid vaccines. The paper ended up being retracted - albeit, with the findings essentially to be re-discovered by Sattar et al. (2023) who did not seem to be aware of Ref. (Jiang and Mei, 2021).

The goal of Ref. (Sattar et al., 2023) was specifically to measure the extent of subcellular localization of S mRNA and protein. Potential processes explaining the mechanisms of the translocation were in part obtained via machine-learning models, building on the notion described above, i.e., that the viral genome is transcribed in a discontinuous manner. Since S mRNA was seen to colocalize with the S protein, Sattar et al. believe that the nuclear translocation is mediated by a novel NLS in the S protein. Intriguingly, this NIAID study (Sattar et al., 2023) also found that this new NLS motif was present at the polybasic FCS. This was surprising since the specificity of the amino acid motif, a furin cleavage motif, was not expected to also fulfill the characteristics of an NLS motif.

The crucial point here is that the inserted sequence—which above was investigated from the perspective of an FCS—also creates an

TABLE 5 An analysis of the controversial Ambati et al. postulate regarding the integration of a sequence encompassing the SARS-CoV-2 FCS has identified critical gaps which should be a key priority for synthetic biology risk assessment, especially from a criminal perspective.

Category	Main finding
At the sequence-analysis level	• Genetic changes may lead to multiple and unexpected biological mechanisms, as seen here with the double FCS/NLS functionality
	• It is possible that dangerous sequences (e.g., here the FCS) are, via their reverse complements, characterized as benign (here, the MSH3 gene)
	• Biorisk assessment is complicated by unknown reading frames and reverse complement sequences which can allow dangerous sequences to be obscured
	• The potential for criminal exploitation of such dangerous sequences has not been adequately appreciated
	• Given that the likelihood of finding matches and sequence homologies is high, as shown in the Commentary by Dubuy and Lachuer (2022) to Ref. (Ambati et al., 2022), this creates a largely underappreciated biosecurity vulnerability to camouflage dangerous sequences
	• Short genetic sequences can lead to erroneous interpretations when a) it appears there is a homology between sequences - implying relationships of organisms - that is artefactual and simply by chance, or when b) true homologies involving the negative sequence are not readily recognized
	• This has critical implications for well-intended research programs to be hijacked and diverted into covert bioweapon development programs
Research objectives and goals	• Various apparently distinct research objectives and goals with intrinsically benign features may lead to a convergence with unique DURC potential
	• Synbio products such as synthetic RNAs may be able to interact with the man-made and the natural world in ways that have not been sufficiently appreciated
	• In recent years, research has shown the increasing role of host immunity, evolutionary pressure, genome function, and viral fitness as key factors driving the genetic recombination of positive-strand RNA viruses
	• Even if specific recombination events are deemed unlikely to arise in nature, this does not mean that the same could not be intentionally targeted in clandestine by lab-imposed evolutionary pressure
	• Since genetic recombination of viruses contributes substantially to the emergence of new viral lineages, expansion in host tropism, adaptations to new environments, increased virulence and pathogenesis, and escape to vaccination, it seems plausible that the development of more dangerous viruses through recombination with synthetic RNAs is substantially enhanced in a susceptible laboratory environment
Risk spectrum and assessment	• It is imperative to consider risk management across the full risk spectrum, also regarding novel actor types and motives. In addition to unintentional risks, the potential for deliberate misuse may extend beyond more traditional GoF/DURC scenarios and traditional bioterrorists
	• Risk management that identifies where and how risk scales most rapidly, e.g., in certain "high risk" or otherwise susceptible experimental contexts or with increased use of technology (Heinemann et al., 2021), may inevitably inform bad actors, who may thereby learn critical information about vulnerabilities, weak spots, and most attractive targets
	• A failure to appreciate emerging attack potentials fostered by the convergence of new ICT-based technologies and under-appreciated molecular mechanisms may enable the deployment of nefarious "Trojan horses," especially if nobody suspects them

unprecedented NLS. Specifically, the novel "PRRA" FCS is subsumed within the longer sequence "NSPRRARSV" - with "PRRARSV" being a novel NLS. The astonishing fact is that both of these are functional: the FCS is key to allowing SARS-CoV-2 to infect human cells and the NLS shuttles viral proteins and mRNA, and possibly the whole genome, into the nucleus.

To the Ambati et al. hypothesis, this is significant, because of the following.

- The double NLS/FCS functionality is believed to have drastically enhanced the pathogenicity and infectivity of the new virus; this is in line with natural selection of the fittest CoV genome which, as summarized above, is now believed to be generated and selected by frequent and continuous recombination events.
- It seems feasible that the research aims to examine the nuclear translocation of viral proteins in the context of potential inhibitors as outlined in the previous section, has created the laboratory framework conducive to viral evolution and recombination.
- Specifically, experiments that assessed the fate of CoVs in the presence of the controllable shuttle protein MSH3, agents that can hinder nuclear transport via NLS-acetylation, or established inhibitors of viral nuclear transport such as ivermectin, may have created enough pressure on these viruses that this could have fostered the development and selection of viral mutants that can transport their proteins into the nucleus in some new/improved ways.

In sum, the experiments to develop inhibitors of viral nucleocytoplasmic transport as envisioned in this section could have created substantial pressure on the virus. Escape mutants may indeed have involved novel/improved features for nuclear

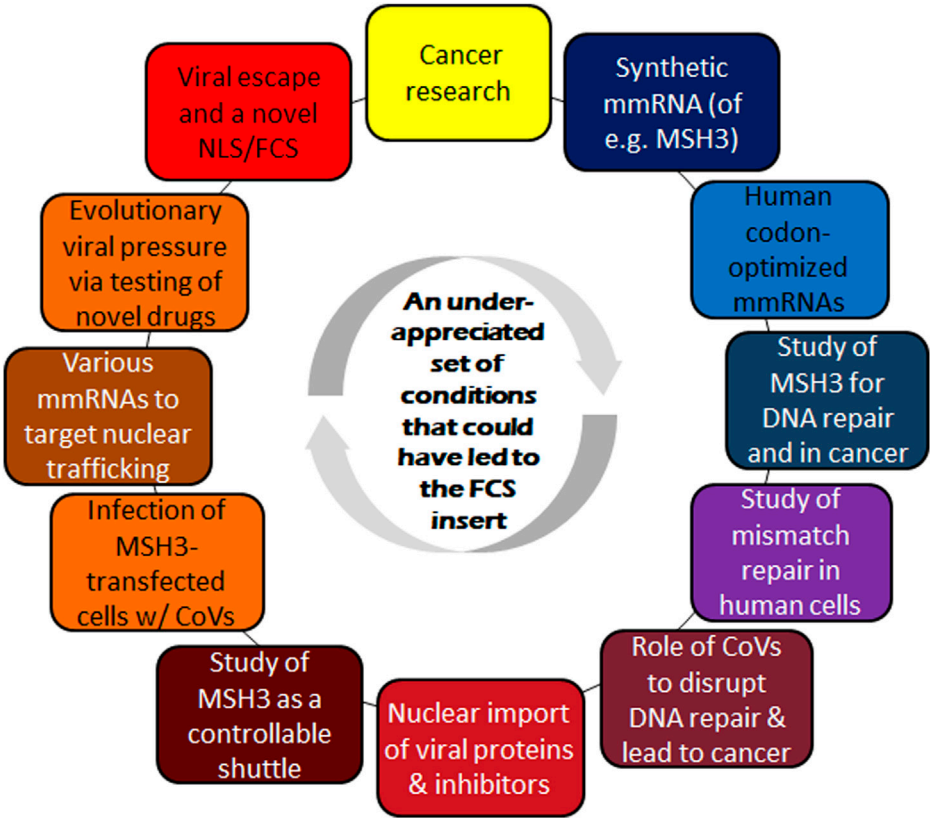

FIGURE 2
Postulated interrelationship/convergence of seemingly unrelated research orientations. Ambati et al. (2022) focus on the unexpected occurrence of a patented sequence in the SARS-CoV-2 genome and offer some ideas of what type of experiments could have led to the purported RNA integration. From the outset, it seems difficult to envision under which circumstances the different constituents postulated by Ambati et al., ranging from research involving SARS-like viruses to cancer research, could have converged in a unifying set of experiments to allow the required molecular events to happen. Even though Ambati and collaborators believe this may have been facilitated by a laboratory accident or a deliberate act, the odds of the implied viral recombination event may have been rather small. To address these issues, a rational approach was taken to show that it could have been possible nonetheless. Several hypothetical aspects and scenarios were envisioned that could have combined various seemingly disconnected research orientations and which could also have substantially increased the odds of the specific viral recombination event as postulated by Ambati et al. The figure summarizes the main pillars of this hypothetical framework.

translocation, e.g., afforded by a novel NLS that is an FCS as well. In this light, the above-described research objectives could explain the recombination event of Ambati et al. (summarized in Table 2). Importantly, even though such recombination may be rare naturally, as has been suggested, under specific experimental settings as postulated above, the extensive evolutionary pressure may have fostered the survival of exactly those rare viral escape mutants with such a unique insert encompassing the FCS.

4 Special considerations for biosafety and biosecurity

A first goal of this article was to scrutinize the feasibility of the postulated mechanism by Ambati and collaborators and to envision specific laboratory settings that could increase the odds of such events. The Ambati et al. postulate, covering only the FCS, cannot resolve the viral origin question *per se*. Nevertheless, the evidence developed above regarding the implicated genetic recombination events points to the existence of biorisks which have not been sufficiently appreciated, especially for their potential for future malicious exploitation.

4.1 Gaps in existing biorisk regulation

Biorisk management has long been divided into biosafety and biosecurity, where, informally, the former targets accidental/unintentional vulnerabilities and the latter, deliberate ones[8]. It has been recognized that whilst biosafety and biosecurity are inextricably linked, they are governed by different legal, policy, and regulatory regimes. Albeit, "[b]oth aim to keep dangerous

8 More precisely, "Biosafety provides policies and practices to prevent the unintentional or accidental release of specific biological agents and toxins, whereas biosecurity provides policies and practices to prevent the intentional or negligent release of biological materials or the acquisition of knowledge, tools, or techniques that could be used to cause harm," (National Research Council, 2015).

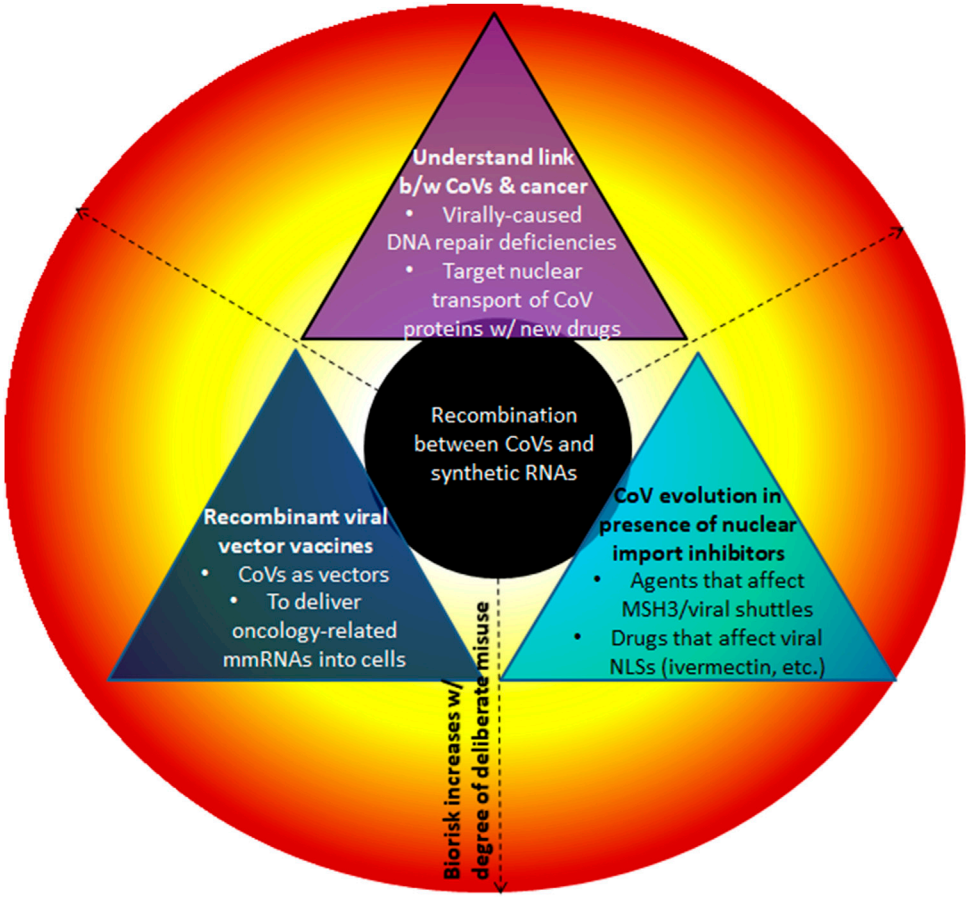

FIGURE 3
Main research orientations fostering CoV recombination in a laboratory context as motivated by the purported presence of a proprietary sequence in SARS-CoV-2. This analysis shows that there are indeed several ways in which the core postulate by Ambati et al. could have been realized in a laboratory setting. Above, it was argued that recombination between SARS-like viruses and other RNA could have happened via three main types of research experiments, and where MSH3 could be involved, either as a positive control, novel therapeutic agent, or contaminant: 1) Experiments to better elucidate the various DNA repair pathways potentially compromised by nuclear CoV proteins and their role in cancer, 2) The development and testing of the therapeutic potential of synthetic mRNAs as gene therapy agents to mitigate the harmful effects of nuclear import of specific CoV proteins, including delivery vehicles to bring these into human cells. 3) Testing and assessment of CoV evolution and escape in the presence of the tested cancer therapeutics/antivirals. MSH3 may have been of special interest as it is itself a DNA repair protein that may also shuttle into the cytoplasm as part of a cellular defense mechanism, and since the shuttling of MSH3 is controllable via (de)acetylation of its intrinsic NLS. Modified MSH3 or drugs that target NLS-based nucleocytoplasmic trafficking of CoVs could have fostered the evolution and escape of viral mutants with a novel NLS/FCS and improved nuclear transport profile.

pathogens safely and securely inside the areas where they are used and stored . . . " (National Research Council, 2015).

Over the years, risk assessors have known that regulation has been vastly complicated by the nomenclature related to DU, DURC, and GoF. Also, it has become increasingly clear that because of new technologies, societal issues, and others, many facets are incompletely understood, allow different interpretations, and that risk assessment is not free from subjectivity either (Bulletin of the Atomic Scientists, 2023). The above, while it intersects biosafety and biosecurity, falls outside existing regulations, because of the following.

4.1.1 Beyond stated pathogen/biological weapons research

Existing biorisk policy, legislation, and regulatory guidelines focus on agents which from the outset suggest some hazardous potential (e.g., 'biological agents and toxins,' 'pathogens,' 'bioterrorists,' 'bioweapons'). Apparently triggered by the Covid pandemic, we now see intense global efforts with an increased focus on pathogen research[9]. However, the above raises concern that specific components of research with rather different objectives, including those that certainly would be classified as benevolent, may converge to harbor under-appreciated GoF/DURC vulnerabilities, raising the prospect of the criminal genesis of dangerous pathogens in clandestine.

9 e.g., https://thebulletin.org/pathogens-project/,https://www.who.int/news/item/26-04-2023-who-launches-new-initiative-to-improve-pandemic-preparedness

4.1.2 Infeasibility to calculate biorisk

While traditionally biorisk policy has focused on the likelihood and potential impact of a range of risks (The Royal Society and the International Council for the Life Sciences, 2009), requiring both biosafety *and* biosecurity (National Research Council, 2015), the above highlights several challenges in doing so. Without awareness of the discussed vulnerabilities, their feasibility and consequences have been under-appreciated and there are no mitigation measures in place, especially against deliberate misuse. The general lack of security-by-design and by-default of the underlying technologies leads to the potential for a crime harvest, so that the above mechanisms or routes to harm could be exploited as a Trojan horse in the form of novel exploits that are largely unpredictable.

4.1.3 A blurring of biosafety and biosecurity

Above, it was argued that certain experimental conditions may result in various viral recombination events with a range of outcomes. Nonetheless, the implicated biorisks may not fall into a clearly defined category such as "accidental" *versus* "deliberate," and the same applies to potential actors. Biological risk itself comprises a spectrum, ranging from unintended/accidental to targeted malicious misuse, and encompasses naturally occurring diseases, re-emerging infectious diseases, unintended consequences of research, laboratory accidents, lack of awareness, negligence, and deliberate misuse (The Royal Society and the International Council for the Life Sciences, 2009).

Thus, a binary distinction between 'unintentional' and 'deliberate' may be difficult, even more so as synthetic biology has increasingly utilized digital technologies (e.g., cloud, mobile, cyber-physical/biological systems). In fact, in (Mueller, 2020), I first argued that in such contexts, the notions of safety and security cannot be readily separated, and this dilemma is further exacerbated by the convergence of fields, knowledge gaps, DU interpretations, and the insurmountable inherent gap between biology, computerized technology, and web interfaces.

4.2 Potentials for a crime harvest and related dangers

Risk assessment of dangerous organisms and pathogens has stressed the importance of taking into account their weaponization potential, the capability (including both scientific knowledge, tacit knowledge, and technological know-how) and intent of an adversary, and the potential consequence of an intentional release or misuse (National Research Council, 2015).

However, a major difficulty to quantify criminal or terrorist risk has been described via the limited historical precedent of biological weapons misuse (The Royal Society and the International Council for the Life Sciences, 2009; National Research Council, 2015). Key factors in this regard, including 'expected outcome,' 'feasibility of attacks,' and 'motives,' align with those made by the information-security community (Mueller and Barros Lourenco, 2023) - which over the decades has gained extensive experience with intentional forms of crime. Below, these will be specifically analyzed in the context of RNA recombination as discussed above.

4.2.1 Factors that increase the potentiality of misuse

Whilst the majority of the life science community is highly conscientious, under-appreciated risks such as the above have not received much attention, especially from a security perspective. Table 3 summarizes key aspects that can drive criminal exploitation of these new vulnerabilities.

4.2.2 Susceptibility and outcome

The current lack of rigorous cyber-biosecurity risk management practices and a poor security mindset have made the entire biotechnology sector vulnerable to exploitation. For example, according to a Forbes article[10], pharma and biotech companies are affected by more cybersecurity breaches than any other industry, with some of the high-profile attacks in recent years involving espionage and intellectual property theft related to COVID-19 vaccine development and attacks on technology involving DNA sequencers. Security risk analyses are also plagued by sociopolitical influences as demonstrated by the ongoing debates involving the pandemic origins and what this means for future events. While there is no sound rationale that the pandemic was deliberately initiated, the numerous controversies may in fact inform bad actors (Mueller, 2023).

The novel vulnerabilities depicted above include cancer research, drug development, and viral research, which constitute highly lucrative assets whose compromise can have systemic implications with enormous social, health, and economic sequelae (further detailed in Table 3).

4.2.3 Actors, motives, and capability

Bad actors may, in addition to gaining physical access to laboratory processes or devices, also mount their nefarious activities by exploiting gaps that are facilitated by the convergence of the underlying technologies (Jean et al., 2018; Mueller, 2020). Both factors combined increase the attack surface to realize viral recombination events as discussed above, via the covert disruption of confidentiality, integrity, and availability (CIA triad) of cyber-physical and bio-related processes, for example, through the swapping of biological/chemical/physical entities and/or their digitized description, mislabeling, masquerading, or other camouflaging attacks, including those fostered by the interrelationship and gap between computerized/automated descriptions, applications, web interfaces, and the actual entities (devices, processes, biomatter, etc.), ranging from research and planning, across the supply chain, to the final biological/bioengineered outcome in question.

Related work on cyberbiosecurity by The European Union Agency for Cybersecurity (Mueller and Barros Lourenco, 2023) has identified key motives that can drive attacks in the life sciences as they are fostered by computerized and networked technologies which are extended to the present context of viral recombination in Table 4.

10 https://www.forbes.com/sites/forbesbusinesscouncil/2021/03/18/how-the-pharmaceutical-industry-can-secure-networks-to-avoid-cyberattacks/?sh=2a5bffdb1eb3

4.3 A criminal context may turn things on their head

Traditionally, biorisk adversaries have been limited to specific groups with extensive skill and interest in creating bioweapons. However, the above vulnerabilities may be susceptible to a larger group of actors, requiring less know-how and tacit knowledge for their exploitation (Table 3; Table 4). Notably, actors could aim to facilitate interactions between the man-made world (e.g., synthetic RNAs) and the 'living' world (e.g., viruses) without aiming for a specific outcome. In the context of drug or vaccine development, viral recombination events can significantly impair research outcomes and product quality and derail a competitor, even if the adversary cannot target a particular type of recombination with specific RNAs.

Secondly, in addition to just waiting for a chance outcome, which could be fostered by covert ingression of RNA contaminants for instance, bad actors may even benefit from biorisk analyses which may expose which determinants could increase the likelihood or scope of a specific outcome. In this sense, information that may be regarded as useful to facilitate benevolent R&D may have an unrecognized DURC component nonetheless. For example, insights derived from the development of recombination-resistant CoVs for live-attenuated vaccines (Graham et al., 2018), may inadvertently also reveal factors that increase the odds of viral recombination.

More generally, a biosafety analysis that identifies where and how risk is most effectively targeted may likewise inform bad actors, revealing where a successful attack could provide "the greatest bang for the buck." In this light, it is unclear how to align biosafety risk mitigation with security principles without providing exploitable information ("side channels" (Mueller and Barros Lourenco, 2023)) and pointing bad actors to unrecognized DURC potentials, weak spots, or most attractive targets.

5 Conclusion

This work envisioned a hypothetical framework that enables underappreciated vulnerabilities of CoV recombination in a lab, and which, at least theoretically, could have led to the integration of the SARS-CoV-2 FCS. Specifically, this article identified several uncertainties that arose in the context of the Ambati et al. controversy and found several gaps in current biorisk assessment and policy (summarized in Table 5) which could inform future threat actors.

It has been suggested that the odds of the particular RNA recombination indicated by Ambati et al. may be low as this would require two crossover events very close together. Nonetheless, recent research about the recombinability of RNA viruses stresses the foundational role of evolutionary pressure in both the selection and maintenance of viral recombination events, a factor that is of great relevance in a lab environment. Therefore, due to the convergence of particular research objectives and experimental conditions as postulated above, the type of recombination as envisioned by Ambati et al. cannot be ruled out, particularly in a criminal context.

From the outset, it seems difficult to see how the research settings implicated by Ambati et al. could align with the Moderna patent and result in the necessary laboratory experiments to facilitate the hypothesized viral recombination. Whilst an inadvertent or intentional act may still have been possible, the chance of the particular viral recombination may have been rather low. To address this, above, the research objectives implied by Ambati et al. were further refined. A logical rationale was developed for how individual goals, ranging from cancer research, viral vector vaccines, and CoVs, to new oncology-related therapeutics, could have converged into one laboratory objective and set of experiments (summarized in Figure 2).

As detailed above, a core pillar of Moderna's cancer research may have been to target the nuclear transport of CoV proteins, the latter of which is a well-established pathway to disrupt DNA repair. Given that MSH3 is involved in double-strand break repair via homologous recombination, is able to facilitate nuclear-cytosolic shuttling of proteins, but can also induce DNA repair deficiency when over-/underexpressed, it is reasonable to envision an experimental context wherein MSH3 was tested to a) better elucidate the role of CoV nuclear import and its role in cancer, b) test drugs that prevent the import of viral proteins, and c) specifically target MSH3 for clinical applications. Indeed, only recently, Tseng-Rogenski et al. (2020) speculated that MSH3 could shuttle into the cytoplasm as a part of cellular defense mechanisms to detect invading pathogens that contain DNA and noted the necessity of further studies of this finding. With MSH3, the right concentration and cellular localization are critically important and aberrations lead to severe forms of disease (Tseng-Rogenski et al., 2020). Interestingly, blocking of the MSH3 import function happens via acetylation of its inherent NLS which has been identified as a molecular toggle in broader contexts (Williams et al., 2020).

Essential to the framework hypothesized above is the link between CoV infection and cancer development, and how this could have been targeted by modified mRNAs (which may have included mRNA acetylation as well). Based on the shuttling properties of MSH3 and its putative role in cellular defense, it is feasible to assume that modified MSH3 has been studied as a potential agent to prevent the nucleocytoplasmic trafficking of CoVs. Therapeutic agents that have shown to direct MSH3 shuttling (Williams et al., 2020) may have had direct impact on NLS-driven nuclear trafficking of CoVs as well. Likewise, efficacy testing of select agents such as ivermectin or novel drugs developed to impair the nuclear translocation of CoVs may also have created substantial pressure on these viruses, favored the development of escape mutants with improved nuclear transport profiles, and specifically led to viral mutants harboring SARS-CoV-2's unique NLS/FCS insert.

With CoVs in particular, viral escape has long been known to be either a mutation- or recombination-driven process, a fact that is

demonstrated by numerous research efforts that aim to render live-attenuated CoV vaccines recombination refractory (Graham et al., 2018). Given that naturally, the heritability of a recombination event is mediated by the replication fitness of the resulting progeny genome (Graham et al., 2018) and that more recently, selection and fitness have been regarded as key in recombination (Bentley and Evans, 2018; Alnaji et al., 2022; Wang et al., 2022), it is likely that the same applies to the laboratory-induced selective pressure during the analysis of viruses described above which could have led to the insertion of both an NLS and an FCS.

The notion that the insert surrounding the SARS-CoV-2 FCS could have resulted from laboratory recombination, even though naturally the two required crossover events may be regarded as happening with low frequency, is also in line with the observation that the FCS itself has been shown to appear in steps during serial passaging, as known particularly for the H5N1 flu virus[11]. Therefore, as for general drivers of pathogenicity, those dictating recombination in a laboratory environment are likely governed by different timelines than those known for viral evolution in the wild.

In conclusion, Figure 3 summarizes various circumstances envisioned above that *could have* favored the type of viral recombination as postulated by Ambati et al., and which constitute an unrecognized biorisk for future events. The fact that these fall outside of existing GoF/DURC biorisk regulation has critical implications for their potential for deliberate misuse. Even though individual research objectives by themselves may be seen as low risk, convergence repercussions can engender substantial biorisk that may be highly vulnerable to intentional disguise, camouflaging, covert infiltration of contaminants, swapping of biological material, and other crime types.

Recombination plays important roles in the spread, virulence, pathogenesis, and vaccine escape of viruses; for instance, it has been found that the emergence of novel CoVs with enhanced virulence can be explained by recombination events (Graham et al., 2018). Thus, regardless of whether the novel insert in SARS-CoV-2 is the result of recombination as indicated by Ambati et al., the analysis above strongly suggests that bad actors could try to facilitate viral recombination events for various nefarious purposes.

Even though the above shows the *feasibility* of the emergence of the FCS through research projects that are not regarded as risky, this analysis was not done to suggest this is what actually happened, nor was it done to imply Moderna's culpability in terms of conducting experiments that led to the Covid pandemic. Indeed, the focus of the above was the insert encompassing the FCS alone - which is not the only feature that distinguishes SARS-CoV-2 from its closest relatives, as demonstrated by the additional large number of small sequence differences scattered throughout the genome. Although some may wonder if an adversary could have introduced these on purpose, this seems unlikely. While it is true that the generation and genetic modification of CoVs via synthetic genomics platforms have long been possible (Almazán et al., 2000; Boyd et al., 2000; Thiel et al., 2001) using viral isolates, cloned viral DNA, clinical samples, or synthetic DNA, and even though an improved reverse-genetics platform has enabled the rapid reconstruction of SARS-CoV-2 in only a week after receipt of the synthetic DNA fragments (Thao et al., 2020), the unparalleled tragic toll of this virus on everyone worldwide does not support the idea that it was intentionally made and released from a lab.

The above vulnerabilities cannot be resolved by one overall policy framework and governing authority alone as it seems impossible to envision all possible routes to harm (accidental or deliberate) in all possible contexts. While synthetic biology holds the promise to be able to fully predict and control the outcome, the risks, and dangers described here should be an eye-opener as to how little we still know about the (misuse) potentiality of the generated/modified biological products to interact with the rest of the world, or even change nature itself.

The convergence of technologies and disciplines shows it will be imperative to appreciate the most important pillars of science, skepticism, curiosity, and trans-disciplinary knowledge, and foster a change of consciousness that emphasizes the responsibilities and powers of expertise, insights (including intuition), transparency, and commitment of every researcher and organization involved, to effectively help protect the future of humanity and nature in general.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

11 https://theintercept.com/2023/01/19/covid-origin-nih-emails/

References

Almazán, F., González, J. M., Pénzes, Z., Calvo, E., Plana-Durán, J., Enjuanes, L., et al. (2000). Engineering the largest rna virus genome as an infectious bacterial artificial chromosome. *Proc. Natl. Acad. Sci.* 97 (10), 5516–5521. doi:10.1073/pnas.97.10.5516

Alnaji, F. G., Bentley, K., Pearson, A., Woodman, A., Moore, J., Fox, H., et al. (2022). Generated randomly and selected functionally? The nature of enterovirus recombination. *Viruses* 14 (5), 916. doi:10.3390/v14050916

Ambati, B. K., Varshney, A., Palú, G., Uhal, B. D., Uversky, V. N., and AdamBrufsky, M. (2022). Msh3 homology and potential recombination link to sars-cov-2 furin cleavage site. *Front. Virology* 10, 834808. doi:10.3389/fviro.2022.834808

Andersen, K. G., Rambaut, A., Lipkin, W. I., Holmes, E. C., and Garry, R. F. (2020). The proximal origin of sars-cov-2. *Nat. Med.* 26 (4), 450–452. doi:10.1038/s41591-020-0820-9

Bancel, S., Chakraborty, T., De Fougerolles, A., Elbashir, S. M., John, M., Roy, A., et al. (2017). Modified polynucleotides for the production of oncology-related proteins and peptides, US Patent US9587003.

Bentley, K., and Evans, D. J. (2018). Mechanisms and consequences of positive-strand rna virus recombination. *J. General Virology* 99 (10), 1345–1356. doi:10.1099/jgv.0.001142

Boyd, Y., Curtis, K. M., and Baric, R. S. (2000). Strategy for systematic assembly of large rna and dna genomes: Transmissible gastroenteritis virus model. *J. virology* 74 (22), 10600–10611. doi:10.1128/jvi.74.22.10600-10611.2000

Bulletin of the Atomic Scientists (2023). Concluding remarks: Jesse bloom and filippa lentzos at the pathogens project conference. Avaliable At: https://www.youtube.com/watch?v=q20_9R86Vg8&list=PL2poHJyhVgebZbSWn3DaVOBgbP3MVtmHy&index=15.

Calisher, C., Carroll, D., Colwell, R., Ronald, B. C., Peter, D., Drosten, C., et al. (2020). Statement in support of the scientists, public health professionals, and medical professionals of China combatting Covid-19. *Lancet* 395 (10226), e42–e43. doi:10.1016/s0140-6736(20)30418-9

Caly, L., Druce, J. D., Catton, M. G., Jans, D. A., and Wagstaff, K. M. (2020). The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 *in vitro*. *Antivir. Res.* 178, 104787.

Chan, Y. A., and Zhan, S. H. (2022). The emergence of the spike furin cleavage site in sars-cov-2. *Mol. Biol. Evol.* 39 (1), msab327. doi:10.1093/molbev/msab327

Chetverin, A. B. (1999). The puzzle of rna recombination. *FEBS Lett.* 460 (1), 1–5. doi:10.1016/s0014-5793(99)01282-x

Cyranoski, D. (2020). Directive on measures for a high common level of cybersecurity across the union (nis2 directive). Avaliable At: https://www.nature.com/articles/d41586-020-01541-z.

Dubuy, Y., and Lachuer, H. (2022). Commentary: Msh3 homology and potential recombination link to sars-cov-2 furin cleavage site. *Front. Virology* 2, 914888. doi:10.3389/fviro.2022.914888

Elgabry, M., Nesbeth, D., and Johnson, S. (2022). The future of biotechnology crime: A parallel delphi study with non-traditional experts. *Futures* 141, 102970. doi:10.1016/j.futures.2022.102970

Gallaher, W. R. (2020). A palindromic rna sequence as a common breakpoint contributor to copy-choice recombination in sars-cov-2. *Archives Virology* 165 (10), 2341–2348. doi:10.1007/s00705-020-04750-z

Godbold, D., Hewitt, F. C., Kappell, A. D., Scholz, M. B., Stacy, L. A., Treangen, J., et al. (2023). Improved understanding of biorisk for research involving microbial modification using annotated sequences of concern. *Front. Bioeng. Biotechnol.* 11, 1124100. doi:10.3389/fbioe.2023.1124100

Graham, R. L., and Baric, R. S. (2010). Recombination, reservoirs, and the modular spike: Mechanisms of coronavirus cross-species transmission. *J. virology* 84 (7), 3134–3146. doi:10.1128/jvi.01394-09

Graham, R. L., Deming, D. J., Deming, M. E., and Baric, R. S. (2018). Evaluation of a recombination-resistant coronavirus as a broadly applicable, rapidly implementable vaccine platform. *Commun. Biol.* 1 (1), 179. doi:10.1038/s42003-018-0175-7

Harrison, N. L., and Sachs, J. D. (2022). A call for an independent inquiry into the origin of the sars-cov-2 virus. *Proc. Natl. Acad. Sci.* 119 (21), e2202769119. doi:10.1073/pnas.2202769119

Heinemann, J. A., Paull, D. J., Walker, S., and Kurenbach, B. (2021). Differentiated impacts of human interventions on nature: Scaling the conversation on regulation of gene technologies. *Elem. Sci. Anthropocene* 9 (1), 00086. doi:10.1525/elementa.2021.00086

Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (2012). *PCR protocols: A guide to methods and applications*. Michigan: Academic Press.

Jean, P., E Gallegos, J., Murch, R., Buchholz, W. G., and Raman, S. (2018). Cyberbiosecurity: From naive trust to risk awareness. *Trends Biotechnol.* 36 (1), 4–7. doi:10.1016/j.tibtech.2017.10.012

Jiang, H., and Mei, Y. F. (2021). Sars–cov–2 spike impairs dna damage repair and inhibits v (d) j recombination *in vitro*. *Viruses* 13 (10), 2056. doi:10.3390/v13102056

Jocelyn, K. (2023). Nih restarts bat virus grant suspended 3 years ago by trump. Avaliable At: https://www.science.org/content/article/nih-restarts-bat-virus-grant-suspended-3-years-ago-trump.

Kylie, M. W., Rawlinson, S. M., Hearps, A. C., and Jans, D. A. (2011). An alphascreen®-based assay for high-throughput screening for specific inhibitors of nuclear import. *J. Biomol. Screen.* 16 (2), 192–200. doi:10.1177/1087057110390360

Lai, M. M. (1996). Recombination in large rna viruses: Coronaviruses. *seminars VIROLOGY* Vol. 7, 381–388. doi:10.1006/smvy.1996.0046

Leon, C., Kylie, M. W., and Jans, D. A. (2012). Nuclear trafficking of proteins from rna viruses: Potential target for antivirals? *Antivir. Res.* 95 (3), 202–206. doi:10.1016/j.antiviral.2012.06.008

Malone, R. W., Felgner, P. L., and Verma, I. M. (1989). Cationic liposome-mediated rna transfection. *Proc. Natl. Acad. Sci.* 86 (16), 6077–6081. doi:10.1073/pnas.86.16.6077

Marra, G., Iaccarino, I., Lettieri, T., Roscilli, G., Delmastro, P., and Jiricny, J. (1998). Mismatch repair deficiency associated with overexpression of the msh3 gene. *Proc. Natl. Acad. Sci.* 95 (15), 8568–8573. doi:10.1073/pnas.95.15.8568

Mills, D. R., Dobkin, C., and Kramer, F. R. (1978). Template-determined, variable rate of rna chain elongation. *Cell.* 15 (2), 541–550. doi:10.1016/0092-8674(78)90022-3

Mueller, S., and Barros Lourenco, M. (2023). "Cybersecurity in life sciences," in *cyberbiosecurity: Cybersecurity research and innovation brief of the the European Union agency for cybersecurity* (ENISA).

Mueller, S. (2020). Facing the 2020 pandemic: What does cyberbiosecurity want us to know to safeguard the future? *Biosaf. Health* 3, 11–21. doi:10.1016/j.bsheal.2020.09.007

Mueller, S. (2023). "Potentials of pathogen research through the lens of cyberbiosecurity, or what threat actors can learn from the Covid-19 pandemic," in *Cyberbiosecurity* (Springer, Cham), 147–171.

Murch, R., and DiEuliis, D. (2019). Editorial: Mapping the cyberbiosecurity enterprise. *Front. Bioeng. Biotechnol.* 7, 235. doi:10.3389/fbioe.2019.00235

Murch, R. S., So, W. K., Buchholz, W. G., Raman, S., and Peccoud, J. (2018). Cyberbiosecurity: An emerging new discipline to help safeguard the bioeconomy. *Front. Bioeng. Biotechnol.* 6, 39. doi:10.3389/fbioe.2018.00039

Murch, R. S. (2015). Bioattribution needs a coherent international approach to improve global biosecurity. *Front. Bioeng. Biotechnol.* 3, 80. doi:10.3389/fbioe.2015.00080

National Research Council (2015). *Potential Risks and Benefits of Gain-of-Function Research: Summary of a Workshop*. Washington, DC: National Academies Press. doi:10.17226/21666

Negroni, M., Ricchetti, M., and Pascal, N. (1995). Homologous recombination promoted by reverse transcriptase during copying of two distinct RNA templates. *Proc. Natl. Acad. Sci.* 92 (15), 6971–6975. doi:10.1073/pnas.92.15.6971

Ozawa, M., Fujii, K., Muramoto, Y., Yamada, S., Yamayoshi, S., Takada, A., et al. (2007). Contributions of two nuclear localization signals of influenza a virus nucleoprotein to viral replication. *J. virology* 81 (1), 30–41. doi:10.1128/jvi.01434-06

Pease, K. (1997). 14 predicting the future: The roles of routine activity and rational choice theory. *Ration. choice situational crime Prev. Theor. Found.* 233, 14.

Piplani, S., Singh, P. K., Winkler, D. A., and Petrovsky, N. (2021). *In silico* comparison of sars-cov-2 spike protein-ace2 binding affinities across species and implications for virus origin. *Sci. Rep.* 11 (1), 13063–13113. doi:10.1038/s41598-021-92388-5

Reardon, S. (2023). Stricter us guidelines for 'gain-of-function' research are on the way-maybe. *Nature* 614 (7947), 206–207. doi:10.1038/d41586-023-00257-0

Romeu, A. R., and Ollé, E. (2021). Sars-cov-2 and the secret of the furin site. Avaliable At: https://www.preearth.net/pdfs/the-furin-site-and-CGGCGG.pdf.

Rowe, C. L., Fleming, J. O., Meera, J. N., Palmenberg, A. C., and Baker, S. C. (1997). Generation of coronavirus spike deletion variants by high-frequency recombination at regions of predicted rna secondary structure. *J. virology* 71 (8), 6183–6190. doi:10.1128/jvi.71.8.6183-6190.1997

Ryan, E. L., Hollingworth, R., and Grand, R. J. (2016). Activation of the dna damage response by rna viruses. *Biomolecules* 6 (1), 2. doi:10.3390/biom6010002

Sattar, S., Kabat, J., Jerome, K., Feldmann, F., Bailey, K., and Mehedi, M. (2023). Nuclear translocation of spike mrna and protein is a novel feature of sars-cov-2. *Front. Microbiol.* 14 (155), 1073789. doi:10.3389/fmicb.2023.1073789

Sola, I., Mateos-Gomez, P. A., Almazan, F., Zuniga, S., and Enjuanes, L. (2011). Rna-rna and rna-protein interactions in coronavirus replication and transcription. *RNA Biol.* 8 (2), 237–248. doi:10.4161/rna.8.2.14991

Thao, T. T. N., Labroussaa, F., Ebert, N., V'kovski, P., Stalder, H., Portmann, J., et al. (2020). Rapid reconstruction of sars-cov-2 using a synthetic genomics platform. *Nature* 582 (7813), 561–565. doi:10.1038/s41586-020-2294-9

The European Commission (2023). Directive on measures for a high common level of cybersecurity across the union (nis2 directive). Avaliable At: https://digital-strategy.ec.europa.eu/en/policies/nis2-directive.

The Royal Society and the International Council for the Life Sciences (2009). New approaches to biological risk assessment. Avaliable At: https://royalsociety.org/topics-policy/publications/2009/biological-risk/.

Thiel, V., Herold, J., Schelle, B., and Siddell, S. G. (2001). Infectious rna transcribed *in vitro* from a cdna copy of the human coronavirus genome cloned in vaccinia virus. *J. General Virology* 82 (6), 1273–1281. doi:10.1099/0022-1317-82-6-1273

Tseng-Rogenski, S. S., Munakata, K., Mehta, S., Koi, M., Zheng, W., Zhang, Y., et al. (2020). The human dna mismatch repair protein msh3 contains nuclear localization and export signals that enable nuclear-cytosolic shuttling in response to inflammation. *Mol. Cell. Biol.* 40 (13), 000299–e120. doi:10.1128/MCB.00029-20

van Oers, J., Edwards, Y., Chahwan, R., Zhang, W., Smith, C., Pechuan, X., et al. (2014). The mutsβ complex is a modulator of p53-driven tumorigenesis through its functions in both dna double-strand break repair and mismatch repair. *Oncogene* 33 (30), 3939–3946. doi:10.1038/onc.2013.365

Wang, H., Cui, X., Cai, X., and An, T. (2022). Recombination in positive-strand rna viruses. *Front. Microbiol.* 13, 870759. doi:10.3389/fmicb.2022.870759

Williams, G. M., Paschalis, V., Ortega, J., Muskett, F. W., Hodgkinson, J. T., Li, G.-M., et al. (2020). HDAC3 deacetylates the DNA mismatch repair factor MutSβ to stimulate triplet repeat expansions. *Proc. Natl. Acad. Sci.* 117 (38), 23597–23605.

Wolff, J. A., Malone, R. W., Williams, P., Wang, C., Acsadi, G., Jani, A., et al. (1990). Direct gene transfer into mouse muscle *in vivo*. *Science* 247 (4949), 1465–1468. doi:10.1126/science.1690918

Yang, S. N. Y., Atkinson, S. C., Wang, C., Lee, A., Bogoyevitch, M. A., Borg, N. A., et al. (2020). The broad spectrum antiviral ivermectin targets the host nuclear transport importin α/β1 heterodimer. *Antivir. Res.* 177, 104760. doi:10.1016/j.antiviral.2020.104760

Yang, Y., Yan, W., Hall, A. B., and Jiang, X. (2021). Characterizing transcriptional regulatory sequences in coronaviruses and their role in recombination. *Mol. Biol. Evol.* 38 (4), 1241–1248. doi:10.1093/molbev/msaa281

Zapatka, M., Borozan, I., Brewer, D. S., Iskar, M., Adam, G., Malik, A., et al. (2020). The landscape of viral associations in human cancers. *Nat. Genet.* 52 (3), 320–330. doi:10.1038/s41588-019-0558-9

Zúniga, S., Cruz, J. L., Sola, I., Mateos-Gómez, P. A., Palacio, L., and Enjuanes, L. (2010). Coronavirus nucleocapsid protein facilitates template switching and is required for efficient transcription. *J. virology* 84 (4), 2169–2175. doi:10.1128/jvi.02011-09

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Review
PUBLISHED 08 August 2023
DOI 10.3389/fbioe.2023.1238917

OPEN ACCESS

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RECEIVED 12 June 2023
ACCEPTED 31 July 2023
PUBLISHED 08 August 2023

CITATION
Buyel JF (2023), Product safety aspects of plant molecular farming.
Front. Bioeng. Biotechnol. 11:1238917.
doi: 10.3389/fbioe.2023.1238917

Product safety aspects of plant molecular farming

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Plant molecular farming (PMF) has been promoted since the 1990s as a rapid, cost-effective and (most of all) safe alternative to the cultivation of bacteria or animal cells for the production of biopharmaceutical proteins. Numerous plant species have been investigated for the production of a broad range of protein-based drug candidates. The inherent safety of these products is frequently highlighted as an advantage of PMF because plant viruses do not replicate in humans and vice versa. However, a more nuanced analysis of this principle is required when considering other pathogens because toxic compounds pose a risk even in the absence of replication. Similarly, it is necessary to assess the risks associated with the host system (e.g., the presence of toxic secondary metabolites) and the production approach (e.g., transient expression based on bacterial infiltration substantially increases the endotoxin load). This review considers the most relevant host systems in terms of their toxicity profile, including the presence of secondary metabolites, and the risks arising from the persistence of these substances after downstream processing and product purification. Similarly, we discuss a range of plant pathogens and disease vectors that can influence product safety, for example, due to the release of toxins. The ability of downstream unit operations to remove contaminants and process-related toxic impurities such as endotoxins is also addressed. This overview of plant-based production, focusing on product safety aspects, provides recommendations that will allow stakeholders to choose the most appropriate strategies for process development.

KEYWORDS

endotoxins, expression strategy, host selection, production process, toxic metabolites, virus removal

1 Introduction

Plants and plant cells can be used to produce active pharmaceutical ingredients, including small-molecule drug candidates and recombinant proteins (Eidenberger et al., 2023). Although recombinant proteins can be produced by many different host systems, the post-translational modifications (PTMs) carried out by plants (particularly glycosylation) can result in superior product activity (Tekoah et al., 2013; Gengenbach et al., 2019), or they can be humanized using state-of-the-art genetic engineering tools (Strasser et al., 2008; Jansing et al., 2018). The same tools can be used to modify host plant species such as tobacco (*Nicotiana tabacum*) (Menary et al., 2020a), converting them into designer hosts optimized for biopharmaceutical production (Fraser et al., 2020; Buyel et al., 2021; Huang and Puchta,

Abbreviations: CEGS, controlled environment growth systems; CHO, Chinese hamster ovary; HCP, host cell protein; PMF, plant molecular farming; PTM, post-translational modification.

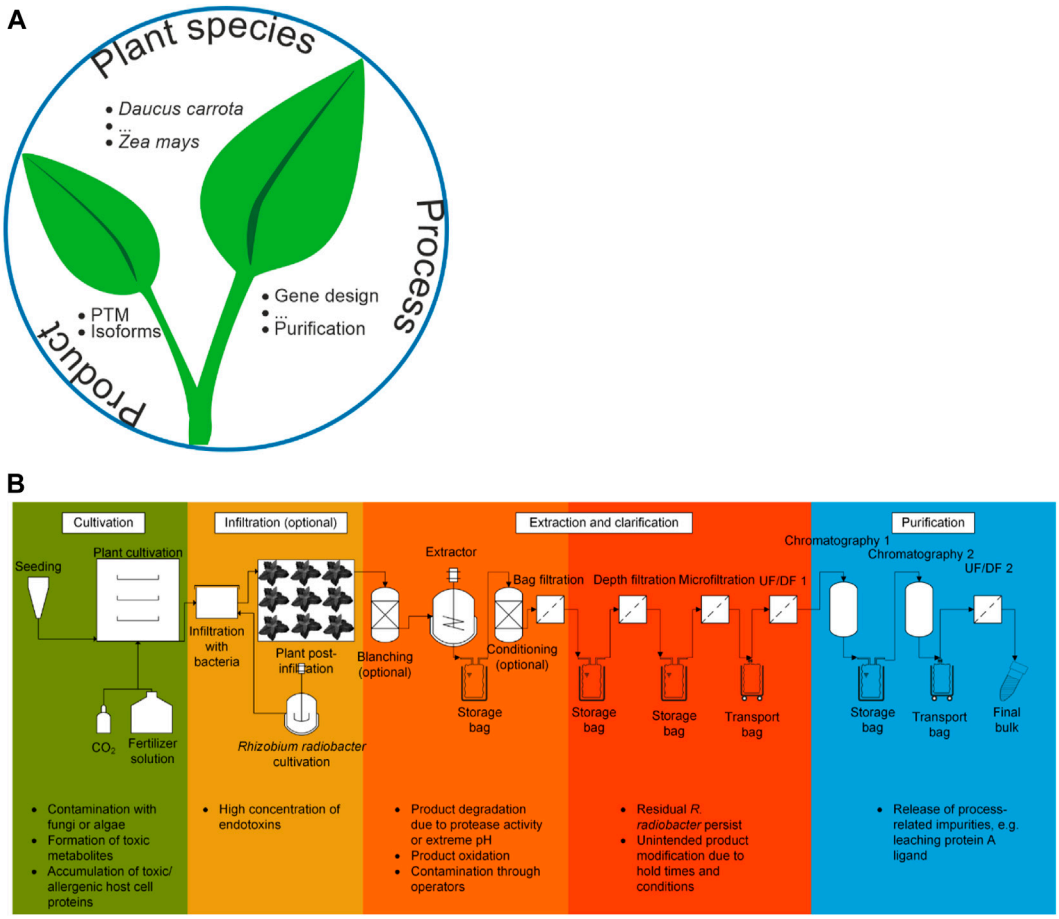

FIGURE 1
Product safety aspects of plant molecular farming. **(A)** The three major aspects that determine final product safety as discussed in this review, including some examples. **(B)** Generic process scheme for the production of recombinant proteins in plants. The cultivation is depicted as a (fully) controlled environment growth system (Section 3.3.2) but other settings can be used, such as greenhouses. The scheme can be adapted to a transgenic expression strategy by omitting the infiltration and *Rhizobium radiobacter* cultivation steps. It can also be converted to a plant cell suspension culture process by replacing the cultivation of whole plants with a bioreactor train. In the latter case, infiltration may still be relevant if plant cell packs are used for expression (Rademacher et al., 2019). Some potential risk factors are highlighted at each process step.

2021; Uranga et al., 2021). One example of this approach is the modification of tobacco metabolism to eliminate nicotine biosynthesis (Schachtsiek and Stehle, 2019). The production strategy can be tailored to prioritize speed (transient expression) or scalability (transgenic plants) as required for specific products and market expectations (Buyel et al., 2017; Tusé et al., 2020). Once an ideal host and production strategy have been identified, downstream processing platform technologies can be selected to ensure high product purity (Buyel et al., 2015a; Ma et al., 2015), including compliance with good manufacturing practices (GMP) even when using basic facilities for cultivation, such as greenhouses (Ma et al., 2015; Ward et al., 2021). The number of dedicated virus removal steps is often lower in PMF processes compared to those based on mammalian cells because plant cells do not support the replication of human viruses (Commandeur and Twyman, 2005; Ma et al., 2015).

These principles suggest that plants and plant cells could be widely used to produce safe biopharmaceuticals in compliance with regulatory requirements and manufacturing standards (Hundleby et al., 2022). Nevertheless, only a small number of PMF products have been approved thus far, and given the diverse production platforms involved, each of them may be regarded as unique. In contrast, microbial and animal cells have been used to produce many different approved recombinant biopharmaceutical proteins (Walsh and Walsh, 2022). Therefore, it is important to identify key factors for the design of cost-efficient, scalable, sustainable and especially safe plant-based manufacturing processes for biopharmaceutical proteins, ultimately allowing the industry to adopt the technology without reservation (Menary et al., 2020b).

This review discusses the safety aspects of PMF, covering a diverse range of plant species (hosts), processes and products (Figure 1) and the associated risks (Table 1). We first consider the impact of host selection, which determines whether the presence of toxic metabolites and proteins must be taken into account. Next, we address production processes, including plant cultivation conditions, expression strategies, and purification operations. Then we turn to the product and its modification within the plant, which links back to host selection. We conclude by assessing the potential of breeding and genetic engineering to address some of the key safety concerns. This article does not

TABLE 1 Sources of risk in plant molecular farming that particularly affect product safety.

Process property	Source			
	Toxins	Pathogens	Oncogenes	Product modifications
Plant species	Endogenous metabolites, lectins	Toxins from algae and bacteria, attraction of disease vectors	n.a	Host-specific glycosylation
Cultivation conditions	Toxins from microbial contamination, higher metabolite levels	Contamination with microorganisms or animals	n.a	Proteolytic degradation or truncation
Expression strategy	Endotoxins from *R. radiobacter*[a]	Residual *R. radiobacter*[a]	Promoter sequences or coding sequences of recombinant proteins	Incomplete PTMs due to overexpression
Subcellular targeting	n.a	n.a	n.a	Incomplete processing, aberrant PTMs
Harvesting	n.a	Contamination via personnel	n.a	Oxidation or degradation due to storage
Extraction conditions	Increased metabolite solubility	No microorganism inactivation	Increased host DNA solubility and size	Oxidation
Purification strategy	Insufficient removal	Insufficient removal	Insufficient removal	Insufficient removal of inactive product isoforms or degradation products
Storage	Re-contamination	Re-contamination	n.a	Degradation, oxidation, truncation, or aggregation

[a]*Rhizobium radiobacter* was formerly known as *Agrobacterium tumefaciens*; n.a.—not applicable; PTMs, post-translational modifications.

consider the environmental or work-related safety of PMF (Knödler et al., 2023a), such as the release of transgenic pollen into the environment, which has been discussed elsewhere (Commandeur and Twyman, 2005). Whereas the focus of this review is biopharmaceuticals, similar considerations apply to products such as food and feed additives, albeit with differences in the mode of manufacturing and utilization. For example, food and feed additives are generally produced on a larger scale than pharmaceuticals and must remain functional after oral delivery (Barzee et al., 2022), whereas pharmaceuticals can be formulated for many different delivery modes, including oral, intravenous and intramuscular. Similarities between pharmaceutical PMF and non-pharmaceutical applications are highlighted where appropriate.

2 Host-related safety aspects of plant molecular farming

2.1 Host-specific harmful metabolites and proteins

2.1.1 Small-molecule metabolites

Host cell components are defined as process-related impurities in all expression systems (Argentine et al., 2007; Arfi et al., 2016; Jones et al., 2021). In some cases, such molecules are directly toxic, such as the lipopolysaccharides known as endotoxins produced by Gram-negative bacteria (Section 3.1) (Serdakowski London et al., 2012). In contrast to these large cell wall components that are easily detected in specific assays, plants and plant cells also contain diverse metabolites with a wide dynamic range of concentrations, including pigments (e.g., chlorophyll) and polyphenols (Moore et al., 2014; Wang et al., 2019). The specific purpose or benefit of these complex small molecules may not readily be apparent, but they are often intrinsically bioactive (Acamovic and Brooker, 2005; Wink, 2009; Napagoda et al., 2022). Accordingly, they are exploited as food additives, cosmetic ingredients and pharmaceuticals, such as the extraction of the anti-cancer drug paclitaxel from medicinal plants (Pereira et al., 2012; Buyel, 2018) and derived cell cultures (Ochoa-Villarreal et al., 2016). However, where such bioactive compounds are present in PMF hosts used for the production of recombinant proteins, they are treated as impurities that must be removed during purification (Table 2). For example, nicotine is purified from tobacco for use as a pharmaceutical, including nicotine replacement therapy and the treatment of mild cognitive impairment (Sanchez-Ramos, 2020; Kheawfu et al., 2021), but when tobacco is used to produce recombinant monoclonal antibodies the nicotine is an unwanted impurity (Ma et al., 2015).

Solanaceous plants like tobacco, pepper (*Capsicum annuum*), potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) are attractive for PMF applications because they produce large amounts of biomass [e.g., 100,000–500,000 kg ha^{-1} a^{-1} for tobacco (Stoger et al., 2002; Huebbers and Buyel, 2021)]. However, they also contain undesirable or even toxic alkaloids like capsaicin, solanine, anabasine and nicotine (Green et al., 2013; Stephan et al., 2017; Günthardt et al., 2018). The latter has an estimated median lethal dose (LD_{50}) of 6–13 mg kg^{-1} body mass in humans (peroral uptake; intravenous probably less) (Mayer, 2014), but concentrations as low as 0.025 mg kg^{-1} may trigger biological reactions such as altered leg extensor torque (Mündel et al., 2017). In the case of anabasine, the teratogenic potential rather than acute toxicity is the major concern (Keeler et al., 1984; Green et al., 2013), even though it is difficult to identify a suitable model system (Welch et al., 2014).

Similarly, cyanogenic glycosides are amino acid-derived compounds found in many plants, including crops, at various stages during their life cycle, depending on the nutrient supply (Gleadow and Møller, 2014; Lechtenberg et al., 2005-2010). This

TABLE 2 Examples of toxic compounds found in the plant hosts used for PMF and associated microorganisms.

Molecule name	Molecule type	Molecular mass (Da)	Host species	Trivial name	Concentration (mg kg^{-1} fresh biomass)	Dose (mg kg^{-1} body mass)	Dose type (–)	Reference species	Route of administration	Ref
Anabasine	Alkaloid	162	*N. tabacum*	Tobacco	250	11–16	LD_{50}	Mouse	i.v	Sisson and Severson (1990), Lee et al. (2006)
Ciguatoxin	Polyether	1,100–1,300	*G. toxicus*	n.a	n.a	0.0003	LD_{50}	Mouse	n.a	Lewis (2000), Fusetani and Kem (2009)
Cyanogenic glycosides	Glycosides	250–900	Diverse, e.g., *Eucalyptus cladocalyx*	Sugar gum	4,000–15,000	4.3	LD_{50}	Rat	i.p	Toxic Rep Ser (1993)
						15	LD_{50}	Rat	p.o	
						4.9–5.9	LD_{50}	Mouse	i.p	
						2.9	LD_{lo}	Human	p.o	
Gluten	Storage protein	30,000–100,000	cereal crops, e.g., *T. aestivum*	Diverse, e.g., wheat	40,000–90,000	10–100 [mg per person per day]	"safe range"	Human	p.o	Hischenhuber et al. (2006), Cohen et al. (2019), Pronin et al. (2020)
Nicotine	Alkaloid	162	*N. tabacum*	Tobacco	20,000–50,000	6–13	LD_{50}	Human	p.o	Mayer, 2014; Henry et al. (2019)
Saxitoxins	Complex heterocyclic compound	299	Dinoflagellates, e.g., *L. wollei*	Diverse, n.a	n.a	0.0005	NOAEL	Human	p.o	Weirich and Miller (2014)
Viscumin	Ribosome-inactivating protein	62,628	*V. album*	Mistletoe	n.a	0.002[a]	LD_{50}	Mouse	i.v	Olsnes et al. (1982)

[a]assuming ~0.03 kg body mass per mouse; LD_{50}, median lethal dose; LD_{lo}, minimal lethal dose; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; NOAEL, no observed adverse effect level; p.o., peroral; s.c., subcutaneous.

10.3389/fbioe.2023.1238917

TABLE 3 Overview of safety aspects of selected plant host species used in molecular farming.

Plant species	Trivial name	Frequent cultivation strategy	Frequent expression strategy	Examples of harmful agents		
				Metabolites	Proteins[a]	Pathogen-related
Daucus carota	Carrot	Suspension culture	Transgenic	Carotatoxin Crosby and Aharonson (1967)	*Dau c 1* Hendrich et al. (2023)	n.f
Hordeum vulgare	Barley	Intact plants	Transgenic	Hordenine Liu and Lovett (1993)	*Protease inhibitors, 15-kDa* Mena et al. (1992); Wróblewska et al. (2022)	Mycotoxins Drakopoulos et al. (2021)
Lactuca sativa	Lettuce	Intact plants	Transgenic	n.f	*EP1-like protein* Sekiya et al. (2020), *thaumatin-like protein* Muñoz-García et al. (2013), *aspartyl protease* Muñoz-García et al. (2013), *sesquiterpene lactones*, e.g., lactucin Paulsen and Andersen (2016), *Lac s 1* Hartz et al. (2007)	Mycotoxins, e.g., tentoxin and tenuazonic acid Kłapeć et al. (2021); Miranda-Apodaca et al. (2023)
Nicotiana benthamiana	Australian tobacco	Intact plants	Transient	Alkaloids, e.g., nicotine Hayashi et al. (2020)	No specific reports, but probably similar to tobacco	Sphinganine-analog mycotoxins Rivas-San Vicente et al. (2013)
Nicotiana tabacum	Tobacco	Intact plants; suspension culture	Transgenic	Alkaloids, e.g., nicotine Hayashi et al. (2020)	Allergies reported but allergen unknown, probably pollen-related Ortega et al. (1999); Bonamonte et al. (2016)	Mycotoxins el-Maghraby and Abdel-Sater (1993)
Oryza sativa	Rice	Intact plants; suspension culture	Transgenic	n.f	*Glyoxalase I* Usui et al. (2001), *Ory s1* Sharma et al. (2009)	Diverse, e.g., aflatoxin B1 Rofiat et al. (2015) and zearalenone Joo et al. (2019)
Zea mays	Maize	Intact plants	Transgenic	n.f	*Lipid transfer protein* Pastorello et al. (2000)	Fumonisins Duvick (2001)

[a]allergens are in italics; n.f., none found.

complex group of molecules has probably emerged in defense against herbivores, and their toxicity stems from the release of hydrogen cyanide upon contact with specific β-glucosidases. Depending on the plant species and tissue, cyanogenic glycosides may be present at concentrations up to ~8 g kg^{-1} dry plant matter. For example, the concentration in young *Eucalyptus cladocalyx* leaves is twice that of old leaves (Gleadow and Woodrow, 2000). Similar concentrations are found in bamboo (*Bambusa vulgaris*, 1–8 g kg^{-1}) (Nyirenda et al., 2021). Some plant compounds are even more toxic, including saponins and glycoalkaloids (Wink, 2009; Napagoda et al., 2022; Rasool et al., 2022).

It is therefore necessary to remove such metabolites during product purification, depleting them not only below the level of toxicity but below the minimum effect level, which may be unknown or difficult to determine. Furthermore, the specific compound in a plant extract that triggers a given biological reaction (such as the impairment of immune responses) may not yet be known (Harwanto et al., 2022; Urbański et al., 2023). Establishing and updating systematic databases of plant-derived toxins (Günthardt et al., 2018) can help to ascertain the risks associated with certain plant hosts in a rational manner. The corresponding quantitative assays are also necessary for the successful, targeted and rational development of safe processes.

One practical example of metabolite removal is the production of monoclonal antibodies in tobacco for human clinical testing. Nicotine was depleted below the limit of detection by applying a simple two-stage purification process consisting of capture chromatography using protein A resin and a polishing step using ceramic hydroxyapatite (Ma et al., 2015). This was possible primarily because the size (or mass) of the monoclonal antibody product and nicotine (i.e., three orders of magnitude) as well as their surface properties differ substantially (e.g., in terms of charge and hydrophobicity). Similar results have been reported by others (Fu et al., 2010). Efficient separation can be more challenging if the product is also a small molecule, especially if the physicochemical properties of the product and impurities are similar (e.g., in terms of solubility). Specifically, this would rule out the use of porous membrane-based unit operations such as ultrafiltration/diafiltration, which can remove small-molecule impurities during buffer exchange operations when purifying larger proteins (Opdensteinen et al., 2018). It is therefore useful to select host plants in which there are no known toxic metabolites or where such metabolites are easy to separate from the product (Table 3). Accordingly, several food plants or cell cultures derived from them have been used for the production of safe biopharmaceuticals, including carrot (*Daucus carota*), lettuce (*Lactuca sativa*), maize (*Zea mays*), barley (*Hordeum vulgare*) and rice (*Oryza sativa*) (Xu et al., 2011; Grabowski et al., 2014; Mirzaee et al., 2022; Ganesan et al., 2023). But even food crops can contain low concentrations of toxic alkaloids that need to be

removed during processing, such as lupinin from lupin (*Lupinus mutabilis*) (Kaiser et al., 2020; Griffiths et al., 2021). Conventional breeding and genetic modification can be used to deplete or even fully remove such metabolites, as discussed in more detail later (see Section 5). Overall, the risk posed by plant-derived small-molecule impurities is low if the product is a recombinant protein because purification schemes typically include size-based fractionation steps to remove protein aggregates and degradation products, and these steps also ensure the removal of alkaloids and other bioactive metabolites.

2.1.2 Plant host cell proteins

Plants not only contain toxic metabolites but also some harmful proteins. The most toxic proteins are ribosome-inactivating toxins like ricin or viscumin but the plants that produce such toxins are not used as PMF hosts (Olsnes et al., 1982; Worbs et al., 2011). However, other lectins such as rice bran agglutinin (UniProt ID Q0JF21; ~22 kDa) or pea (*Pisum sativum*) lectin (UniProt ID P02867; ~30 kDa) are present in PMF food crops (Miyoshi et al., 2001; Kabir et al., 2013). These proteins can arrest the cell cycle, inhibit proliferation or trigger apoptosis in animals and therefore confer a relevant safety risk that should be monitored (Jiang et al., 2015). Due to their size, they may co-purify with products such as cyanovirin-N (~11 kDa) (Opdensteinen et al., 2018), but should be easy to separate from large proteins like antibodies (~150 kDa) (Ma et al., 2015). However, the carbohydrate-binding activity of many lectins causes them to bind glycosylated target proteins, which can result in co-purification. Similar nonspecific interactions have been reported between Chinese hamster ovary (CHO) host cell proteins (HCPs) and monoclonal antibodies (Li, 2022). Conditions that suppress such interactions should be identified during downstream process development.

The presence of glutens is another protein-based risk, which is particularly relevant when using cereal crops as PMF hosts (Ito, 2015; Abedi and Pourmohammadi, 2020). Glutens are diverse proteins that can be classified as glutenins or gliadins (also known as Osborne fractions) (Osborne, 1907; Biesiekierski, 2017). These proteins are not toxic *per se*, but they are present at much higher concentrations than most toxic proteins and are potent allergens. In wheat (*Triticum aestivum*), 40–90 g of gluten is present per kilogram of wheat flour (Pronin et al., 2020). Glutens can trigger immune responses at concentrations of ~12 mg kg^{-1} body mass in humans (Lähdeaho et al., 2011; Cabanillas, 2020; Taraghikhah et al., 2020). Whereas some tolerance may be built up in celiac disease patients (Elli et al., 2020), a safety threshold of 10–100 mg per person per day has been proposed (Hischenhuber et al., 2006; Cohen et al., 2019). Importantly, glutens are soluble in water and are stored in the seeds, where recombinant proteins tend to be targeted in cereals because this enhances product stability (Tosi et al., 2011; Arcalis et al., 2014). The concentration of these allergens in primary seed extracts is therefore high. Glutens are thermostable (Biesiekierski, 2017) and range in molecular mass from ~30 to >100 kDa (Tosi et al., 2011), so they can be difficult to separate from target proteins by blanching/heating (Buyel et al., 2016) or ultrafiltration/diafiltration (Opdensteinen et al., 2018). Although the presence of gluten is challenging in terms of downstream process development, the overall safety impact is low. Specifically, glutens are easy to detect (Schubert-Ullrich et al., 2009) and pharmaceutical proteins must exceed 95% purity (Jin et al., 2018). In the unlikely event that a PMF product contains 5% gluten, and large doses of the product are required (e.g., 0.05 g anti-Ebola antibody per kilogram of body mass every 3 days (Davey et al., 2016)), a 70-kg patient would be exposed to an average of ~175 mg gluten per day, which would be about twice the safe threshold. Although it is unlikely that a single compound would account for all impurities in a product, this estimate underlines the importance of removing even compounds that may be regarded moderate safety risks, such as allergens. This applies especially in cases where high doses (up to several grams per person) of product are required, as might be the case in post-exposure prophylaxis, the treatment of acute disease (Taylor et al., 2021; Hwang et al., 2022), or cancer therapy (Hendrikx et al., 2017). It is also relevant for non-antibody products that cannot be captured by affinity chromatography, and non-pharmaceutical products such as food additives where any form of chromatography would too expensive.

Importantly, the number, abundance and activity of hazardous proteins can be reduced, in some cases to below the level of detection, through process development (see Section 3.4) and genetic engineering strategies (see Section 5). For example, the majority of plant host cell proteins can be removed by anion exchange chromatography (Buyel and Fischer, 2014a; Bernau et al., 2022).

2.2 Contamination by disease vectors and plant pathogen products

In addition to harmful molecules produced by plants, PMF hosts may also attract pests and pathogens that can directly harm humans or produce toxic proteins and metabolites, which is an active area of research in the context of food safety (Fletcher et al., 2013; Sobiczewski and Iakimova, 2022). For example, fungi that infect cereals produce (ergot) alkaloids and carcinogenic mycotoxins (Hulvová et al., 2013; Florea et al., 2017; Sweany et al., 2022), the latter including aflatoxin B1 which is toxic at micromolar concentrations (Bianco et al., 2012; Marchese et al., 2018). Similarly, prokaryotic blue green algae (cyanoprokaryota) such as *Lyngbya wollei* and eukaryotic green algae (chlorophyta) such as dinoflagellates (e.g., *Ostreopsis siamensis* and *Gambierdiscus toxicus*) can colonize human environments (Hofbauer, 2021) such as personal aquariums, irrigation/drainage gullies or flood tables (see Section 3.3) and the corresponding fertilizer reservoirs. Algae can spread through the air, and also proliferate in soil or on the stone wool blocks often used to support plant growth in PMF. The risk to biomanufacturing reflects the ability of algae to produce allergens and toxins such as ciguatoxin and maitotoxin (both from *G. toxicus*) that cause diarrhea and vomiting in humans (Friedman et al., 2017; Hofbauer, 2021) or even death (Ohizumi and Yasumoto, 1983). Specifically, the LD_{50} of ciguatoxin in mice is ~250 ng kg^{-1} when administered intraperitoneally (Lewis, 2000) and maitotoxin has a minimal lethal dose of ~170 ng kg^{-1} (Bagnis et al., 1980; Ohizumi and Yasumoto, 1983). Likewise, cyanoprokaryota produce saxitoxins such as *L. wollei* toxin-1, with a no observed adverse effect level (NOAEL) of ~500 ng kg^{-1} body mass following peroral uptake in humans (Weirich and Miller, 2014). For intravenous pharmaceuticals, the NOAEL is likely to be lower.

Some fungal (*Alternaria infectoria*) and bacterial (*Erwinia persinicus*) pathogens of plants may cause opportunistic infections in humans. For example, *Rhizobium radiobacter*, which is widely used for transient expression in PMF applications, can cause bacteremia and keratitis as recently reviewed (Kim et al., 2020). However, the number of reported cases is extremely low (<50 in the available literature) despite the ubiquitous nature of the species in soil and artificial environments such as laboratories (Dessaux and Faure, 2018; Zhu et al., 2020). Furthermore, most of the patients suffering from a sporadic disease that seemed to be related to *R. radiobacter* infection were immunocompromised or the infection site was related to surgery or the eye (Kim et al., 2020), where the adaptive immune system is particularly weak (Akpek and Gottsch, 2003). Accordingly, the risk of infection with plant pathogens appears to be minimal for humans, especially given that pharmaceutical products undergo (several) sterile filtration steps or even more stringent size-based separation (e.g., ultrafiltration/diafiltration or size-exclusion chromatography) that will remove any intact cells (Holtz et al., 2015; Ma et al., 2015). Endotoxins present a more relevant, process-related risk specifically associated with *R. radiobacter* and transient expression (see Section 3.2).

In contrast to such bacteria, plant viruses do not infect or replicate in human cells and are therefore unlikely to cause diseases. Plant viruses can be found in association with humans but the link is thought to be indirect–for example, tobacco mosaic virus RNA was found in human saliva, but its presence was attributed to smoking rather than an infection (Balique et al., 2012). Similarly, tobacco DNA was detected in ventilator-associated pneumonia patients who were smokers (Bousbia et al., 2010).

Plants may also attract insects that can be vectors of human diseases. For example, volatiles (especially terpenoids) released from certain plant species can attract mosquitos such as *Anopheles gambiae* (Nyasembe et al., 2012; Nikbakhtzadeh et al., 2014), a key malaria vector. Furthermore, the plant species on which the mosquitos feed can also affect the viability of *Plasmodium falciparum* (Hien et al., 2016), one of the parasites that causes malaria. Specifically, mosquitos feeding on fruits of *Mangifera indica* instead of cuttings from *Thevetia neriifolia* or a glucose control were ~50% less likely to survive over 7 days and the mean number of developing oocysts in the guts of infected female mosquitos was reduced by ~60%. Certain plant species or cultivation conditions may also attract rodents that carry pathogens.

The risk attributed to plant pathogens, insects and other animals in the context of PMF is low. For example, PMF crops such as tobacco only produce low concentrations of terpenoids that are unlikely to attract mosquitoes (Lücker et al., 2004). In moderate climate zones, such disease vectors are in any case unlikely to be present in the vicinity of a manufacturing site. In general, PMF cultivation conditions do not support many of the pathogens discussed above, and the natural microbiome of plants can reduce the fitness of pathogens such as *P. falciparum* (Bassene et al., 2020). As discussed below, many facilities and process design options exist to minimize or even exclude risks associated with pests and pathogens, including UV lamps or ozone generators to inactivate algae and bacteria in irrigation systems or carried by personnel, a controlled environment, and traps (e.g., mouse traps and yellow sticky traps for insects) to protect the cultivation area from pathogens spread by animals.

3 Potential risks arising from bioprocess design

The decisions made during process design can greatly affect product safety. Certain process steps are directly intended to focus on safety, including low-pH hold steps for virus inactivation (Mazzer et al., 2015), but other choices can have unintentional effects and should be avoided or mitigated.

3.1 Expression cassette elements

Oncogenes or parts thereof may be used as products or as building blocks for expression vectors, including regulatory elements to enhance product accumulation. As a product-related example, oncogenic protein E7 from human papillomaviruses binds to the retinoblastoma protein and is necessary to maintain the viability of papillomavirus-induced tumors, as found in the commonly-used HeLa cell line (Nishimura et al., 2006). However, the E7 protein has also been produced in plants (and many other host systems) as a vaccine candidate for the treatment of infections with human papillomavirus 16 (Venuti et al., 2009; Buyel et al., 2012). Accordingly, the vaccine product could potentially contain residual host cell DNA including sequences encoding the oncogenic recombinant protein. In the specific case of E7, the coding sequence had been mutated to render the protein non-tumorigenic and thus mitigate this risk (Smahel et al., 2001). However, such solutions require precise knowledge about the protein binding/interaction sites, which may not always be available.

The risks associated with such oncogenic DNA can also include regulatory sequences, and the likelihood that residual DNA could transform animal cells and ultimately trigger tumor development has been debated (Peden et al., 2006). Models have been built to assess the associated risk (Yang et al., 2010), which seems to be negligible based on evidence from multiple studies (Palladino et al., 1987; Dortant et al., 1997). Specifically, 1–10 g of residual host DNA was deemed necessary for tumor induction (Sheng et al., 2008), whereas the regulatory threshold for host cell genomic DNA in pharmaceutical products is ~0.1–1.0 ng per dose (Wang et al., 2012). Given that sensitive PCR-based detection methods are available and that DNA is a highly charged polymer that can be removed efficiently by anion exchange chromatography (Stone et al., 2018), the risk associated with residual DNA is small. If necessary, an enzymatic treatment step can reduce the residual DNA burden further and thus improve product safety (Kawka et al., 2021).

3.2 Expression strategy and endotoxins

Endotoxins are a well-known risk factor in biomanufacturing because they are strong activators and modulators of the human

immune system, leading to septic shock (Opal, 2010). Such toxins are abundant in processes where Gram-negative bacteria are used as hosts (Petsch and Anspach, 2000), but they are also relevant in PMF due to the deliberate use of bacteria for gene transfer and also the presence of adventitious bacteria on or within plant tissues. In the scalable transgenic system (Buyel et al., 2017), the endotoxin load is typically low because the bacteria used for gene transfer are killed after the transgenes are stably integrated into the plant nuclear or plastid genome (Herrera-Estrella et al., 2005). Therefore, the only Gram-negative bacteria present will be those naturally occurring on the plant surface, such as *Pseudomonas* spp. (Compant et al., 2019). In contrast, transient expression (Tusé et al., 2020) requires that plants are infiltrated with the Gram-negative bacterium *R. radiobacter* (Spiegel et al., 2019), and the stress involved in this process also stimulates endotoxin production as well as secondary metabolite synthesis in plants (Buyel et al., 2015b). Accordingly, the concentration of endotoxins can increase 200-fold to ~10^4 EU per milligram of total protein (Arfi et al., 2016), which is ~3×10^4 EU mL^{-1}. This is in the same range as *Escherichia coli* lysate (10^3–10^5 EU mL^{-1}) (Szermer-Olearnik and Boratyński, 2015), and is above the regulatory threshold of 5 EU kg^{-1} body mass h^{-1} (Hirayama and Sakata, 2002), even assuming microgram doses of protein (Cummings et al., 2014). Accordingly, endotoxins have to be removed, particularly when the product is manufactured by transient expression. Dedicated methods such as phase separation (Aida and Pabst, 1990), affinity capture (Anspach, 2001), chromatography (Serdakowski London et al., 2012) and ultrafiltration/diafiltration (Jang et al., 2009) can be used for this purpose, as demonstrated for a range of plant extracts (Arfi et al., 2016). However, many of these steps are typically included in common downstream processing schemes (Ma et al., 2015; Opdensteinen et al., 2018; Knödler et al., 2023b), so additional effort is not usually required for endotoxin removal, as long as the process is monitored carefully.

A hybrid approach is the use of inducible transgene expression (Mortimer et al., 2015; Hahn-Löbmann et al., 2019). This strategy has the same development times as the transgenic approach but facilitates time-bound product expression, thereby minimizing toxic effects of the latter on plant development and growth. In terms of product safety, it is similar to transgenic plants (low endotoxin levels and absence of *R. radiobacter*) and the induction agent should be selected to ensure efficiency (active at low concentrations), easy removal (e.g., ideally a small molecule such as ethanol) and lack of toxicity.

3.3 Cultivation conditions

Importantly, regardless of the expression strategy and cultivation conditions, PMF products can be manufactured without animal-derived components because defined or vegan fertilizer/media can be used for the cultivation of plants, plant cells and *R. radiobacter* (Houdelet et al., 2017; Leth and McDonald, 2017; Geng et al., 2019; Kang et al., 2022). Therefore, contamination with pathogens and harmful agents such as prions in substrates can be ruled out, which increases the safety of PMF products.

3.3.1 Plant cell suspension cultures

Bioreactors suitable for conventional microbial and mammalian cell cultures can also be used for plant cells [and even plant tissues and intact plants (Murthy et al., 2023)] with little or no modification (Holland et al., 2013). These reactors provide a high degree of process containment and minimize or even eliminate some of the risks discussed above (Huang and McDonald, 2012). For example, bacteria that colonize plant surfaces will not be found in a bioreactor. Nevertheless, care must be taken when inoculating and harvesting the reactors, especially when large volumes (i.e., several liters) are handled during the late stages of a typical reactor seed train, because sterility can be difficult to ensure, as is well known for other bioprocesses (Müller et al., 2022). The contamination risk can be reduced if, for example, orbitally shaken (Raven et al., 2014) or airlift/bubble reactors (Wilson and Roberts, 2012) are used because these contain fewer moving parts, grommets and fittings than stirred-tank reactors (Werner et al., 2018). Similarly, single-use reactors can reduce cross-product contamination risks (Raven et al., 2011). The use of photobioreactors also enhances safety because the autotrophic cultivation of plant cells in such reactors does not require organic carbon sources in the culture medium (Legrand et al., 2021), effectively depleting it of a substrate necessary for the growth many contaminating bacteria, yeast and fungi (although phototrophic bacteria and algae remain a contamination risk). The cultivation of plant cells in photobioreactors also requires the accumulation of chlorophylls and other pigments, and these compounds may unintentionally interact with product molecules (see Section 3.4.2). Another drawback of photobioreactors is that they typically use non-standard designs, such as tubular geometry, to ensure sufficient illumination (Chanquia et al., 2022).

Plant cells in suspension often have a tendency to adhere to even stainless-steel surfaces in a bioreactor (Holland et al., 2017). This not only limits the bioprocess operation time but may also interfere with cellular metabolism by limiting the oxygen and/or nutrient supply in the resulting cell clusters. These suboptimal conditions can lead to cell stress, autophagy (e.g., of peroxisomes) and cell death (Voitsekhovskaja et al., 2014; Tyutereva et al., 2018; Ma et al., 2022), which may cause (partial) product degradation or modification, ultimately increasing product heterogeneity and reducing activity. Also, if cells begin to decompose in these surface aggregates and then re-enter the bulk fermentation broth, the molecules they release may trigger unwanted signaling cascades in the living cells, reducing overall productivity (Salguero-Linares and Coll, 2023). Therefore, production cell line development and cultivation protocols should focus on low adhesion and low aggregation properties as well as monitoring strategies to ensure that product quality is not compromised.

3.3.2 Cultivation of intact plants

Plant cultivation in the open field is currently suitable only for small-molecule pharmaceutical products like morphine, which is extracted from opium poppy straw (Krikorian and Ledbetter, 1975). These molecules have a simple structure, a well-defined conformation, and are typically isolated using organic solvents that have the added value of acting as disinfectants (Kuyukina et al., 2014). Therefore, product quality control is straightforward

(e.g., LC-MS analysis) and any contaminants are effectively removed by the harsh extraction conditions.

In contrast, to date protein-based pharmaceuticals have been produced in plants grown indoors. This avoids any unpredictable effects of the variable external environment and ensures compliance with GMP requirements. Specifically, recombinant protein extraction typically relies on aqueous buffers (Buyel et al., 2015a) that do not inactivate pathogens introduced by pest insects and rodents (see Section 2.2). Additional factors that bar open field cultivation are heavy metal ions, pesticides and anthropogenic toxic pollutants that can contaminate soils (Sigmund et al., 2022; D'Angelo et al., 2013) and plant tissues, and potentially the final product (Zeng et al., 2019; Zhang et al., 2020).

Such risks can be averted if plants are cultivated in greenhouses, as reported for several GMP-compliant processes for the production of monoclonal antibodies and vaccines (Ma et al., 2015; Ward et al., 2021). Also, well-defined growth supports such as stone wool blocks can be used in this setting and can be combined with automated hydroponic irrigation systems. The closed environment facilitates effective pest control and allows strictly regulated personnel access, which minimizes the risk of contamination with pathogens. However, process control is still limited in such a setting. For example, yields of the same antibody product can fluctuate between 2 and 6 g per 200-kg batch of plants due to seasonal effects and variable weather (Sack et al., 2015). More importantly, product integrity can be compromised by protease activity, for example, under conditions of intense light or high temperature that cannot be mitigated by climate control (Knödler et al., 2019). Even if climate control maintains cultivation conditions within specifications, leaves can become hotter than the surrounding environment due to intensive insolation (Huebbers and Buyel, 2021). Intense light can also trigger the synthesis of potentially harmful metabolites (Buyel et al., 2015b; Thoma et al., 2020) (see Section 2.1) that need to be removed during downstream processing. Because greenhouses are typically non-sterile environments, it is likely that algae will start to grow on surfaces and in the fertilizer solution, especially if the tanks and gullies/flood tables are not properly covered. As discussed above, these prokaryotic and eukaryotic algae can be harmful or may secrete toxic compounds (see Section 2.2). Therefore, in-line UV light or ozone generators should be installed to reduce the impact of algae (Sharrer and Summerfelt, 2007).

Closed cultivation facilities achieve an even higher degree of process control than greenhouses. These facilities are designed to eliminate any environmental impact on plant growth by providing a complete artificial climate: temperature, humidity and irrigation as well as light and potentially gas composition (Farhangi et al., 2023). The terminology used for closed cultivation facilities can be misleading and ambiguous. For example, they are often called "vertical farms" because multiple vertically-stacked cultivation layers can improve cost-efficiency, but single-layer designs can be used as well (Huebbers and Buyel, 2021). The alternative term "indoor farm" or "indoor agriculture" is also imprecise because this could be extended to include greenhouses. Therefore, a more precise term may be (fully) "controlled environment growth systems" (CEGS).

Regardless of terminology, digital integration ensures control over individual parameters such as fertilizer composition and light (Huebbers and Buyel, 2021; Kaur et al., 2023) but requires sensors or even sensor networks that account for the discrete characters of individual plants (Huebbers and Buyel, 2021). For example, the metabolite and lipid composition of plants can be modulated by selecting specific light wavelengths for illumination (Rihan et al., 2022), and can thus help to reduce the concentration of potentially harmful metabolites like alkaloids (see Section 2.1.1). Furthermore, CEGS can be fully automated so that human intervention and ultimately the risk of contamination with human pathogens is minimized (Wirz et al., 2012; Huebbers and Buyel, 2021; Ren et al., 2023). The high degree of automation/mechanization in such systems, and the close proximity of the corresponding devices and plants, increases the likelihood that the product will come into contact with auxiliary and operating materials such as lubricants. Therefore, all devices should be designed to minimize such risks. This includes the selection of appropriate building materials, including steels compatible with food or pharmaceutical applications and plastics devoid of leachables (Jenke, 2002; Cuadros-Rodríguez et al., 2020; Zimmermann et al., 2021).

CEGS are overall the safest environment in which to produce biopharmaceutical proteins by PMF. The technology is scalable (e.g., several hundred kg of biomass can be processed per week (Holtz et al., 2015)), but the high investment and energy costs remain a significant bottleneck (Huebbers and Buyel, 2021; van Delden et al., 2021). This is especially relevant if the PMF products are not intended for pharmaceutical use, where the cost pressure on manufacturing is greater and low-investment infrastructure may be the only option to build economically viable processes.

3.4 Product extraction and purification

Although product purification is a key GMP requirement in PMF, at least for products that will be injected, purification also introduces some risks that must be mitigated during process design. A typical downstream processing sequence in PMF starts with harvesting and optional conditioning (e.g., washing (Ma et al., 2015) or blanching (Buyel et al., 2014a); Figure 1B). This is followed by initial extraction, which may involve further conditioning steps such as pH adjustment or flocculation (Buyel and Fischer, 2014b; Buyel and Fischer, 2014c; Buyel and Fischer, 2014d). The next major operation is clarification, which typically involves multiple filtration steps (Buyel and Fischer, 2014e), leading to product purification by two-phase extraction (Platis et al., 2008), membrane separation (Opdensteinen et al., 2018), chromatography (Buyel et al., 2012), or combinations thereof. The overall risk is that the sequence of downstream unit operations does not achieve the necessary purity due to the insufficient removal of process-related and/or product-related impurities, but each downstream operation poses specific risks to product safety that should be monitored and minimized during process development.

3.4.1 Harvesting and conditioning

Manual harvesting processes carry an inherent safety risk because human operators come into close contact with the plant biomass containing the pharmaceutical product and may transfer pathogens. Therefore, personal protective equipment (in this case from the perspective of protecting the harvested biomass and product) should be worn at all times, including coats, gloves, hair nets and masks. In addition, the health of the operators should be monitored and staff should be encouraged to report any signs of illness to allow replacement and/or rescheduling. Although these are

common routines for GMP-compliant processes based on cell cultures, they are also important in the context of intact plants because International Conference on Harmonisation (ICH) guidelines such as Q7[1] (Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients) apply only to steps after plant harvesting and initial extraction. Even though plants do not support the replication of human viruses as discussed above, the harvesting of intact plants and parts such as leaves should ideally be fully automated as implemented in several CEGS, specifically indoor vertical farming (Wirz et al., 2012), because this minimizes operator-based contamination risks.

In some processes, the harvested biomass undergoes a thermal pretreatment described as blanching, in which the plant biomass is submerged in a hot (50°C–90°C) (Buyel et al., 2014a), potentially slightly acidic buffer (Opdensteinen et al., 2020), which will remove 50%–95% of tobacco host cell proteins (Buyel et al., 2016). This is an asset in terms of product safety but risks include partial or complete irreversible product denaturation accompanied by altered activity. An ill-designed blanching step may even increase protease activity and product degradation (Menzel et al., 2016; Menzel et al., 2018). Such conditioning steps should therefore be designed and implemented carefully, including the cross-checking of product activity in suitable assays.

3.4.2 Extraction and further conditioning

Manual harvesting is usually accompanied by the manual transfer of biomass to the extraction device, so the protective measures discussed above should be maintained for this subsequent step. Biopharmaceuticals are usually extracted from plants and plant cells by homogenizing the biomass in the presence of a buffer (Buyel et al., 2015a). The latter controls pH and redox conditions to stabilize the product and to prevent unwanted modification by oxidation or reaction with plant-derived pigments and phenolic compounds. Oxidation and other unwanted reactions can also be suppressed by extraction in a nitrogen atmosphere (Ma et al., 2015). Protease inhibitors can be added during small-scale extraction (Menzel et al., 2016) or co-expressed in the plant cells (which also increases product accumulation (Jutras et al., 2016; Grosse-Holz et al., 2018)), but product integrity can be maintained simply by ensuring that all buffers are cooled to ~10°C (Ma et al., 2015). Buffer cooling is beneficial because nothing is added to the process, but additional equipment will be required for this step. In contrast, any inhibitors are additional contaminants that need to be removed later. The same holds true for extraction techniques that do not require buffers at all, such as the use of a screw press (Buyel and Fischer, 2014d). However, the conditions in the resulting green juice can negatively affect the product and its activity, for example, due to the low pH (e.g., ~5.5 in case of tobacco). As for blanching, the implementation of such methods should therefore be accompanied by a careful assessment of the impact on product stability and activity.

An extract can then be conditioned to facilitate subsequent clarification and purification by pH adjustment as well as the addition of flocculants and/or filter aids. The latter are often large, inert cellulose fibers that are easily removed during subsequent purification steps (Buyel et al., 2014b), whereas flocculants are highly charged polymers (Buyel and Fischer, 2014c) that can bind to proteins (Jurjevec et al., 2023) and escape detection. The highest purity grades should therefore be used with pharmaceutical products to ensure product safety. Although non-pharmaceutical products like food additives also need to comply with good manufacturing practices (Manning, 2018), the purity and safety requirements are usually less stringent, and this should be taken into account when selecting the reagents in order to align raw material quality and safety requirements.

3.4.3 Clarification and purification

Clarification (mostly filtration) and purification steps typically remove particles, including viruses, as well as soluble host cell components such as proteins. Therefore, both operations inherently increase the safety of biopharmaceuticals. Nevertheless, processes based on bacteria or animal cell hosts have revealed that both steps also introduce risks in terms of product safety. For example, equipment can release leachables and extractables and should be selected to minimize these risks, taking into account the properties of plant extracts such as the presence of phenolic compounds. Specifically, protein A, the common affinity ligand for antibody capture, can be found as a process-related impurity in antibody elution fractions (Carter-Franklin et al., 2007) and exposure to phenolics can result in the permanent discoloration of chromatography resins.

Another major risk to product safety is presented by the hold times that are required during extract processing. For example, even many continuous processes use intermediate storage tanks that allow time buffering between individual downstream steps (such as two in-series filters) to compensate for fluctuations in volumetric fluxes. The flow regime in such tanks is far from an ideal plug flow and will create a broad residence time distribution (Sencar et al., 2020; Lali et al., 2022). Therefore, some of the product will be held substantially longer in the process than might be expected based on the average residence time. This is critical during the early purification stages, when host cell proteases or oxidases are still abundant and can act on and modify the product, potentially compromising its activity and safety. Cooling the process intermediates can reduce such unwanted enzyme activities but this requires additional equipment. A fully continuous process without hold tanks requires sophisticated process control, and this is vulnerable to errors that create new product safety risks. Comprehensive risk management is therefore necessary during process development (Sparrow et al., 2013; Zalai et al., 2013; Qiu et al., 2015; Luo et al., 2021).

4 Safety-relevant product properties

The target product profile of biopharmaceuticals produced by PMF is based on the same aspects stipulated for other bioprocesses, such as efficacy and safety[2]. Both depend on the molecular properties of the product, such as sequence integrity, folding, and

1 https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-7-good-manufacturing-practice-active-pharmaceutical-ingredients-step-5_en.pdf

2 https://www.who.int/observatories/global-observatory-on-health-research-and-development/analyses-and-syntheses/target-product-profile/links-to-who-tpps-and-ppcs

PTMs such as disulfide bonds, phosphorylation and glycosylation. The latter can affect folding, but in the context of product safety the major concern is immunogenicity because plant *N*-linked glycans differ from those found in mammals in several fundamental ways, including the presence of xylose residues (not found in mammals) and the linkage of fucose via an α3 glycosidic bond (an α6 bond is found in mammals) (Strasser, 2016). Furthermore, *O*-linked glycans in plants are mostly found on hydroxyproline residues whereas serine and threonine are the preferential targets in mammals. These non-native glycans can trigger immune responses when recombinant human proteins produced by PMF are injected (Bardor et al., 2003; Jin et al., 2008). However, there is no evidence that the elicitation of anti-glycan antibodies is harmful (Shaaltiel and Tekoah, 2016; Rup et al., 2017). The situation may be different in patients with a history of allergy (Schwestka et al., 2021), who may be especially sensitive to plant-derived glycan structures. Similarly, persons with allergies against egg proteins, including glycoproteins such as ovotransferrin and ovomucoid (Hwang et al., 2014), may exhibit mild but unwanted allergy-like side effects (not anaphylaxis) when receiving influenza vaccines produced in eggs (James et al., 1998; Gruenberg and Shaker, 2011). To address this, various plant (cell) lines have been developed that lack plant-specific glycosyltransferases (Strasser et al., 2004; Jansing et al., 2018) and in some cases also incorporate human enzymes to make the glycans not only human-compatible but fully humanized (Castilho et al., 2013; Montero-Morales and Steinkellner, 2018). The corresponding PMF products may achieve greater activity, as reported for at least one vaccine candidate (Pantazica et al., 2023).

5 Breeding and genetic engineering targets to address safety aspects

Breeding and genetic engineering can be used to reduce several of the safety risks discussed above, and not only by the modification of glycans. Specifically, plant proteases can be inactivated to enhance product integrity (e.g., to minimize degradation and aggregation) or they can be expressed in a targeted manner to ensure precise processing, such as the removal of leader sequences, as demonstrated for transforming growth factor β1 (Goulet et al., 2012; Wilbers et al., 2016). Similarly, enzyme cascades synthesizing toxic compounds can be interrupted, as demonstrated by the creation of nicotine-free tobacco (Schachtsiek and Stehle, 2019). One can also learn from other host systems and knock out host cell proteins that are difficult to remove during downstream processing (Chiu et al., 2017), including but not limited to those that are toxic or allergenic as discussed above. These and other options such as the use of chaperones to promote correct protein folding or modifications to prevent oxidation, all of which improve the performance of host species in terms of product yield, activity and safety, have been reviewed in detail elsewhere (Buyel et al., 2021; Singh et al., 2021). The CRISPR/Cas9 system and its regulatory implications have been thoroughly assessed in the context of PMF (Bortesi and Fischer, 2015; Eckerstorfer et al., 2019; Fiaz et al., 2021).

Importantly, such safety-improving genetic engineering steps must be balanced against, for example, the viability and productivity of the resulting plant (cell) line. For example, it is desirable to knock out proteases as discussed above because they can trigger the (partial) degradation of a PMF product, thus reducing its activity (Donini et al., 2015; Mandal et al., 2016; Menzel et al., 2018). However, proteases fulfil essential biological functions and may be required for germination and plant growth (Martinez et al., 2019; van der Hoorn and Klemenčič, 2021), which are important to achieve a high product yield. Therefore, process engineering rather than genetic engineering may be more suitable in some instances.

Furthermore, single knockouts may not be sufficient due to redundancies in metabolic pathways. For example, the morphine biosynthesis pathway branches when it reaches the intermediate thebaine, which may be converted to morphine via codeinone or morphinone (Ziegler et al., 2009; Onoyovwe et al., 2013). Therefore, at least one enzyme in each branch must be knocked out to block morphine synthesis completely. In this context, inactivating a certain enzyme cascade may result in a re-direction of the metabolic flux to other metabolites that can be toxic too.

6 Conclusion and outlook

Plants, like all other biological hosts, present certain product safety risks due to their natural components (e.g., toxic metabolites), cultivation conditions, and differences in PTMs. It is important to monitor these risks when operating GMP-compliant manufacturing processes and to implement a suitable risk management strategy. Such a strategy should focus on identifying and prioritizing risks based on the specifics of a given process, e.g., protein (Bracewell et al., 2015) or low-molecular-mass impurities (Luo et al., 2021). Because prioritization will depend on product properties and the characteristics of the manufacturing process, it is not useful to provide general recommendations other than the established concepts and heuristics such as failure mode effects (and criticality) analysis (FME(C)A) or hazard analysis and critical control points (HACCP) as outlined in the ICH Q9 guidelines[3]. However, such a risk assessment could benefit greatly from structured and curated databases that aggregate, for example, information on phytotoxins (Günthardt et al., 2018), because knowledge and ultimately product safety will increase as more and more processes are developed. Also, general knowledge about such impurities and contaminants (i.e., excluding specific process steps or conditions) should have a pre-competitive character and should thus be disclosable by the companies involved. Financing the curation and maintenance of such a database is more likely to be a bottleneck.

When looking at the individual safety aspects discussed in Sections 2–5, none of the risks is grave enough to prevent the use of plants for PMF applications. Indeed, such risks are easily mitigated by implementing established risk management and process design principles. Overall, plants can be regarded as safe

3 https://www.ema.europa.eu/en/documents/scientific-guideline/international-conference-harmonisation-technical-requirements-registration-pharmaceuticals-human-use_en-3.pdf

host systems for PMF, and the selection of food or feed crops can exclude many of the risks associated with hosts that produce intrinsic toxic components.

Author contributions

JB planned the manuscript, analyzed the literature, wrote and revised the text and secured the funding.

Acknowledgments

I wish to thank Dr. Richard M. Twyman for editorial assistance.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Abedi, E., and Pourmohammadi, K. (2020). The effect of redox agents on conformation and structure characterization of gluten protein: An extensive review. *Food Sci. Nutr.* 8 (12), 6301–6319. [PMID: 33312518]. doi:10.1002/fsn3.1937

Acamovic, T., and Brooker, J. D. (2005). Biochemistry of plant secondary metabolites and their effects in animals. *Proc. Nutr. Soc.* 64 (3), 403–412. [PMID: 16048675]. doi:10.1079/PNS2005449

Aida, Y., and Pabst, M. J. (1990). Removal of endotoxin from protein solutions by phase-separation using Triton X-114. *J. Immunol. Methods* 132 (2), 191–195. doi:10.1016/0022-1759(90)90029-U

Akpek, E. K., and Gottsch, J. D. (2003). Immune defense at the ocular surface. *Eye (Lond)* 17 (8), 949–956. [PMID: 14631402]. doi:10.1038/sj.eye.6700617

Manning, L. (2018). *Food & drink - good manufacturing practice: A guide to its responsible management*. Seventh edition. Oxford, London: Wiley; Institute of Food Science and Technology.

Anspach, F. B. (2001). Endotoxin removal by affinity sorbents. *J. Biochem. Biophysical Methods* 49 (1-3), 665–681. [PMID: 11694310]. doi:10.1016/S0165-022X(01)00228-7

Arcalis, E., Ibl, V., Peters, J., Melnik, S., and Stoger, E. (2014). The dynamic behavior of storage organelles in developing cereal seeds and its impact on the production of recombinant proteins. *Front. plant Sci.* 5 (439), 439–512. [PMID: 25232360]. doi:10.3389/fpls.2014.00439

Arfi, Z. A., Hellwig, S., Drossard, J., Fischer, R., and Buyel, J. F. (2016). Polyclonal antibodies for specific detection of tobacco host cell proteins can be efficiently generated following RuBisCO depletion and the removal of endotoxins. *Biotechnol. J.* 11 (4), 507–518. doi:10.1002/biot.201500271

Argentine, M. D., Owens, P. K., and Olsen, B. A. (2007). Strategies for the investigation and control of process-related impurities in drug substances. *Adv. Drug Deliv. Rev.* 59 (1), 12–28. [PMID: 17189658]. doi:10.1016/j.addr.2006.10.005

Bagnis, R., Chanteau, S., Chungue, E., Hurtel, J. M., Yasumoto, T., and Inoue, A. (1980). Origins of ciguatera fish poisoning: A new dinoflagellate, Gambierdiscus toxicus adachi and fukuyo, definitively involved as a causal agent. *Toxicon* 18 (2), 199–208. [PMID: 7190330]. doi:10.1016/0041-0101(80)90074-4

Balique, F., Colson, P., and Raoult, D. (2012). Tobacco mosaic virus in cigarettes and saliva of smokers. *J. Clin. Virol.* 55 (4), 374–376. [PMID: 22959216]. doi:10.1016/j.jcv.2012.08.012

Bardor, M., Faveeuw, C., and Fitchette, A. C. (2003). Immunoreactivity in mammals of two typical plant glyco-epitopes, core alpha(1,3)-fucose and core xylose. *Glycobiology* 13 (6), 427–434. doi:10.1093/glycob/cwg024

Barzee, T. J., El Mashad, H. M., Cao, L., Chio, A., Pan, Z., and Zhang, R. (2022). Cell-cultivated food production and processing: A review. *Food Bioeng.* 1 (1), 4–25. doi:10.1002/fbe2.12009

Bassene, H., Niang, E. H. A., Fenollar, F., Doucoure, S., Faye, O., Raoult, D., et al. (2020). Role of plants in the transmission of Asaia sp., which potentially inhibit the Plasmodium sporogenic cycle in Anopheles mosquitoes. *Sci. Rep.* 10 (1), 7144. [PMID: 32346047]. doi:10.1038/s41598-020-64163-5

Bernau, C. R., Knödler, M., Emonts, J., Jäpel, R. C., and Buyel, J. F. (2022). The use of predictive models to develop chromatography-based purification processes. *Front. Bioeng. Biotechnol.* 10, 1009102. doi:10.3389/fbioe.2022.1009102

Bianco, G., Russo, R., Marzocco, S., Velotto, S., Autore, G., and Severino, L. (2012). Modulation of macrophage activity by aflatoxins B1 and B2 and their metabolites aflatoxins M1 and M2. *Toxicon* 59 (6), 644–650. [PMID: 22402176]. doi:10.1016/j.toxicon.2012.02.010

Biesiekierski, J. R. (2017). What is gluten? *J. Gastroenterol. Hepatol.* 32 (1), 78–81. [PMID: 28244676]. doi:10.1111/jgh.13703

Bonamonte, D., Vestita, M., Filoni, A., Mastrolonardo, M., Angelini, G., and Foti, C. (2016). Tobacco-induced contact dermatitis. *Eur. J. Dermatol* 26 (3), 223–231. [PMID: 27020490]. doi:10.1684/ejd.2016.2771

Bortesi, L., and Fischer, R. (2015). The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnol. Adv.* 33 (1), 41–52. [PMID: 25536441]. doi:10.1016/j.biotechadv.2014.12.006

Bousbia, S., Papazian, L., La Scola, B., and Raoult, D. (2010). Detection of plant DNA in the bronchoalveolar lavage of patients with ventilator-associated pneumonia. *PLoS ONE* 5 (6), e11298. [PMID: 20585574]. doi:10.1371/journal.pone.0011298

Bracewell, D. G., Francis, R., and Smales, C. M. (2015). The future of host cell protein (HCP) identification during process development and manufacturing linked to a risk-based management for their control. *Biotechnol. Bioeng.* 112 (9), 1727–1737. doi:10.1002/bit.25628

Buyel, J. F., Bautista, J. A., Fischer, R., and Yusibov, V. M. (2012). Extraction, purification and characterization of the plant-produced HPV16 subunit vaccine candidate E7 GGG. *J. Chromatogr. B* 880 (1), 19–26. [PMID: 22134037]. doi:10.1016/j.jchromb.2011.11.010

Buyel, J. F., Buyel, J. J., Haase, C., and Fischer, R. (2015b). The impact of *Pseudomonas syringae* type III effectors on transient protein expression in tobacco. *Plant Biol. (Stuttg)* 17 (2), 484–492. [PMID: 25243954]. doi:10.1111/plb.12264

Buyel, J. F., and Fischer, R. (2014d). A juice extractor can simplify the downstream processing of plant-derived biopharmaceutical proteins compared to blade-based homogenizers. *Process Biochem.* 50 (5), 859–866. doi:10.1016/j.procbio.2015.02.017

Buyel, J. F., and Fischer, R. (2014c). Flocculation increases the efficacy of depth filtration during the downstream processing of recombinant pharmaceutical proteins produced in tobacco. *Plant Biotechnol. J.* 12 (2), 240–252. [PMID: 24165151]. doi:10.1111/pbi.12132

Buyel, J. F., and Fischer, R. (2014a). Generic chromatography-based purification strategies accelerate the development of downstream processes for biopharmaceutical proteins produced in plants. *Biotechnol. J.* 9 (4), 566–577. [PMID: 24478119]. doi:10.1002/biot.201300548

Buyel, J. F., and Fischer, R. (2014e). Scale-down models to optimize a filter train for the downstream purification of recombinant pharmaceutical proteins produced in tobacco leaves. *Biotechnol. J.* 9 (3), 415–425. [PMID: 24323869]. doi:10.1002/biot.201300369

Buyel, J. F., and Fischer, R. (2014b). Synthetic polymers are more effective than natural flocculants for the clarification of tobacco leaf extracts. *J. Biotechnol.* 195, 37–42. doi:10.1016/j.jbiotec.2014.12.018

Buyel, J. F., Gruchow, H. M., Boes, A., and Fischer, R. (2014a). Rational design of a host cell protein heat precipitation step simplifies the subsequent purification of recombinant proteins from tobacco. *Biochem. Eng. J.* 88 (15), 162–170. doi:10.1016/j.bej.2014.04.015

Buyel, J. F., Hubbuch, J., and Fischer, R. (2016). Comparison of tobacco host cell protein removal methods by blanching intact plants or by heat treatment of extracts. *Jove-J Vis. Exp.* e54343 (114), 54343. doi:10.3791/54343

Buyel, J. F., Opdensteinen, P., and Fischer, R. (2014b). Cellulose-based filter aids increase the capacity of depth filters during the downstream processing of plant-derived biopharmaceutical proteins. *Biotechnol. J.* 10 (4), 584–591. doi:10.1002/biot.201400611

Buyel, J. F. (2018). Plants as sources of natural and recombinant anti-cancer agents. *Biotechnol. Adv.* 36 (2), 506–520. [PMID: 29408560]. doi:10.1016/j.biotechadv.2018.02.002

Buyel, J. F., Stöger, E., and Bortesi, L. (2021). Targeted genome editing of plants and plant cells for biomanufacturing. *Transgenic Res.* 30 (4), 401–426. [PMID: 33646510]. doi:10.1007/s11248-021-00236-z

Buyel, J. F., Twyman, R. M., and Fischer, R. (2015a). Extraction and downstream processing of plant-derived recombinant proteins. *Biotechnol. Adv.* 33 (6), 902–913. [PMID: 25922318]. doi:10.1016/j.biotechadv.2015.04.010

Buyel, J. F., Twyman, R. M., and Fischer, R. (2017). Very-large-scale production of antibodies in plants: The biologization of manufacturing. *Biotechnol. Adv.* 35 (4), 458–465. [PMID: 28347720]. doi:10.1016/j.biotechadv.2017.03.011

Cabanillas, B. (2020). Gluten-related disorders: Celiac disease, wheat allergy, and nonceliac gluten sensitivity. *Crit. Rev. Food Sci. Nutr.* 60 (15), 2606–2621. [PMID: 31402697]. doi:10.1080/10408398.2019.1651689

Carter-Franklin, J. N., Victa, C., McDonald, P., and Fahrner, R. (2007). Fragments of protein A eluted during protein A affinity chromatography. *J. Chromatogr. A* 1163 (1-2), 105–111. [PMID: 17643441]. doi:10.1016/j.chroma.2007.06.012

Castilho, A., Neumann, L., Gattinger, P., Strasser, R., Vorauer-Uhl, K., Sterovsky, T., et al. (2013). Generation of biologically active multi-sialylated recombinant human EPOFc in plants. *PloS one* 8 (1), e54836. [PMID: 23372778]. doi:10.1371/journal.pone.0054836

Chanquia, S. N., Vernet, G., and Kara, S. (2022). Photobioreactors for cultivation and synthesis: Specifications, challenges, and perspectives. *Eng. Life Sci.* 22 (12), 712–724. [PMID: 36514531]. doi:10.1002/elsc.202100070

Chiu, J., Valente, K. N., Levy, N. E., Min, L., Lenhoff, A. M., and Lee, K. H. (2017). Knockout of a difficult-to-remove CHO host cell protein, lipoprotein lipase, for improved polysorbate stability in monoclonal antibody formulations. *Biotechnol. Bioeng.* 114 (5), 1006–1015. [PMID: 27943242]. doi:10.1002/bit.26237

Cohen, I. S., Day, A. S., and Shaoul, R. (2019). Gluten in celiac disease-more or less? *Rambam Maimonides Med. J.* 10 (1), e0007. [PMID: 30720425]. doi:10.5041/RMMJ.10360

Commandeur, U., and Twyman, R. M. (2005). "Biosafety aspects of molecular farming in plants," in *Molecular farming* (Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA), 251–266.

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: Ecology, functions, and emerging trends in microbial application. *J. Adv. Res.* 19, 29–37. [PMID: 31341667]. doi:10.1016/j.jare.2019.03.004

Crosby, D. G., and Aharonson, N. (1967). The structure of carotatoxin, a natural toxicant from carrot. *Tetrahedron* 23 (1), 465–472. [PMID: 6037290]. doi:10.1016/S0040-4020(01)83330-5

Cuadros-Rodríguez, L., Lazúen-Muros, M., Ruiz-Samblás, C., and Navas-Iglesias, N. (2020). Leachables from plastic materials in contact with drugs. State of the art and review of current analytical approaches. *Int. J. Pharm.* 583, 119332. [PMID: 32360549]. doi:10.1016/j.ijpharm.2020.119332

Cummings, J. F., Guerrero, M. L., Moon, J. E., Waterman, P., Nielsen, R. K., Jefferson, S., et al. (2014). Safety and immunogenicity of a plant-produced recombinant monomer hemagglutinin-based influenza vaccine derived from influenza A (H1N1)pdm09 virus: A phase 1 dose-escalation study in healthy adults. *Vaccine* 32 (19), 2251–2259. doi:10.1016/j.vaccine.2013.10.017

D'Angelo, E. (2013). "Methods for determining toxic organic compounds in wetlands," in *Methods in biogeochemistry of wetlands*. Editors R. DeLaune, K. Reddy, and C. Richardson (Madison, WI, USA: John Wiley & Sons, Inc), 815–839.

Davey, R. T., Dodd, L., and Proschan, M. A. (2016). A randomized, controlled trial of ZMapp for ebola virus infection. *N. Engl. J. Med.* 375 (15), 1448–1456. [PMID: 27732819]. doi:10.1056/NEJMoa1604330

Dessaux, Y., and Faure, D. (2018). Niche construction and exploitation by Agrobacterium: How to survive and face competition in soil and plant habitats. *Curr. Top. Microbiol.* 418, 55–86.] [PMID: 29556826]. doi:10.1007/82_2018_83

Donini, M., Lombardi, R., Lonoce, C., Di Carli, M., Marusic, C., Morea, V., et al. (2015). Antibody proteolysis: A common picture emerging from plants. *Bioengineered* 6 (5), 299–302. [PMID: 26186119]. doi:10.1080/21655979.2015.1067740

Dortant, P. M., Claassen, I. J., van Kreyl, C. F., van Steenis, G., and Wester, P. W. (1997). Risk assessment on the carcinogenic potential of hybridoma cell DNA: Implications for residual contaminating cellular DNA in biological products. *Biologicals* 25 (4), 381–390. [PMID: 9467034]. doi:10.1006/biol.1997.0114

Drakopoulos, D., Sulyok, M., Krska, R., Logrieco, A. F., and Vogelgsang, S. (2021). Raised concerns about the safety of barley grains and straw: A Swiss survey reveals a high diversity of mycotoxins and other fungal metabolites. *Food control.* 125, 107919. doi:10.1016/j.foodcont.2021.107919

Duvick, J. (2001). Prospects for reducing fumonisin contamination of maize through genetic modification. *Environ. Health Persp* 109, 337–342. [PMID: 11359705]. doi:10.1289/ehp.01109s2337

Eckerstorfer, M. F., Engelhard, M., Heissenberger, A., Simon, S., and Teichmann, H. (2019). Plants developed by new genetic modification techniques-comparison of existing regulatory frameworks in the EU and non-EU countries. *Front. Bioeng. Biotechnol.* 7, 26. [PMID: 30838207]. doi:10.3389/fbioe.2019.00026

Eidenberger, L., Kogelmann, B., and Steinkellner, H. (2023). Plant-based biopharmaceutical engineering. *Nat. Rev. Bioeng.* 1, 426–439. doi:10.1038/s44222-023-00044-6

el-Maghraby, O. M., and Abdel-Sater, M. A. (1993). Mycoflora and natural occurrence of mycotoxins in tobacco from cigarettes in Egypt. *Zentralbl Mikrobiol.* 148 (4), 253–264. [PMID: 8368025]. doi:10.1016/s0232-4393(11)80101-0

Elli, L., Bascuñán, K., Di Lernia, L., Bardella, M. T., Doneda, L., Soldati, L., et al. (2020). Safety of occasional ingestion of gluten in patients with celiac disease: A real-life study. *BMC Med.* 18 (1), 42. [PMID: 32172690]. doi:10.1186/s12916-020-1511-6

Farhangi, H., Mozafari, V., Roosta, H. R., Shirani, H., and Farhangi, M. (2023). Optimizing growth conditions in vertical farming: Enhancing lettuce and basil cultivation through the application of the taguchi method. *Sci. Rep.* 13 (1), 6717. [PMID: 37185295]. doi:10.1038/s41598-023-33855-z

Fiaz, S., Aslam Khan, S., and Anis, G. B. (2021). Application of genome engineering methods for quality improvement in important crops. In: S. Upadhyay, *Genome engineering for crop improvement*. 1st edition. [Erscheinungsort nicht ermittelbar], Boston, MA: Wiley, 43–68.

Fletcher, J., Leach, J. E., Eversole, K., and Tauxe, R. (2013). Human pathogens on plants: Designing a multidisciplinary strategy for research. *Phytopathology* 103 (4), 306–315. IA] [PMID: 23406434]. doi:10.1094/phyto-09-12-0236-ia

Florea, S., Panaccione, D. G., and Schardl, C. L. (2017). Ergot alkaloids of the family clavicipitaceae. *Phytopathology* 107 (5), 504–518. PMID: 28168931]. doi:10.1094/phyto-12-16-0435-rvw

Fraser, P. D., Aharoni, A., Hall, R. D., Huang, S., Giovannoni, J. J., Sonnewald, U., et al. (2020). Metabolomics should be deployed in the identification and characterization of gene-edited crops. *Plant J.* 102 (5), 897–902. [PMID: 31923321]. doi:10.1111/tpj.14679

Friedman, M. A., Fernandez, M., Backer, L. C., Dickey, R., Bernstein, J., Schrank, K., et al. (2017). An updated review of ciguatera fish poisoning: Clinical, epidemiological, environmental, and public health management. *Mar. Drugs* 15 (3), 72. [PMID: 28335428]. doi:10.3390/md15030072

Fu, H., Machado, P. A., Hahm, T. S., Kratochvil, R. J., Wei, C. I., and Lo, Y. M. (2010). Recovery of nicotine-free proteins from tobacco leaves using phosphate buffer system under controlled conditions. *Bioresour. Technol.* 101 (6), 2034–2042. doi:10.1016/j.biortech.2009.10.045

N. Fusetani and W. Kem (Editors) (2009). *Marine toxins as research tools* (Berlin: Springer).

Ganesan, P. K., Kulchar, R. J., Kaznica, P., Montoya-Lopez, R., Green, B. J., Streatfield, S. J., et al. (2023). Optimization of biomass and target protein yield for Phase III clinical trial to evaluate Angiotensin Converting Enzyme 2 expressed in lettuce chloroplasts to reduce SARS-CoV-2 infection and transmission. *Plant Biotechnol. J.* 21 (2), 244–246. [PMID: 36314106]. doi:10.1111/pbi.13954

Geng, Y., Cao, G., Wang, L., and Wang, S. (2019). Effects of equal chemical fertilizer substitutions with organic manure on yield, dry matter, and nitrogen uptake of spring maize and soil nitrogen distribution. *PLoS ONE* 14 (7), e0219512. [PMID: 31287845]. doi:10.1371/journal.pone.0219512

Gengenbach, B. B., Keil, L. L., Opdensteinen, P., Müschen, C. R., Melmer, G., Lentzen, H., et al. (2019). Comparison of microbial and transient expression (tobacco plants and plant-cell packs) for the production and purification of the anticancer mistletoe lectin viscumin. *Biotechnol. Bioeng.* 116 (9), 2236–2249. [PMID: 31140580]. doi:10.1002/bit.27076

Gleadow, R. M., and Møller, B. L. (2014). Cyanogenic glycosides: Synthesis, physiology, and phenotypic plasticity. *Annu. Rev. Plant Biol.* 65, 155–185.] [PMID: 24579992]. doi:10.1146/annurev-arplant-050213-040027

Gleadow, R. M., and Woodrow, I. E. (2000). Temporal and spatial variation in cyanogenic glycosides in Eucalyptus cladocalyx. *Tree Physiol.* 20 (9), 591–598. [PMID: 12651423]. doi:10.1093/treephys/20.9.591

Goulet, C., Khalf, M., Sainsbury, F., D'Aoust, M. A., and Michaud, D. (2012). A protease activity-depleted environment for heterologous proteins migrating towards the leaf cell apoplast. *Plant Biotechnol. J.* 10 (1), 83–94. doi:10.1111/j.1467-7652.2011.00643.x

Grabowski, G. A., Golembo, M., and Shaaltiel, Y. (2014). Taliglucerase alfa: An enzyme replacement therapy using plant cell expression technology. *Mol. Genet. Metab.* 112 (1), 1–8. doi:10.1016/j.ymgme.2014.02.011

Green, B. T., Lee, S. T., Welch, K. D., and Panter, K. E. (2013). Plant alkaloids that cause developmental defects through the disruption of cholinergic neurotransmission. *Birth Defects Res. C Embryo Today* 99 (4), 235–246. [PMID: 24339035]. doi:10.1002/bdrc.21049

Griffiths, M. R., Strobel, B. W., Hama, J. R., and Cedergreen, N. (2021). Toxicity and risk of plant-produced alkaloids to Daphnia magna. *Environ. Sci. Eur.* 33 (1), 10. doi:10.1186/s12302-020-00452-0

Grosse-Holz, F., Madeira, L., Zahid, M. A., Songer, M., Kourelis, J., Fesenko, M., et al. (2018). Three unrelated protease inhibitors enhance accumulation of pharmaceutical recombinant proteins in Nicotiana benthamiana. *Plant Biotechnol. J.* 16 (10), 1797–1810. [PMID: 29509983]. doi:10.1111/pbi.12916

Gruenberg, D. A., and Shaker, M. S. (2011). An update on influenza vaccination in patients with egg allergy. *Curr. Opin. Pediatr.* 23 (5), 566–572.] [PMID: 21881505]. doi:10.1097/MOP.0b013e32834ac7a3

Günthardt, B. F., Hollender, J., Hungerbühler, K., Scheringer, M., and Bucheli, T. D. (2018). Comprehensive toxic plants-phytotoxins database and its application in assessing aquatic micropollution potential. *J. Agric. Food Chem.* 66 (29), 7577–7588. [PMID: 29944838]. doi:10.1021/acs.jafc.8b01639

Hahn-Löbmann, S., Stephan, A., Schulz, S., Schneider, T., Shaverskyi, A., Tusé, D., et al. (2019). Colicins and salmocins - new classes of plant-made non-antibiotic food antibacterials. *Front. Plant Sci.* 10, 437. [PMID: 31024601]. doi:10.3389/fpls.2019.00437

Hartz, C., Del San Miguel-Moncín, M. M., Cisteró-Bahíma, A., Fötisch, K., Metzner, K. J., Fortunato, D., et al. (2007). Molecular characterisation of Lac s 1, the major allergen from lettuce (Lactuca sativa). *Mol. Immunol.* 44 (11), 2820–2830. [PMID: 17349693]. doi:10.1016/j.molimm.2007.01.030

Harwanto, D., Negara, B. F. S. P., Tirtawijaya, G., Meinita, M. D. N., and Choi, J-S. (2022). Evaluation of toxicity of crude phlorotannins and phloroglucinol using different model organisms. *Toxins (Basel)* 14 (5), 312. [PMID: 35622559]. doi:10.3390/toxins14050312

Hayashi, S., Watanabe, M., Kobayashi, M., Tohge, T., Hashimoto, T., and Shoji, T. (2020). Genetic manipulation of transcriptional regulators alters nicotine biosynthesis in tobacco. *Plant Cell Physiol.* 61 (6), 1041–1053. [PMID: 32191315]. doi:10.1093/pcp/pcaa036

Hendrich, J. M., Wangorsch, A., Rödel, K., Jacob, T., Mahler, V., and Wöhrl, B. M. (2023). Allergenicity and IgE recognition of new dau c 1 allergens from carrot. *Mol. Nutr. Food Res.* 67 (3), e2200421. [PMID: 36458641]. doi:10.1002/mnfr.202200421

Hendrikx, J. J. M. A., Haanen, J. B. A. G., Voest, E. E., Schellens, J. H. M., Huitema, A. D. R., and Beijnen, J. H. (2017). Fixed dosing of monoclonal antibodies in oncology. *Oncologist* 22 (10), 1212–1221. [PMID: 28754722]. doi:10.1634/theoncologist.2017-0167

Henry, J. B., Vann, M. C., and Lewis, R. S. (2019). Agronomic practices affecting nicotine concentration in flue-cured tobacco: A review. *Agron. J.* 111 (6), 3067–3075. doi:10.2134/agronj2019.04.0268

Herrera-Estrella, L., Simpson, J., and Martinez-Trujillo, M. (2005). Transgenic plants: An historical perspective. *Methods Mol. Biol.* 286, 003–032.] [PMID: 15310910]. doi:10.1385/1-59259-827-7:003

Hien, D. F. D. S., Dabiré, K. R., Roche, B., Diabaté, A., Yerbanga, R. S., Cohuet, A., et al. (2016). Plant-Mediated effects on mosquito capacity to transmit human malaria. *PLoS Pathog.* 12 (8), e1005773. [PMID: 27490374]. doi:10.1371/journal.ppat.1005773

Hirayama, C., and Sakata, M. (2002). Chromatographic removal of endotoxin from protein solutions by polymer particles. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 781 (1-2), 419–432. [PMID: 12450672]. doi:10.1016/S1570-0232(02)00430-0

Hischenhuber, C., Crevel, R., Jarry, B., Maki, M., Moneret-Vautrin, D. A., Romano, A., et al. (2006). Review article: Safe amounts of gluten for patients with wheat allergy or coeliac disease. *Aliment. Pharmacol. Ther.* 23 (5), 559–575. [PMID: 16480395]. doi:10.1111/j.1365-2036.2006.02768.x

Hofbauer, W. K. (2021). Toxic or otherwise harmful algae and the built environment. *Toxins (Basel)* 13 (7), 465. [PMID: 34209446]. doi:10.3390/toxins13070465

Holland, T., Blessing, D., Hellwig, S., and Sack, M. (2013). The in-line measurement of plant cell biomass using radio frequency impedance spectroscopy as a component of process analytical technology. *Biotechnol. J.* 8 (10), 1231–1240. doi:10.1002/biot.201300125

Holland, T., and Buyel, J. F. (2017). "Bioreactor-based production of glycoproteins in plant cell suspension cultures," in *Recombinant glycoprotein production*. Editors V. Picanço e Castro, and K. Swiech (New York: Springer), 129–146.

Holtz, B. R., Berquist, B. R., Bennett, L. D., Kommineni, V. J., Munigunti, R. K., White, E. L., et al. (2015). Commercial-scale biotherapeutics manufacturing facility for plant-made pharmaceuticals. *Plant Biotechnol. J.* 13 (8), 1180–1190. [PMID: 26387511]. doi:10.1111/pbi.12469

Houdelet, M., Galinski, A., Holland, T., Wenzel, K., Schillberg, S., and Buyel, J. F. (2017). Animal component-free Agrobacterium tumefaciens cultivation media for better GMP-compliance increases biomass yield and pharmaceutical protein expression in Nicotiana benthamiana. *Biotechnol. J.* 12 (4), 1600721. [PMID: 28221723]. doi:10.1002/biot.201600721

Huang, T-K., and Puchta, H. (2021). Novel CRISPR/cas applications in plants: From prime editing to chromosome engineering. *Transgenic Res.* 30 (4), 529–549. [PMID: 33646511]. doi:10.1007/s11248-021-00238-x

Huang, T. K., and McDonald, K. A. (2012). Bioreactor systems for *in vitro* production of foreign proteins using plant cell cultures. *Biotechnol. Adv.* 30 (2), 398–409. [PMID: 21846499]. doi:10.1016/j.biotechadv.2011.07.016

Huebbers, J. W., and Buyel, J. F. (2021). On the verge of the market - plant factories for the automated and standardized production of biopharmaceuticals. *Biotechnol. Adv.* 46, 107681. [PMID: 33326816]. doi:10.1016/j.biotechadv.2020.107681

Hulvová, H., Galuszka, P., Frébortová, J., and Frébort, I. (2013). Parasitic fungus Claviceps as a source for biotechnological production of ergot alkaloids. *Biotechnol. Adv.* 31 (1), 79–89. [PMID: 22261014]. doi:10.1016/j.biotechadv.2012.01.005

Hundleby, P. A. C., D'Aoust, M-A., Finkle, C., Atkins, J., and Twyman, R. M. (2022). Regulation of molecular farming products. *Methods Mol. Biol.* 2480, 313–333. [PMID: 35616870]. doi:10.1007/978-1-0716-2241-4_17

Hwang, H. S., Kim, B. S., Park, H., Park, H-Y., Choi, H-D., and Kim, H. H. (2014). Type and branched pattern of N-glycans and their structural effect on the chicken egg allergen ovotransferrin: A comparison with ovomucoid. *Glycoconj. J.* 31 (1), 41–50.] [PMID: 24014058]. doi:10.1007/s10719-013-9498-2

Hwang, Y-C., Lu, R-M., Su, S-C., Chiang, P. Y., Ko, S. H., Ke, F. Y., et al. (2022). Monoclonal antibodies for COVID-19 therapy and SARS-CoV-2 detection. *J. Biomed. Sci.* 29 (1), 1. [PMID: 34983527]. doi:10.1186/s12929-021-00784-w

Ito, K. (2015). Grain and legume allergy. *Chem. Immunol. Allergy* 101, 145–151. [PMID: 26022874]. doi:10.1159/000375468

James, J. M., Zeiger, R. S., Lester, M. R., Fasano, M. B., Gern, J. E., Mansfield, L. E., et al. (1998). Safe administration of influenza vaccine to patients with egg allergy. *J. Pediatr.* 133 (5), 624–628. [PMID: 9821418]. doi:10.1016/s0022-3476(98)70101-5

Jang, H., Kim, H-S., Moon, S-C., Lee, Y. R., Yu, K. Y., Lee, B. K., et al. (2009). Effects of protein concentration and detergent on endotoxin reduction by ultrafiltration. *Biochem. Mol. Biol. Rep.* 42 (7), 462–466. [PMID: 19643046]. doi:10.5483/bmbrep.2009.42.7.462

Jansing, J., Sack, M., Augustine, S. M., Fischer, R., and Bortesi, L. (2018). CRISPR/Cas9-mediated knockout of six glycosyltransferase genes in Nicotiana benthamiana for the production of recombinant proteins lacking beta-1,2-xylose and core alpha-1,3-fucose. *Plant Biotechnol. J.* 17, 350–361. [PMID: 29969180]. doi:10.1111/pbi.12981

Jenke, D. (2002). Extractable/leachable substances from plastic materials used as pharmaceutical product containers/devices. *Pda J. Pharm. Sci. Tech.* 56 (6), 332–371. [PMID: 12497770].

Jiang, Q. L., Zhang, S., Tian, M., Xie, T., and Chen, D. Y. (2015). Plant lectins, from ancient sugar-binding proteins to emerging anti-cancer drugs in apoptosis and autophagy. *Cell Prolif.* 48 (1), 17–28. [PMID: 25488051]. doi:10.1111/cpr.12155

Jin, C., Altmann, F., Strasser, R., Mach, L., Schahs, M., Kunert, R., et al. (2008). A plant-derived human monoclonal antibody induces an anti-carbohydrate immune response in rabbits. *Glycobiology* 18 (3), 235–241. [PMID: 18203810]. doi:10.1093/glycob/cwm137

Jin, J., Tarrant, R. D., Bolam, E. J., Angell-Manning, P., Soegaard, M., Pattinson, D. J., et al. (2018). Production, quality control, stability, and potency of cGMP-produced Plasmodium falciparum RH5.1 protein vaccine expressed in Drosophila S2 cells. *NPJ Vaccines* 3, 32. [PMID: 30131879]. doi:10.1038/s41541-018-0071-7

Jones, M., Palackal, N., Wang, F., Gaza-Bulseco, G., Hurkmans, K., Zhao, Y., et al. (2021). High-risk" host cell proteins (HCPs): A multi-company collaborative view. *Biotechnol. Bioeng.* 118 (8), 2870–2885. [PMID: 33930190]. doi:10.1002/bit.27808

Joo, Y., Ok, H. E., Kim, J., Lee, S. Y., Jang, S. K., Park, K. H., et al. (2019). A statistical model for determining zearalenone contamination in rice (Oryza sativa L) at harvest and its prediction under different climate change scenarios in South Korea. *Appl. Biol. Chem.* 62 (1), 38. doi:10.1186/s13765-019-0447-z

Jurjevec, A., Brocard, C., Striedner, G., Cserjan-Puschmann, M., and Hahn, R. (2023). Polyethyleneimine efficiently extracts recombinant cytoplasmatic green fluorescent protein produced in *Escherichia coli* with high purity. *J. Biotechnol.* 371-372, 41–49. [PMID: 37285941]. doi:10.1016/j.jbiotec.2023.06.001

Jutras, P. V., Marusic, C., Lonoce, C., Deflers, C., Goulet, M. C., Benvenuto, E., et al. (2016). An accessory protease inhibitor to increase the yield and quality of a tumour-targeting mAb in nicotiana benthamiana leaves. *PloS one* 11 (11), e0167086. [PMID: 27893815]. doi:10.1371/journal.pone.0167086

Kabir, S. R., Nabi, M. M., Haque, A., Rokon, U. Z., Mahmud, Z. H., and Reza, M. A. (2013). Pea lectin inhibits growth of Ehrlich ascites carcinoma cells by inducing apoptosis and G2/M cell cycle arrest *in vivo* in mice. *Phytomedicine* 20 (14), 1288–1296. [PMID: 23867650]. doi:10.1016/j.phymed.2013.06.010

Kaiser, N., Douches, D., Dhingra, A., Glenn, K. C., Herzig, P. R., Stowe, E. C., et al. (2020). The role of conventional plant breeding in ensuring safe levels of naturally occurring toxins in food crops. *Trends Food Sci. Technol.* 100, 51–66. doi:10.1016/j.tifs.2020.03.042

Kang, S-M., Adhikari, A., Bhatta, D., Gam, H. J., Gim, M. J., Son, J. I., et al. (2022). Comparison of effects of chemical and food waste-derived fertilizers on the growth and nutrient content of lettuce (Lactuca sativa L). *Resources* 11 (2), 21. doi:10.3390/resources11020021

Kaur, G., Upadhyaya, P., and Chawla, P. (2023). Comparative analysis of IoT-based controlled environment and uncontrolled environment plant growth monitoring system for hydroponic indoor vertical farm. *Environ. Res.* 222, 115313. [PMID: 36709025]. doi:10.1016/j.envres.2023.115313

Kawka, K., Wilton, A. N., Madadkar, P., Medina, M. F. C., Lichty, B. D., Ghosh, R., et al. (2021). Integrated development of enzymatic DNA digestion and membrane chromatography processes for the purification of therapeutic adenoviruses. *Sep. Purif. Technol.* 254, 117503. doi:10.1016/j.seppur.2020.117503

Keeler, R. F., Crowe, M. W., and Lambert, E. A. (1984). Teratogenicity in swine of the tobacco alkaloid anabasine isolated from Nicotiana glauca. *Teratology* 30 (1), 61–69. [PMID: 6484853]. doi:10.1002/tera.1420300109

Kheawfu, K., Kaewpinta, A., Chanmahasathien, W., Rachtanapun, P., and Jantrawut, P. (2021). Extraction of nicotine from tobacco leaves and development of fast dissolving

nicotine extract film. *Membr. (Basel)* 11 (6), 403. [PMID: 34071396]. doi:10.3390/membranes11060403

Kim, J-S., Yoon, S-J., Park, Y-J., Kim, S-Y., and Ryu, C-M. (2020). Crossing the kingdom border: Human diseases caused by plant pathogens. *Environ. Microbiol.* 22 (7), 2485–2495. [PMID: 32307848]. doi:10.1111/1462-2920.15028

Kłapeć, T., Wójcik-Fatla, A., Farian, E., Kowalczyk, K., Cholewa, G., Cholewa, A., et al. (2021). Levels of filamentous fungi and selected mycotoxins in leafy and fruit vegetables and analysis of their potential health risk for consumers. *Ann. Agric. Environ. Med.* 28 (4), 585–594. [PMID: 34969215]. doi:10.26444/aaem/143031

Knödler, M., Opdensteinen, P., Sankaranarayanan, R. A., Morgenroth, A., Buhl, E. M., Mottaghy, F. M., et al. (2023b). Simple plant-based production and purification of the assembled human ferritin heavy chain as a nanocarrier for tumor-targeted drug delivery and bioimaging in cancer therapy. *Biotechnol. Bioeng.* 120 (4), 1038–1054. [PMID: 36539373]. doi:10.1002/bit.28312

Knödler, M., Reunious, P. W., and Buyel, J. F. (2023a). Risk assessment and bioburden evaluation of Agrobacterium tumefaciens-mediated transient protein expression in plants using the CaMV35S promoter. *Bmc Biotechnol.* 23 (1), 14. [PMID: 37286972]. doi:10.1186/s12896-023-00782-w

Knödler, M., Rühl, C., Emonts, J., and Buyel, J. F. (2019). Seasonal weather changes affect the yield and quality of recombinant proteins produced in transgenic tobacco plants in a greenhouse setting. *Front. plant Sci.* 10, 1245. doi:10.3389/fpls.2019.01245

Krikorian, A. D., and Ledbetter, M. C. (1975). Some observations on the cultivation of opium poppy (Papaver somniferum L) for its latex. *Bot. Rev.* 41 (1), 30–103. [PMID: 11631688]. doi:10.1007/BF02860836

Kuyukina, M. S., Ivshina, I. B., Korshunova, I. O., and Rubtsova, E. V. (2014). Assessment of bacterial resistance to organic solvents using a combined confocal laser scanning and atomic force microscopy (CLSM/AFM). *J. Microbiol. Methods* 107, 23–29. [PMID: 25193441]. doi:10.1016/j.mimet.2014.08.020

Lähdeaho, M-L., Mäki, M., Laurila, K., Huhtala, H., and Kaukinen, K. (2011). Small-bowel mucosal changes and antibody responses after low- and moderate-dose gluten challenge in celiac disease. *BMC Gastroenterol.* 11, 129. [PMID: 22115041]. doi:10.1186/1471-230X-11-129

Lali, N., Satzer, P., and Jungbauer, A. (2022). Residence time distribution in counter-current protein A affinity chromatography using an inert tracer. *J. Chromatogr. A* 1683, 463530. [PMID: 36206581]. doi:10.1016/j.chroma.2022.463530

Lechtenberg, M. (2005-2010). "Cyanogenic glycosides and biogenetically related compounds in higher plants and animals," in *Encyclopedia of life sciences*. Editors John Wiley, and L. Sons (London, New York: John Wiley & Sons), 1–18.

Lee, S. T., Wildeboer, K., Panter, K. E., Kem, W. R., Gardner, D. R., Molyneux, R. J., et al. (2006). Relative toxicities and neuromuscular nicotinic receptor agonistic potencies of anabasine enantiomers and anabaseine. *Neurotoxicol Teratol.* 28 (2), 220–228. [PMID: 16488116]. doi:10.1016/j.ntt.2005.12.010

Legrand, J., Artu, A., and Pruvost, J. (2021). A review on photobioreactor design and modelling for microalgae production. *React. Chem. Eng.* 6 (7), 1134–1151. doi:10.1039/D0RE00450B

Leth, I. K., and McDonald, K. A. (2017). Media development for large scale Agrobacterium tumefaciens culture. *Biotechnol. Prog.* 33 (5), 1218–1225. [PMID: 28556626]. doi:10.1002/btpr.2504

Lewis, R. J. (2000). Ion channel toxins and therapeutics: From cone snail venoms to ciguatera. *Ther. Drug Monit.* 22 (1), 61–64.] [PMID: 10688261]. doi:10.1097/00007691-200002000-00013

Li, Y. (2022). Two major mechanisms contributing to copurification of CHO host cell proteins and strategies to minimize their negative impact. *Protein Expr. Purif.* 197, 106113. [PMID: 35598695]. doi:10.1016/j.pep.2022.106113

Liu, D. L., and Lovett, J. V. (1993). Biologically active secondary metabolites of barley. II. Phytotoxicity of barley allelochemicals. *J. Chem. Ecol.* 19 (10), 2231–2244.] [PMID: 24248572]. doi:10.1007/BF00979660

Lücker, J., Schwab, W., van Hautum, B., Blaas, J., van der Plas, L. H. W., Bouwmeester, H. J., et al. (2004). Increased and altered fragrance of tobacco plants after metabolic engineering using three monoterpene synthases from lemon. *Plant Physiol.* 134 (1), 510–519. [PMID: 14718674]. doi:10.1104/pp.103.030189

Luo, H., Li, Y., Robbins, D., Wang, S., Xi, G., Cox, M., et al. (2021). Safety risk management for low molecular weight process-related impurities in monoclonal antibody therapeutics: Categorization, risk assessment, testing strategy, and process development with leveraging clearance potential. *Biotechnol. Prog.* 37 (3), e3119. [PMID: 33373106]. doi:10.1002/btpr.3119

Ma, J., Rukh, G., Ruan, Z., Xie, X., Ye, Z., and Liu, D. (2022). Effects of hypoxia stress on growth, root respiration, and metabolism of phyllostachys praecox. *Life (Basel)* 12 (6), 808. [PMID: 35743839]. doi:10.3390/life12060808

Ma, J. K., Drossard, J., Lewis, D., Altmann, F., Boyle, J., Christou, P., et al. (2015). Regulatory approval and a first-in-human phase I clinical trial of a monoclonal antibody produced in transgenic tobacco plants. *Plant Biotechnol. J.* 13 (8), 1106–1120. [PMID: 26147010]. doi:10.1111/pbi.12416

Mandal, M. K., Ahvari, H., Schillberg, S., and Schiermeyer, A. (2016). Tackling unwanted proteolysis in plant production hosts used for molecular farming. *Front. plant Sci.* 7, 267. [PMID: 27014293]. doi:10.3389/fpls.2016.00267

Marchese, S., Polo, A., Ariano, A., Velotto, S., Costantini, S., and Severino, L. (2018). Aflatoxin B1 and M1: Biological properties and their involvement in cancer development. *Toxins (Basel)* 10 (6), 214. [PMID: 29794965]. doi:10.3390/toxins10060214

Martinez, M., Gómez-Cabellos, S., Giménez, M. J., Barro, F., Diaz, I., and Diaz-Mendoza, M. (2019). Plant proteases: From key enzymes in germination to allies for fighting human gluten-related disorders. *Front. Plant Sci.* 10, 721. [PMID: 31191594]. doi:10.3389/fpls.2019.00721

Mayer, B. (2014). How much nicotine kills a human? Tracing back the generally accepted lethal dose to dubious self-experiments in the nineteenth century. *Arch. Toxicol.* 88 (1), 5–7. [PMID: 24091634]. doi:10.1007/s00204-013-1127-0

Mazzer, A. R., Perraud, X., Halley, J., O'Hara, J., and Bracewell, D. G. (2015). Protein A chromatography increases monoclonal antibody aggregation rate during subsequent low pH virus inactivation hold. *J. Chromatogr. A* 1415, 83–90. [PMID: 26346187]. doi:10.1016/j.chroma.2015.08.068

Mena, M., Sanchez-Monge, R., Gomez, L., Salcedo, G., and Carbonero, P. (1992). A major barley allergen associated with baker's asthma disease is a glycosylated monomeric inhibitor of insect alpha-amylase: cDNA cloning and chromosomal location of the gene. *Plant Mol. Biol.* 20 (3), 451–458. [PMID: 1421148]. doi:10.1007/BF00040604

Menary, J., Amato, M., Sanchez, A. C., Hobbs, M., Pacho, A., and Fuller, S. S. (2020a). New hope for a "cursed" crop? Understanding stakeholder attitudes to plant molecular farming with modified tobacco in europe. *Front. Plant Sci.* 11, 791. [PMID: 32595677]. doi:10.3389/fpls.2020.00791

Menary, J., Hobbs, M., Mesquita de Albuquerque, S., Pacho, A., Drake, P. M. W., Prendiville, A., et al. (2020b). Shotguns vs Lasers: Identifying barriers and facilitators to scaling-up plant molecular farming for high-value health products. *PloS one* 15 (3), e0229952. [PMID: 32196508]. doi:10.1371/journal.pone.0229952

Menzel, S., Holland, T., Boes, A., Spiegel, H., Fischer, R., and Buyel, J. F. (2018). Downstream processing of a plant-derived malaria transmission-blocking vaccine candidate. *Protein Expres Purif.* 152, 122–130. [PMID: 30059744]. doi:10.1016/j.pep.2018.07.012

Menzel, S., Holland, T., Boes, A., Spiegel, H., Bolzenius, J., Fischer, R., et al. (2016). Optimized blanching reduces the host cell protein content and substantially enhances the recovery and stability of two plant-derived malaria vaccine candidates. *Front. plant Sci.* 7 (159), 159–215. doi:10.3389/fpls.2016.00159

Miranda-Apodaca, J., Artetxe, U., Aguado, I., Martin-Souto, L., Ramirez-Garcia, A., Lacuesta, M., et al. (2023). Stress response to climate change and postharvest handling in two differently pigmented lettuce genotypes: Impact on *Alternaria alternata* invasion and mycotoxin production. *Plants (Basel)* 12 (6), 1304. [PMID: 36986993]. doi:10.3390/plants12061304

Mirzaee, M., Osmani, Z., Frébortová, J., and Frébort, I. (2022). Recent advances in molecular farming using monocot plants. *Biotechnol. Adv.* 58, 107913. [PMID: 35081454]. doi:10.1016/j.biotechadv.2022.107913

Miyoshi, N., Koyama, Y., Katsuno, Y., Hayakawa, S., Mita, T., Ohta, T., et al. (2001). Apoptosis induction associated with cell cycle dysregulation by rice bran agglutinin. *J. Biochem.* 130 (6), 799–805. [PMID: 11726280]. doi:10.1093/oxfordjournals.jbchem.a003051

Montero-Morales, L., and Steinkellner, H. (2018). Advanced plant-based glycan engineering. *Front. Bioeng. Biotechnol.* 6, 81. [PMID: 29963553]. doi:10.3389/fbioe.2018.00081

Moore, B., Andrew, R. L., Kulheim, C., and Foley, W. J. (2014). Explaining intraspecific diversity in plant secondary metabolites in an ecological context. *New Phytol.* 201 (3), 733–750. doi:10.1111/Nph.12526

Mortimer, C. L., Dugdale, B., and Dale, J. L. (2015). Updates in inducible transgene expression using viral vectors: From transient to stable expression. *Curr. Opin. Biotechnol.* 32, 85–92. [PMID: 25437638]. doi:10.1016/j.copbio.2014.11.009

Müller, J., Ott, V., Eibl, D., and Eibl, R. (2022). Seed train intensification using an ultra-high cell density cell banking process. *Processes* 10 (5), 911. doi:10.3390/pr10050911

Mündel, T., Machal, M., Cochrane, D. J., and Barnes, M. J. (2017). A randomised, placebo-controlled, crossover study investigating the effects of nicotine gum on strength, power and anaerobic performance in nicotine-naïve, active males. *Sports Med. Open* 3 (1), 5.] [PMID: 28092056]. doi:10.1186/s40798-016-0074-8

Muñoz-García, E., Luengo-Sánchez, O., Haroun-Díaz, E., Maroto, A. S., Palacín, A., Díaz -Perales, A., et al. (2013). Identification of thaumatin-like protein and aspartyl protease as new major allergens in lettuce (Lactuca sativa). *Mol. Nutr. Food Res.* 57 (12), 2245–2252. [PMID: 23983075]. doi:10.1002/mnfr.201300139

Murthy, H. N., Joseph, K. S., Paek, K. Y., and Park, S. Y. (2023). Bioreactor systems for micropropagation of plants: Present scenario and future prospects. *Front. Plant Sci.* 14, 1159588. [PMID: 37152119]. doi:10.3389/fpls.2023.1159588

Napagoda, M. (2022). "9 Poisonous plants and their toxic metabolites," in *Chemistry of natural products*. Editors M. Napagoda, and L. Jayasinghe (Berlin, Germany: De Gruyter), 173–188.

Nikbakhtzadeh, M. R., Terbot, J. W., Otienoburu, P. E., and Foster, W. A. (2014). Olfactory basis of floral preference of the malaria vector *Anopheles gambiae* (Diptera:

Culicidae) among common african plants. *J. Vector Ecol.* 39 (2), 372–383. [PMID: 25424267]. doi:10.1111/jvec.12113

Nishimura, A., Nakahara, T., Ueno, T., Sasaki, K., Yoshida, S., Kyo, S., et al. (2006). Requirement of E7 oncoprotein for viability of HeLa cells. *Microbes Infect.* 8 (4), 984–993. [PMID: 16500131]. doi:10.1016/j.micinf.2005.10.015

Nyasembe, V. O., Teal, P. E. A., Mukabana, W. R., Tumlinson, J. H., and Torto, B. (2012). Behavioural response of the malaria vector *Anopheles gambiae* to host plant volatiles and synthetic blends. *Parasit. Vectors* 5, 234. [PMID: 23069316]. doi:10.1186/1756-3305-5-234

Nyirenda, K. K. (2021). "Toxicity potential of cyanogenic glycosides in edible plants," in *Medical toxicology*. Editors P. Erkekoglu, and T. Ogawa (London, UK: IntechOpen).

Ochoa-Villarreal, M., Howat, S., Hong, S., Jang, M. O., Jin, Y. W., Lee, E. K., et al. (2016). Plant cell culture strategies for the production of natural products. *Bmb Rep.* 49 (3), 149–158. [PMID: 26698871]. doi:10.5483/bmbrep.2016.49.3.264

Ohizumi, Y., and Yasumoto, T. (1983). Contraction and increase in tissue calcium content induced by maitotoxin, the most potent known marine toxin, in intestinal smooth muscle. *Br. J. Pharmacol.* 79 (1), 3–5. [PMID: 6871549]. doi:10.1111/j.1476-5381.1983.tb10485.x

Olsnes, S., Stirpe, F., Sandvig, K., and Pihl, A. (1982). Isolation and characterization of viscumin, a toxic lectin from Viscum album L. (mistletoe). *J. Biol. Chem.* 257 (22), 13263–13270. [PMID: 7142144]. doi:10.1016/S0021-9258(18)33440-9

Onoyovwe, A., Hagel, J. M., Chen, X., Khan, M. F., Schriemer, D. C., and Facchini, P. J. (2013). Morphine biosynthesis in opium poppy involves two cell types: Sieve elements and laticifers. *Plant Cell* 25 (10), 4110–4122. [PMID: 24104569]. doi:10.1105/tpc.113.115113

Opal, S. M. (2010). Endotoxins and other sepsis triggers. *Contrib. Nephrol.* 167, 14–24. [PMID: 20519895]. doi:10.1159/000315915

Opdensteinen, P., Clodt, J. I., Müschen, C. R., Filiz, V., and Buyel, J. F. (2018). A combined ultrafiltration/diafiltration step facilitates the purification of cyanovirin-N from transgenic tobacco extracts. *Front. Bioeng. Biotechnol.* 6, 206. [PMID: 30687700]. doi:10.3389/fbioe.2018.00206

Opdensteinen, P., Lobanov, A., and Buyel, J. F. (2020). A combined pH and temperature precipitation step facilitates the purification of tobacco-derived recombinant proteins that are sensitive to extremes of either parameter. *Biotechnol. J.* 16, 2000340. In preparation. doi:10.1002/biot.202000340

Ortega, N., Quiralte, J., Blanco, C., Castillo, R., Alvarez, M. J., and Carrillo, T. (1999). Tobacco allergy: Demonstration of cross-reactivity with other members of solanaceae family and mugwort pollen. *Ann. Allergy Asthma Immunol.* 82 (2), 194–197. [PMID: 10071524]. doi:10.1016/S1081-1206(10)62596-3

Osborne, T. B. (1907). *The proteins of the wheat kernel*. Washington: Carnegie Inst.

Palladino, M. A., Levinson, A. D., Svedersky, L. P., and Obijeski, J. F. (1987). Safety issues related to the use of recombinant DNA-derived cell culture products. I. Cellular components. *Dev. Biol. Stand* 66, 13–22. [PMID: 3582742].

Pantazica, A-M., van Eerde, A., Dobrica, M-O., Caras, I., Ionescu, I., Costache, A., et al. (2023). The "humanized" N-glycosylation pathway in CRISPR/Cas9-edited Nicotiana benthamiana significantly enhances the immunogenicity of a S/preS1 Hepatitis B Virus antigen and the virus-neutralizing antibody response in vaccinated mice. *Plant Biotechnol. J.* 21 (6), 1176–1190. [PMID: 36779605]. doi:10.1111/pbi.14028

Pastorello, E. A., Farioli, L., Pravettoni, V., Ispano, M., Scibola, E., Trambaioli, C., et al. (2000). The maize major allergen, which is responsible for food-induced allergic reactions, is a lipid transfer protein. *J. Allergy Clin. Immunol.* 106 (4), 744–751. [PMID: 11031346]. doi:10.1067/mai.2000.108712

Paulsen, E., and Andersen, K. E. (2016). Lettuce contact allergy. *Contact Dermat.* 74 (2), 67–75. [PMID: 26289653]. doi:10.1111/cod.12458

Peden, K., Sheng, L., Pal, A., and Lewis, A. (2006). Biological activity of residual cell-substrate DNA. *Dev. Biol.* 123, 45–53. [PMID: 16566436].

Pereira, D. M., Valentao, P., Correia-da-Silva, G., Teixeira, N., and Andrade, P. B. (2012). Plant secondary metabolites in cancer chemotherapy: Where are we? *Curr. Pharm. Biotechno* 13 (5), 632–650. [PMID: 22122478]. doi:10.2174/138920112799857530

Petsch, D., and Anspach, F. B. (2000). Endotoxin removal from protein solutions. *J. Biotechnol.* 76 (2-3), 97–119. [PMID: 10656326]. doi:10.1016/S0168-1656(99)00185-6

Platis, D., Drossard, J., Fischer, R., Ma, J. K. C., and Labrou, N. E. (2008). New downstream processing strategy for the purification of monoclonal antibodies from transgenic tobacco plants. *J. Chromatogr. A* 1211 (1-2), 80–89. doi:10.1016/j.chroma.2008.09.103

Pronin, D., Börner, A., Weber, H., and Scherf, K. A. (2020). Wheat (*Triticum aestivum* L) breeding from 1891 to 2010 contributed to increasing yield and glutenin contents but decreasing protein and gliadin contents. *J. Agric. Food Chem.* 68 (46), 13247–13256. [PMID: 32648759]. doi:10.1021/acs.jafc.0c02815

Qiu, J., Li, K., Miller, K., and Raghani, A. (2015). Risk-based strategy to determine testing requirement for the removal of residual process reagents as process-related impurities in bioprocesses. *Pda J. Pharm. Sci. Tech.* 69 (3), 334–345. [PMID: 26048741]. doi:10.5731/pdajpst.2015.01056

Rademacher, T., Sack, M., Blessing, D., Fischer, R., Holland, T., and Buyel, J. F. (2019). Plant cell packs: A scalable platform for recombinant protein production and metabolic engineering. *Plant Biotechnol. J.* 17 (8), 1560–1566. [PMID: 30672078]. doi:10.1111/pbi.13081

Rasool, F., Nizamani, Z. A., Ahmad, K. S., Parveen, F., Khan, S. A., and Sabir, N. (2022). Phytotoxicological study of selected poisonous plants from Azad Jammu & Kashmir. *PLoS ONE* 17 (5), e0263605. [PMID: 35544538]. doi:10.1371/journal.pone.0263605

Raven, N., Rasche, S., Kuehn, C., Anderlei, T., Klöckner, W., Schuster, F., et al. (2014). Scaled-up manufacturing of recombinant antibodies produced by plant cells in a 200-L orbitally-shaken disposable bioreactor. *Biotechnol. Bioeng.* 112, 308–321. [PMID: 25117428]. doi:10.1002/bit.25352

Raven, N., Schillberg, S., Kirchhoff, J., Brändli, J., Imseng, N., and Eibl, R. (2011). "Growth of BY-2 suspension cells and plantibody production in single-use bioreactors," in *Single-use technology in biopharmaceutical manufacture*. Editors R. Eibl, and D. Eibl (Hoboken, NY, USA: John Wiley & Sons), 252–259.

Ren, G., Wu, H., Bao, A., Lin, T., Ting, K-C., and Ying, Y. (2023). Mobile robotics platform for strawberry temporal-spatial yield monitoring within precision indoor farming systems. *Front. Plant Sci.* 14, 1162435. [PMID: 37180389]. doi:10.3389/fpls.2023.1162435

Rihan, H. Z., Aljafer, N., Jbara, M., McCallum, L., Lengger, S., and Fuller, M. P. (2022). The impact of LED lighting spectra in a plant factory on the growth, physiological traits and essential oil content of lemon balm (melissa officinalis). *Plants (Basel)* 11 (3), 342. [PMID: 35161322]. doi:10.3390/plants11030342

Rivas-San Vicente, M., Larios-Zarate, G., and Plasencia, J. (2013). Disruption of sphingolipid biosynthesis in Nicotiana benthamiana activates salicylic acid-dependent responses and compromises resistance to *Alternaria alternata* f. sp. lycopersici. *Planta* 237 (1), 121–136. [PMID: 22990908]. doi:10.1007/s00425-012-1758-z

Rofiat, A-S., Fanelli, F., Atanda, O., Sulyok, M., Cozzi, G., Bavaro, S., et al. (2015). Fungal and bacterial metabolites associated with natural contamination of locally processed rice (Oryza sativa L) in Nigeria. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 32 (6), 950–959. [PMID: 25767914]. doi:10.1080/19440049.2015.1027880

Rup, B., Alon, S., Amit-Cohen, B. C., Brill Almon, E., Chertkoff, R., Tekoah, Y., et al. (2017). Immunogenicity of glycans on biotherapeutic drugs produced in plant expression systems-The taliglucerase alfa story. *PloS one* 12 (10), e0186211. [PMID: 29088235]. doi:10.1371/journal.pone.0186211

Sack, M., Rademacher, T., Spiegel, H., Boes, A., Hellwig, S., Drossard, J., et al. (2015). From gene to harvest: Insights into upstream process development for the GMP production of a monoclonal antibody in transgenic tobacco plants. *Plant Biotechnol. J.* 13 (8), 1094–1105. doi:10.1111/pbi.12438

Salguero-Linares, J., and Coll, N. S. (2023). Cell death as a defense strategy against pathogens in plants and animals. *PLoS Pathog.* 19 (4), e1011253. [PMID: 37023043]. doi:10.1371/journal.ppat.1011253

Sanchez-Ramos, J. R. (2020). The rise and fall of tobacco as a botanical medicine. *J. Herb. Med.* 22, 100374. [PMID: 32834941]. doi:10.1016/j.hermed.2020.100374

Schachtsiek, J., and Stehle, F. (2019). Nicotine-free, nontransgenic tobacco (Nicotiana tabacum l) edited by CRISPR-Cas9. *Plant Biotechnol. J.* 17 (12), 2228–2230. [PMID: 31206994]. doi:10.1111/pbi.13193

Schubert-Ullrich, P., Rudolf, J., Ansari, P., Galler, B., Führer, M., Molinelli, A., et al. (2009). Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: An overview. *Anal. Bioanal. Chem.* 395 (1), 69–81. [PMID: 19308361]. doi:10.1007/s00216-009-2715-y

Schwestka, J., König-Beihammer, J., Shin, Y-J., Vavra, U., Kienzl, N. F., Grünwald-Gruber, C., et al. (2021). Impact of specific N-glycan modifications on the use of plant-produced SARS-CoV-2 antigens in serological assays. *Front. Plant Sci.* 12, 747500. [PMID: 34646292]. doi:10.3389/fpls.2021.747500

Sekiya, R., Nagano, T., Moriyama, T., Kishi, T., Shinke, H., Yano, E., et al. (2020). Occupational respiratory allergy to lettuce in lettuce farmers. *Clin. Exp. Allergy* 50 (8), 932–941. [PMID: 32542808]. doi:10.1111/cea.13682

Sencar, J., Hammerschmidt, N., and Jungbauer, A. (2020). Modeling the residence time distribution of integrated continuous bioprocesses. *Biotechnol. J.* 15 (8), e2000008. [PMID: 32302457]. doi:10.1002/biot.202000008

Serdakowski London, A., Kerins, B., Tschantz, W. R., and Mackay, K. (2012). Endotoxin removal and prevention for pre-clinical biologics production. *Biotechnol. J.* 7 (12), 1509–1516. [PMID: 23081824]. doi:10.1002/biot.201200220

Shaaltiel, Y., and Tekoah, Y. (2016). Plant specific N-glycans do not have proven adverse effects in humans. *Nat. Biotechnol.* 34 (7), 706–708. [PMID: 27404878]. doi:10.1038/nbt.3556

Sharma, R., Singh, A. K., and Umashankar, V. (2009). Characterization of allergenic epitopes of Ory s1 protein from Oryza sativa and its homologs. *Bioinformation* 4 (1), 12–18. [PMID: 20011147]. doi:10.6026/97320630004012

Sharrer, M. J., and Summerfelt, S. T. (2007). Ozonation followed by ultraviolet irradiation provides effective bacteria inactivation in a freshwater recirculating system. *Aquac. Eng.* 37 (2), 180–191. doi:10.1016/j.aquaeng.2007.05.001

Sheng, L., Cai, F., Zhu, Y., Pal, A., Athanasiou, M., Orrison, B., et al. (2008). Oncogenicity of DNA *in vivo*: Tumor induction with expression plasmids for activated H-ras and c-myc. *Biologicals* 36 (3), 184–197. [PMID: 18218323]. doi:10.1016/j.biologicals.2007.11.003

Sigmund, G., Arp, H. P. H., Aumeier, B. M., Bucheli, T. D., Chefetz, B., Chen, W., et al. (2022). Sorption and mobility of charged organic compounds: How to confront and overcome limitations in their assessment. *Environ. Sci. Technol.* 56 (8), 4702–4710. [PMID: 35353522]. doi:10.1021/acs.est.2c00570

Singh, A. A., Pillay, P., and Tsekoa, T. L. (2021). Engineering approaches in plant molecular farming for global health. *Vaccines (Basel)* 9 (11), 1270. [PMID: 34835201]. doi:10.3390/vaccines9111270

Sisson, V. A., and Severson, R. F. (1990). Alkaloid composition of the nicotiana species. *Contributions Tob. Nicotine Res.* 14 (6), 327–339. doi:10.2478/cttr-2013-0610

Smahel, M., Sima, P., Ludvikova, V., and Vonka, V. (2001). Modified HPV16 E7 genes as DNA vaccine against E7-containing oncogenic cells. *Virology* 281 (2), 231–238. doi:10.1006/viro.2000.0794

Sobiczewski, P., and Iakimova, E. T. (2022). Plant and human pathogenic bacteria exchanging their primary host environments. *J. Hortic. Res.* 30 (1), 11–30. doi:10.2478/johr-2022-0009

Sparrow, P., Broer, I., Hood, E., Eversole, K., Hartung, F., and Schiemann, J. (2013). Risk assessment and regulation of molecular farming - a comparison between europe and us. *Curr. Pharm. Des.* 19 (31), 5513–5530. doi:10.2174/1381612811319310007

Spiegel, H., Boes, A., Perales Morales, C., Rademacher, T., and Buyel, J. F. (2019). Ready to use stocks of Agrobacterium tumefaciens can simplify process development for the production of recombinant proteins by transient expression in plants. *Biotechnol. J.* 14, e1900113. [PMID: 31218827]. doi:10.1002/biot.201900113

Stephan, A., Hahn-Löbmann, S., Rosche, F., Buchholz, M., Giritch, A., and Gleba, Y. (2017). Simple purification of nicotiana benthamiana-produced recombinant colicins: High-yield recovery of purified proteins with minimum alkaloid content supports the suitability of the host for manufacturing food additives. *Int. J. Mol. Sci.* 19 (1), 95. [PMID: 29286298]. doi:10.3390/ijms19010095

Stoger, E., Sack, M., Perrin, Y., Vaquero, C., Torres, E., Twyman, R. M., et al. (2002). Practical considerations for pharmaceutical antibody production in different crop systems. *Mol. Breed.* 9 (3), 149–158. doi:10.1023/A:1019714614827

Stone, M. C., Borman, J., Ferreira, G., and Robbins, P. D. (2018). Effects of pH, conductivity, host cell protein, and DNA size distribution on DNA clearance in anion exchange chromatography media. *Biotechnol. Prog.* 34 (1), 141–149. [PMID: 28884511]. doi:10.1002/btpr.2556

Strasser, R., Altmann, F., Mach, L., Glössl, J., and Steinkellner, H. (2004). Generation of *Arabidopsis thaliana* plants with complex *N*-glycans lacking β1,2-linked xylose and core α1,3-linked fucose. *FEBS Lett.* 561 (1-3), 132–136. doi:10.1016/S0014-5793(04)00150-4

Strasser, R., Stadlmann, J., Schahs, M., Stiegler, G., Quendler, H., Mach, L., et al. (2008). Generation of glyco-engineered Nicotiana benthamiana for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. *Plant Biotechnol. J.* 6 (4), 392–402. doi:10.1111/j.1467-7652.2008.00330.x

Strasser, R. (2016). Plant protein glycosylation. *Glycobiology* 26 (9), 926–939. [PMID: 26911286]. doi:10.1093/glycob/cww023

Sweany, R. R., Breunig, M., Opoku, J., Clay, K., Spatafora, J. W., Drott, M. T., et al. (2022). Why do plant-pathogenic fungi produce mycotoxins? Potential roles for mycotoxins in the plant ecosystem. *Phytopathology* 112 (10), 2044–2051. [PMID: 35502928]. doi:10.1094/phyto-02-22-0053-sym

Szermer-Olearnik, B., and Boratyński, J. (2015). Removal of endotoxins from bacteriophage preparations by extraction with organic solvents. *PLoS ONE* 10 (3), e0122672. [PMID: 25811193]. doi:10.1371/journal.pone.0122672

Taraghikhah, N., Ashtari, S., Asri, N., Shahbazkhani, B., Al-Dulaimi, D., Rostami-Nejad, M., et al. (2020). An updated overview of spectrum of gluten-related disorders: Clinical and diagnostic aspects. *BMC Gastroenterol.* 20 (1), 258. [PMID: 32762724]. doi:10.1186/s12876-020-01390-0

Taylor, P. C., Adams, A. C., Hufford, M. M., La Torre, I. de, Winthrop, K., and Gottlieb, R. L. (2021). Neutralizing monoclonal antibodies for treatment of COVID-19. *Nat. Rev. Immunol.* 21 (6), 382–393. [PMID: 33875867]. doi:10.1038/s41577-021-00542-x

Tekoah, Y., Tzaban, S., Kizhner, T., Hainrichson, M., Gantman, A., Golembo, M., et al. (2013). Glycosylation and functionality of recombinant beta-glucocerebrosidase from various production systems. *Biosci. Rep.* 33, e00071–U272. doi:10.1042/Bsr20130081

Thoma, F., Somborn-Schulz, A., Schlehuber, D., Keuter, V., and Deerberg, G. (2020). Effects of light on secondary metabolites in selected leafy greens: A review. *Front. Plant Sci.* 11, 497. [PMID: 32391040]. doi:10.3389/fpls.2020.00497

Tosi, P., Gritsch, C. S., He, J., and Shewry, P. R. (2011). Distribution of gluten proteins in bread wheat (*Triticum aestivum*) grain. *Ann. Bot.* 108 (1), 23–35. [PMID: 21693664]. doi:10.1093/aob/mcr098

Toxic Rep Ser (1993). NTP toxicity studies of sodium cyanide (CAS No. 143-33-9) administered by dosed water to F344/N rats and B6C3F1 mice. *Toxic. Rep. Ser.* 37, 1. D3 [PMID: 11965236].

Tusé, D., Nandi, S., McDonald, K. A., and Buyel, J. F. (2020). The emergency response capacity of plant-based biopharmaceutical manufacturing-what it is and what it could Be. *Front. Plant Sci.* 11, 594019. [PMID: 33193552]. doi:10.3389/fpls.2020.594019

Tyutereva, E. V., Dobryakova, K. S., Schiermeyer, A., Shishova, M. F., Pawlowski, K., Demidchik, V., et al. (2018). The levels of peroxisomal catalase protein and activity modulate the onset of cell death in tobacco BY-2 cells via reactive oxygen species levels and autophagy. *Funct. Plant Biol.* 45 (2), 247–258. [PMID: 32291039]. doi:10.1071/FP16418

Uranga, M., Vazquez-Vilar, M., Orzáez, D., and Daròs, J-A. (2021). CRISPR-Cas12a genome editing at the whole-plant level using two compatible RNA virus vectors. *CRISPR J.* 4 (5), 761–769. [PMID: 34558964]. doi:10.1089/crispr.2021.0049

Urbański, A., Konopińska, N., Bylewska, N., Gmyrek, R., Spochacz-Santoro, M., Bufo, S. A., et al. (2023). Solanum nigrum fruit extract modulates immune system activity of mealworm beetle, *Tenebrio molitor* L. *Tenebrio Molit. L. Toxins (Basel)* 15 (1), 68. [PMID: 36668887]. doi:10.3390/toxins15010068

Usui, Y., Nakase, M., Hotta, H., Urisu, A., Aoki, N., Kitajima, K., et al. (2001). A 33-kDa allergen from rice (oryza sativa L.japonica). *J. Biol. Chem.* 276 (14), 11376–11381. [PMID: 11139585]. doi:10.1074/jbc.M010337200

van Delden, S. H., SharathKumar, M., Butturini, M., Graamans, L. J. A., Heuvelink, E., Kacira, M., et al. (2021). Current status and future challenges in implementing and upscaling vertical farming systems. *Nat. Food* 2, 944–956. doi:10.1038/s43016-021-00402-w

van der Hoorn, R. A. L., and Klemenčič, M. (2021). Plant proteases: From molecular mechanisms to functions in development and immunity. *J. Exp. Bot.* 72 (9), 3337–3339. [PMID: 33847361]. doi:10.1093/jxb/erab129

Venuti, A., Massa, S., Mett, V., Vedova, L. D., Paolini, F., Franconi, R., et al. (2009). An E7-based therapeutic vaccine protects mice against HPV16 associated cancer. *Vaccine* 27 (25-26), 3395–3397. doi:10.1016/j.vaccine.2009.01.068

Voitsekhovskaja, O. V., Schiermeyer, A., and Reumann, S. (2014). Plant peroxisomes are degraded by starvation-induced and constitutive autophagy in tobacco BY-2 suspension-cultured cells. *Front. Plant Sci.* 5, 629. [PMID: 25477890]. doi:10.3389/fpls.2014.00629

Walsh, G., and Walsh, E. (2022). Biopharmaceutical benchmarks 2022. *Nat. Biotechnol.* 40 (12), 1722–1760. [PMID: 36471135]. doi:10.1038/s41587-022-01582-x

Wang, S., Alseekh, S., Fernie, A. R., and Luo, J. (2019). The structure and function of major plant metabolite modifications. *Mol. Plant* 12 (7), 899–919. [PMID: 31200079]. doi:10.1016/j.molp.2019.06.001

Wang, X., Morgan, D. M., Wang, G., and Mozier, N. M. (2012). Residual DNA analysis in biologics development: Review of measurement and quantitation technologies and future directions. *Biotechnol. Bioeng.* 109 (2), 307–317. [PMID: 21956148]. doi:10.1002/bit.23343

Ward, B. J., Gobeil, P., Séguin, A., Atkins, J., Boulay, I., Charbonneau, P. Y., et al. (2021). Phase 1 randomized trial of a plant-derived virus-like particle vaccine for COVID-19. *Nat. Med.* 27 (6), 1071–1078.] [PMID: 34007070]. doi:10.1038/s41591-021-01370-1

Weirich, C. A., and Miller, T. R. (2014). Freshwater harmful algal blooms: Toxins and children's health. *Curr. Probl. Pediatr. Adolesc. Health Care* 44 (1), 2–24. [PMID: 24439026]. doi:10.1016/j.cppeds.2013.10.007

Welch, K. D., Lee, S. T., Panter, K. E., Gardner, D., Knoppel, E., Green, B., et al. (2014). Studies on the teratogenicity of anabasine in a rat model. *Toxicon* 87, 32–37. [PMID: 24905648]. doi:10.1016/j.toxicon.2014.05.018

Werner, S., Maschke, R. W., Eibl, D., and Eibl, R. (2018). Bioreactor technology for sustainable production of plant cell-derived products. In: T. Bley, and A. Pavlov editors. *Bioprocessing of plant* in vitro *systems*. Cham: Springer International Publishing, 413–432.

Wilbers, R. H., Westerhof, L. B., van Raaij, D. R., Adrichem, M., Prakasa, A. D., Lozano-Torres, J. L., et al. (2016). Co-expression of the protease furin in*Nicotiana benthamiana*leads to efficient processing of latent transforming growth factor-β1 into a biologically active protein. *Plant Biotechnol. J.* 14 (8), 1695–1704. [PMID: 26834022]. doi:10.1111/pbi.12530

Wilson, S. A., and Roberts, S. C. (2012). Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. *Plant Biotechnol. J.* 10 (3), 249–268. doi:10.1111/j.1467-7652.2011.00664.x

Wink, M. (2009). Mode of action and toxicology of plant toxins and poisonous plants. *Mitt. Julius Kühn-Inst* 421, 93–112.

Wirz, H., Sauer-Budge, A. F., Briggs, J., Sharpe, A., Shu, S. D., and Sharon, A. (2012). Automated production of plant-based vaccines and pharmaceuticals. *J. Laboratory Automation* 17 (6), 449–457. doi:10.1177/2211068212460037

Worbs, S., Kohler, K., Pauly, D., Avondet, M. A., Schaer, M., Dorner, M. B., et al. (2011). Ricinus communis intoxications in human and veterinary medicine-a summary of real cases. *Toxins (Basel)* 3 (10), 1332–1372. [PMID: 22069699]. doi:10.3390/toxins3101332

Wróblewska, B., Kubicka, E., Semenowicz, E., Ogrodowczyk, A., Mikołajczyk, A., and Złotkowska, D. (2022). The immune system response to 15-kDa barley protein: A mouse model study. *Nutrients* 14 (20), 4371. [PMID: 36297055]. doi:10.3390/nu14204371

Xu, X., Gan, Q., Clough, R. C., Pappu, K. M., Howard, J. A., Baez, J. A., et al. (2011). Hydroxylation of recombinant human collagen type I alpha 1 in transgenic maize co-expressed with a recombinant human prolyl 4-hydroxylase. *Bmc Biotechnol.* 11, 69. [PMID: 21702901]. doi:10.1186/1472-6750-11-69

Yang, H., Zhang, L., and Galinski, M. (2010). A probabilistic model for risk assessment of residual host cell DNA in biological products. *Vaccine* 28 (19), 3308–3311. [PMID: 20226252]. doi:10.1016/j.vaccine.2010.02.099

Zalai, D., Dietzsch, C., and Herwig, C. (2013). Risk-based process development of biosimilars as part of the quality by design paradigm. *Pda J. Pharm. Sci. Tech.* 67 (6), 569–580. [PMID: 24265299]. doi:10.5731/pdajpst.2013.00943

Zeng, S., Ma, J., Yang, Y., Zhang, S., Liu, G-J., and Chen, F. (2019). Spatial assessment of farmland soil pollution and its potential human health risks in China. *Sci. Total Environ.* 687, 642–653. [PMID: 31220718]. doi:10.1016/j.scitotenv.2019.05.291

Zhang, Z., Wu, X., Wu, Q., Huang, X., Zhang, J., and Fang, H. (2020). Speciation and accumulation pattern of heavy metals from soil to rice at different growth stages in farmland of southwestern China. *Environ. Sci. Pollut. Res. Int.* 27 (28), 35675–35691.] [PMID: 32601861]. doi:10.1007/s11356-020-09711-2

Zhu, X., Li, X., Wang, W., and Ning, K. (2020). Bacterial contamination screening and interpretation for biological laboratory environments. *Med. Microecology* 5, 100021. doi:10.1016/j.medmic.2020.100021

Ziegler, J., Facchini, P. J., Geissler, R., Schmidt, J., Ammer, C., Kramell, R., et al. (2009). Evolution of morphine biosynthesis in opium poppy. *Phytochemistry* 70 (15-16), 1696–1707. [PMID: 19665152]. doi:10.1016/j.phytochem.2009.07.006

Zimmermann, L., Bartosova, Z., Braun, K., Oehlmann, J., Völker, C., and Wagner, M. (2021). Plastic products leach chemicals that induce *in vitro* toxicity under realistic use conditions. *Environ. Sci. Technol.* 55 (17), 11814–11823. [PMID: 34488348]. doi:10.1021/acs.est.1c01103

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Original Research
PUBLISHED 30 August 2023
DOI 10.3389/fbioe.2023.1250298

OPEN ACCESS

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RECEIVED 29 June 2023
ACCEPTED 16 August 2023
PUBLISHED 30 August 2023

CITATION
Trump BD, Cummings CL, Loschin N, Keisler JM, Wells EM and Linkov I (2023), The worsening divergence of biotechnology: the importance of risk culture.
Front. Bioeng. Biotechnol. 11:1250298.
doi: 10.3389/fbioe.2023.1250298

The worsening divergence of biotechnology: the importance of risk culture

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In the last 20 years, the field of biotechnology has made significant progress and attracted substantial investments, leading to different paths of technological modernization among nations. As a result, there is now an international divide in the commercial and intellectual capabilities of biotechnology, and the implications of this divergence are not well understood. This raises important questions about why global actors are motivated to participate in biotechnology modernization, the challenges they face in achieving their goals, and the possible future direction of global biotechnology development. Using the framework of prospect theory, this paper explores the role of risk culture as a fundamental factor contributing to this divergence. It aims to assess the risks and benefits associated with the early adoption of biotechnology and the regulatory frameworks that shape the development and acceptance of biotechnological innovations. By doing so, it provides valuable insights into the future of biotechnology development and its potential impact on the global landscape.

KEYWORDS

biotechnology, governance, divergence, risk culture, prospect theory

1 Introduction

Over the past 2 decades, biotechnology has experienced significant advancements, fueled by large infusions of capital and institutional development. However, this progress has taken place against a backdrop of uncertain social, economic, and risk-based concerns that threaten to derail national technology modernization plans for certain countries or stymie future development altogether. The result is a burgeoning international divergence in commercial and intellectual capabilities, with some nations adopting a slower, more risk averse development pathway while others seek primacy in one or more permutations of biotechnology. The implications of this divergence are unknown, though questions abound regarding what countries might do about it. More directly: why did this divergence arise, why is it worsening, and might future global biotechnology development look like if this trend is unchanged for the next decade?

Addressing these questions requires an understanding of the perceived incentives that global actors have in engaging biotechnology modernization. Such modernization does not happen by accident, requiring hundreds of millions of dollars and a concerted effort to develop the human capital and subsequent market demand to sustain innovation upon the conclusion of initial government investment. The overall requirement to reach this end-stage

is that any commercial-ready materials or composite products possess relatively well-understood risk profiles consistent with domestic requirements and norms, while the characteristics and behavior of such materials is predictable under recommended circumstances.

Unfortunately, reaching these desired endpoints is an uncertain process fraught with many challenges. Overcoming the technical rate-limiting steps that enable scientific progress is not guaranteed. As such, any potential benefits accrued by unlocking a technology's capabilities must be discounted by the potential for failure throughout the development process, as well as any institutional, social, economic, or security concerns that various stakeholders would have in approving of and supporting technology modernization. The risk, governance, and ELSI (ethnical, legal, and social implications) surrounding biotechnology worsen this discounting factor—presenting considerable hurdles that many nations would face in their modernization process. The result of 2 decades of biotechnology development has resulted in a wide and broadening gulf between countries with interest and capability in biotechnology modernization that is likely to worsen without corrective action over the next 20 years.

2 Why does technology divergence happen?

At face value, emerging technologies promise benefits that present societies lack, and offer improvements to quality of living. Often, these improvements are iterative—a refinement or increased efficiency to a current process or capability. Occasionally, the improvements are revolutionary—posing benefits that have little to no corollary within current markets or technological capacities. Generally, evolutionary benefits (e.g., improving crop yield and nutritional value) carry less technical risk and are more likely to succeed, though produce less net societal value than revolutionary benefits (novel treatment for a debilitating illness).

If deemed of interest, developers and governments seek both types of benefits as "early adopters". Defined as actors who invest in the earliest years of a technology's development, and prior to the introduction of marketable products, early adopters reap the benefits of being at the forefront of innovation. These benefits can be multifaceted, including economic gains from commercialization, the prestige of technological leadership, the strategic advantage of possessing proprietary knowledge, and the societal benefits of improved services and products. Importantly, these benefits often influence the trajectory of technology development, with developers and governments strategically investing in areas they believe will yield the highest return on investment.

The early adopter dynamic can also create a feedback loop, where the countries that are the most successful in developing and adopting new biotechnologies attract more investment, talent, and political support for future biotech endeavors. This is particularly true for technologies requiring a massive up-front cost with few barriers to maturation, such as advanced rocketry and the space race, to cases where the ability to innovate is tightly controlled, contested, and of a military nature (e.g., competition for nuclear energy and the atomic bomb in the 1940s and 1950s). Ultimately, the ability of an early adopter to successfully innovate and capture portions of a new market contributes to a self-reinforcing cycle of technology leadership, capturing a greater portion of potential technological and economic benefits from innovation, as well as shaping the global trade and regulatory system to be more in-line with the norms, values, and modernization objectives of early adopter nations.

However, being an early adopter of technology is not without risks. The trajectory of technological progress is notoriously difficult to predict, with a high degree of uncertainty surrounding both the technical feasibility of emerging technologies and the societal response to these technologies. Early adopters must navigate this uncertainty, balancing the potential rewards of successful innovation against the risks of technological failure, public backlash, or unintended consequences.

It is because of these risks that many nations opt for a more risk-averse approach to innovation—particularly when the technology in question or its potential applications clash with local institutions, regulatory instruments, as well as domestic ELSI norms and values. Such hesitancy to innovate in certain areas may persist despite enormous potential benefits, following the precepts of prospect theory on a societal scale (Kahneman and Tversky, 2012). They prioritize the management of potential risks and the prevention of harm over the pursuit of potential benefits and/or the considerable expense of funding technically uncertain scientific endeavors. The regulatory frameworks in these countries often embody the precautionary principle, requiring extensive evidence of safety and efficacy before new technologies can be approved. This approach can slow the pace of technology development and adoption but is seen by these nations as a necessary trade-off to protect public health, the environment, and societal values in the short to intermediate term.

Applications of prospect theory, rooted in behavioral economics and psychology, have gained significant attention in the field of international governance and policy comparisons. Its conceptual framework provides a lens through which to examine decision-making processes and outcomes at both individual and national levels. Traditional risk evaluation methods are prescriptive, such as with guiding biotechnological developments in a manner congruent with rationality and objective economic tradeoffs. However, prospect theory serves as a descriptive counterpoint, acknowledging the reality that decision-making, whether in the pursuit of biotechnological advancements or the formation of governing policies, is not always aligned with predicted rational outcomes. The theory underscores how cognitive biases, such as framing, can lead stakeholders away from objectively beneficial choices in biotechnology—particularly in an environment of heightened uncertainty relative to technology hazards, exposure pathways, and health consequences. Acknowledging these biases offers an opportunity for intervention, enabling the creation of strategies that consider and counteract these biases.

Since Kahneman and Tversky's initial discussion of prospect theory, its applications for international risk governance have entered into various applications. For instance, Mercer (2005) reviewed and applied prospect theory to the field of political science to evaluate decision-making under conditions of uncertainty and policy choices in the realm of international politics. By extension, Levy (1996) employed prospect theory to evaluate governance issues including who conducted a two-level analysis to investigate the interaction between the individual-level

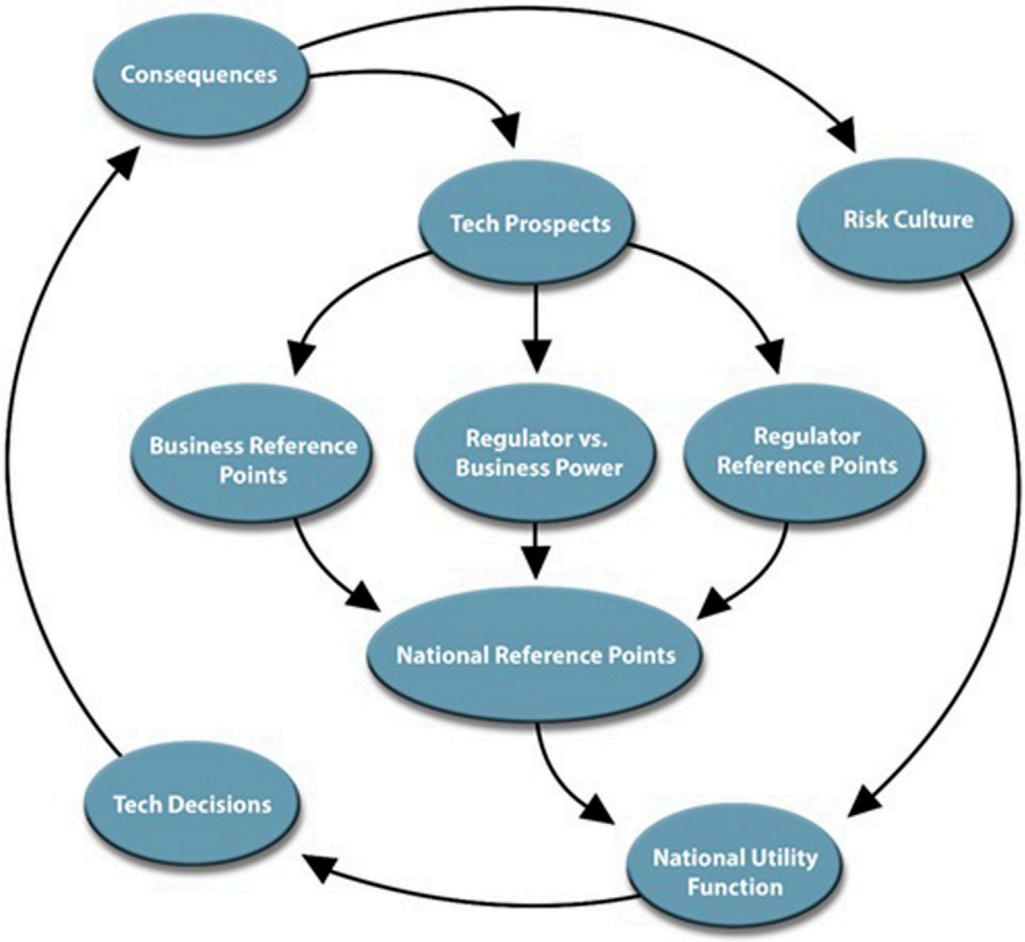

FIGURE 1
Flow diagram illustrating international competition for technology innovation based upon technology prospects and risk culture.

prospect theory and the systemic-level security dilemma to evaluate how political leaders of adversarial states behave differently when they are bargaining over gains than when they are bargaining over losses. More recently, Ross (2020) applied prospect theory to evolving standard operating procedures and decision-making in Afghanistan and explored how psychological biases influence risk calculation and decision-making, emphasizing the significance of reference points and how they are modulated. Further applications of prospect theory have evaluated competing strategic behaviors with regard transjurisdictional water pollution (Yuan et al., 2022) emergency decision-making regarding water diversion in China (Li et al., 2022) and blockchain-based data governance and government policy incentives for manufacturing supply chains (Wei et al., 2023).

From this foundation, we identify that, for biotechnology, prospect theory may best inform the institutional, political, and social values and constraints that frame innovation tradeoffs for countries inform national "risk culture" that, alongside the perceived prospects of a given innovation, inform national desire to engage in a potentially risky technology modernization endeavor (Figure 1). During the earliest stages of technology development, the capabilities and products resulting from potential technology maturation are assessed based upon stated political and institutional goals, as well as desired needs for economic competitiveness, national defense, and overall societal wellbeing (Corona et al., 2006; Titus et al., 2020; Zhang, 2020). In turn, these prospects are evaluated through different regulatory and industry frames, balancing potential returns on technology investment against direct or indirect human and environmental health hazards. These frames, alongside social perceptions and demand for technology innovation, form the impetus of technology modernization platforms that inevitably inform policy (Jasanoff, 1987).

National risk culture is a pervasive influence on both top-down and bottom-up governance, ranging from how regulators and legislators perceive risk of an emerging technology, to the willingness of the public and markets to embrace new technologies, their products, and the benefits associated with marketable innovation (Jasanoff, 2015). Top-down, regulators informed by a risk-averse culture may seek to impose stringent controls on the development and deployment of new technologies, demanding high levels of evidence of safety and efficacy, and prioritizing the avoidance of potential harm. On the other hand, in a risk-tolerant culture, regulators may be more inclined to adopt a flexible approach, allowing innovation to proceed with appropriate

oversight, while continuously monitoring and adjusting regulatory measures in response to new information about risks and benefits. These frames are difficult to change outside of a focusing event, such as a major technology breakthrough, or an international accord (e.g., the Cartagena Protocol on Biosafety) (Lee and Malerba, 2017).

From the bottom-up, the public and market's acceptance of new technologies is also shaped by the prevailing risk culture. Public trust in government institutions is crucial; faith in the government's ability to regulate and monitor new technologies with uncertain and potentially hazardous properties can significantly alter public and market acceptance. Prevailing ethics and cultural values also play a pivotal role. For instance, societies prioritizing environmental sustainability might be more accepting of innovations in green technologies, despite potential risks, than those where economic growth is prioritized above all else. For biotechnology, perceived trustworthiness of policymakers informs social and market enthusiasm for technology modernization efforts. A demonstrative case includes China, which in the aftermath of the "CRISPR-baby scandal", revised hard law codes via the Chinese Ministry of Justice as well as the Ministry Science and Technology established clearer requirements for the handling of human genetic resources (State Council of China, 2019; Araz, 2020). Simultaneously in July 2019, China established the National Science and Technology Ethics Committee to address ELSI concerns of various emerging technologies (Araz, 2020). All regulatory and policy developments exist against a greater backdrop of a dedicated drive for field leadership of biotechnology in the life sciences, including over $100 billion in public funding that has been invested into Chinese biotechnology research, particularly on the life sciences (Moore, 2020). The stringency of ethical and legal proscriptions is debated (Araz, 2020), though the improved *de jure* policy structure alongside substantial financial incentives push Chinese advancement in an area of biotechnology research with heightened risks, and competition against western nations with stringent controls and public skepticism of human subjects research (Akin et al., 2017).

In risk-averse societies, consumers may be skeptical of products derived from new technologies, demanding transparent information about their development and potential risks. This consumer skepticism can influence market dynamics, potentially discouraging investment in innovative but risk-associated technologies. Conversely, in societies with a risk-tolerant culture, there may be greater public and market enthusiasm for new technologies, driving investment and rapid adoption of innovative products. Thus, national risk culture, acting from both top-down and bottom-up, can significantly influence the pace and direction of technology modernization in a country based upon discounted evaluations of likely short-term risks against potential longer-term early adopter benefits.

Technology divergence occurs when risk culture becomes increasingly entrenched for a given innovation, and the potential benefits of the innovation are not perceived as revolutionary enough to contravene regulatory practice and societal expectations (Liebowitz and Margolis, 1995). Once an innovation is tagged as being excessively risky within a certain risk culture, it becomes difficult to reverse this perception, even with emerging evidence of safety or efficacy. One example includes perceptions of engineered agriculture in the European Union, which throughout decades of research and commodification, still encounters both public and regulatory reluctance to approve the importation, planting, and consumption of genetically modified crops that can be traced back to early concerns of GMO safety in the 1990s (Jiang, 2020). Likewise, the pursuit of early actor privileges places increased political and market pressure on successfully translating innovation to markets—even if potential hazards are not fully characterized, exposure pathways are less than certain, and consequences are questionable. Unless a tremendous shift occurs to stimulate development (e.g., the successful launch of Sputnik that ignited the space race) or limit marketability (e.g., the Chernobyl and Fukushima Daiichi nuclear catastrophes), nations are likely to continue on their existing risk culture pathways until interest in the innovation fizzles out, or it switches from an "emerging" to an "emerged" technology, with established markets, safety and security norms, and general best practices and operating procedures.

This process can take years or decades, with the implications of uneven international technology development uncertain until risk culture entrenchment is well underway. Even then, as a technology matures, differences in technology framing can drive practitioners, regulators, and civil societies from different nations to interpret the "winners and losers" of the innovation race differently and create self-fulfilling prophecies. Risk averse nations can point to instances of safety or security breakdown as proof that their wariness of rapid innovation is justified, while early adopters frame their economic, technical, and social benefits from marketable innovation as proof of how aggressive innovation can lift standards of living. While identifying true winners and losers is often difficult, cases emerge where the aggressive innovator is unable to overcome technical or safety hurdles prior to marketability, or when risk averse nations become reliant upon early adopters for some critical and high-demand benefit for their businesses, consumers, and citizens. Both outcomes carry tremendous strategic risk for economic competitiveness and national security.

3 Why is technology divergence worsening for biotechnology?

Biotechnology's progress is marked by a particularly contentious debate that fuels self-reinforcing technology divergence. Simultaneously, emerging biotechnologies like synthetic biology possess unknown, potentially extreme, and possibly irreversible risks (e.g., gene transfer, introduction of invasive species that disrupts local ecosystems, unforeseen harms to human subjects, potential self-sustaining persistence in the environment), while also must contend with decades of difficult debates regarding the safety, security, ethics, and benefits of early research into genetic engineering due to breakthroughs in understanding of DNA and its synthesis (Berg et al., 1975; Barkstrom, 1985; Abels, 2005; Hurlbut, 2015; Parthasarathy, 2015; Bier, 2022). Heated historical debates formed the battlelines by which much of present-day biotechnology and synthetic biology are waged, while the novelties of emerging research foster an even broader risk-reward gamble for countries considering biotechnology modernization.

Historical breakthroughs with significant debate on the future of biotechnology governance included the creation of the first transgenic animal in 1985 (pigs), as well as transgenic corn in

1988 (Klein et al., 1988). These advancements eventually contributed to the rise of genetically modified organisms being sold in markets, such as with the engineered tomato in 1994 (Uzogara, 2000). The development and maturation of genetic engineering during this period focused on addition, deletion, or substitution of specific DNA base pairs, where more substantial genetic modification of cellular systems was limited by technological constraints of the day (Cameron et al., 2014). Debates raged through this early period regarding safety concerns and good governance challenges of engineered products (Kuzma, 2022), as well as the dual-use nature of many biotechnology breakthroughs (Epstein, 2005), their potential misuse (Mueller, 2019), and broader ethical challenges by skeptical publics ranging from religious (Austriaco, 2020), to moral (Midgley, 2000), to commercial (Mitalipov, 2017), to personal preferences for less exposure to genetically engineered organisms (GMOs) (Sagar et al., 2000).

More recently, advancements in automated DNA sequencing, coupled with sophisticated computational tools, have enabled high-throughput methodologies to analyze RNA, proteins, lipids, and metabolites and create extensive libraries of cellular components (Cameron et al., 2014). This step up in genetic research, coupled with continued reduction in cost of genetic sequencing and synthesis, have facilitated a systems engineering approach to biology. From the early 2000s and onward, genetic engineers pondering questions of whether complex cellular networks could be viewed as an engineered system, where deliberate biological engineering of a cell's DNA could yield complex changes to how those systems operate (Cameron et al., 2014). In recent years, this enhanced capability has contributed to an explosion of biotechnology research, affording engineers with greater control over cellular expression, and more precise instruments to engineer and nurture desired changes in the genome (Kozovska et al., 2021). The implications include potentially revolutionary treatments of debilitating disease, to environmental restoration, to various industrial advancements that address critical challenges in the future of global standards of living (El Karoui et al., 2019; Meng and Ellis, 2020; Cubillos-Ruiz et al., 2021). Other scholars have also explored options to make risk management measures more proportionate and adaptive to potential risks, uncertainties, and benefits (Devos et al., 2022).

Yet, despite 2 decades of improved understanding of synthetic biology and other emerging biotechnologies, uncertainty with respect to downstream implications (e.g., unintended exposure to novel genetic material, affecting human health and biodiversity alike) has grown rather than shrunk (Eriksson et al., 2020). This is due in part to the increased reach of biotechnology applications, including examples as species control (e.g., mosquito vectors for human pathogens—(Benelli et al., 2014), to de-extinction (e.g., wooly mammoth—McCauley et al., 2015), to biomining with engineered bacteria (Brune and Bayer, 2012), to the potential elimination of harmful heritable human diseases (Bosley et al., 2015) among many others. Many proposed biotechnology applications are intended for public or environmental release to maximize their beneficial potential, yet equally carry some measure of uncertain risks to proliferate in the environment or incur harms. In some ways, these risks are fundamentally unknowable up-front and require research and application to identify and characterize—creating a Catch-22 for risk averse risk cultures (Carter et al., 2014). And for instances where hazards have been identified, the research requirements to effectively bound risk in a manner consistent with many nations' precautionary attitudes are prohibitive (Kuiken et al., 2014; Wareham and Nardini, 2015; Trump et al., 2018).

The result is a global regulatory environment forced to grapple with extreme uncertainty—including the possibility for global spillovers of biological risk events, however minute on a case-by-case basis. In-turn, such uncertainty limits the governance options of potential innovators: rather than a continuum of policy options that accounts for a rough bounding of technology risk against socioeconomic benefit, effective options are to (a) heavily restrict innovation to the point of no near-term market viability, or (b) permit near-free innovation potential, governed by existing capabilities for overall laboratory safety and material biosecurity (Lyall et al., 2012; Mandel, 2013; Lyall and Tait, 2019). Depending upon frames, perceived incentives, and local risk culture, both postures are individually rational despite being based upon near-identical starting points of uncertainty in risk and benefit.

Absent the pull towards a new set of international norms, values, or codes of conduct, individual nations will inevitably pursue biotechnology modernization platforms with less congruity to others over time. The implications of this include issues of safety (potential for accidents or unintended harmful consequences) and security (potential for deliberate misuse of biotechnology or its products). And, inevitably, this entrenchment will have decades of economic and health implications, where early actors may enjoy dominance over large swathes of a new field given their hard-won knowledge of translating elements of biotechnology into viable products. Likewise, however, exposure to potential novel hazards will be concentrated in aggressively developing nations, until safety protocols can be defined, regulated, and implemented. If successful, nations less willing to engage in early technology development risk becoming "captured" by the market capabilities of others (Wu et al., 2010; Adenle, 2011; Yrjola, et al., 2022). Subsequently, the divergence between a small number of early adopters and a larger body of risk averse nations will worsen the technology pacing problem, whereby the accelerating growth of innovation in the biotechnology space outstrips established best practices for environmental health and safety assessment as well as regulatory practices (Marchant et al., 2011; Fenwick et al., 2017; Trump et al., 2020). Closing this gap is no simple task—compelling *suis generis* hard law across the international landscape is doomed to clash against prevailing political and institutional debates and regulatory instruments. Scholars denote the possibility of soft law approaches as suggesting guidelines for best practice without compelling changes to national regulation, such as within the earliest days of genomics research at the Asilomar Conference on Recombinant DNA (Berg et al., 1975; Abels, 2005). Whether or not such a focusing event as a major conference can incentivize international commitment to biotechnology soft law after decades of national investment and regulatory development remains to be seen, and will likely be more complex, and more expensive in political and economic capital, than in the field's early decades.

4 What does the next decade look like if such divergence is unchanged?

As the foundational life sciences of biotechnology continue to evolve and are increasingly integrated into product development, the task of governing biotechnology concurrently grows more intricate. The incorporation of these advanced scientific principles and techniques into the fabric of product design and development necessitates policies that comprehensively address not just the end products, but also the processes involved in their creation (NAS, 2018; Marris and Calvert, 2020). This expanded policy requirement contributes to an escalation in the complexity of strategic shifts, thus heightening the associated political and economic costs of switching technology policies. The inherent difficulty of navigating these changes serves to consolidate a natural preference for policy *status quo*, barring a significant crisis or catalytic event that necessitates a change (Sun et al., 2022). Consequently, the stakes are raised higher, with the cost of policy inertia becoming a significant factor in the wider discussion on the direction of biotechnology governance (Greer and Trump, 2019). This setting is how technology divergence forms and worsens, and has shaped the past 2 decades of international biotechnology research and development.

Early hints of what might become of the next ten to 20 years of international biotechnology competition are taking shape, though not guaranteed to transpire. Many capabilities are sought through biotechnology research, including those with iterative improvement over conventional product options (e.g., chemical production and synthesis), as well as revolutionary or groundbreaking (e.g., treatments or cures of debilitating diseases that current lack adequate interventions). While these application areas are numerous and growing with each year, they are generally summarized in government pronouncements as falling into various categories: biomanufacturing and biology-based fermentation of compounds, environmental deployment (e.g., environmental sensing and/or remediation by designed microbial systems against environmental targets), systems-enabled biotechnologies (broad-based genomic engineering to enable unique or novel phenotypic expression in a range of plants and animals), and bio-engineering for substances intended for ingestion, treatment, or gene therapy of humans (Titus et al., 2020).

Several governments are clarifying their biotechnology modernization strategies through public pronouncements, indicating the capabilities they seek to acquire. For example, in 2022, the Chinese National Development and Reform Commission (NDRC) shared the "14th Five-Year Plan for Bioeconomic Development" with a focus in synthetic biology in biomedicine, bio-agriculture, bio-manufacturing, and biosecurity, emphasizing China's goal in achieving field leadership in medical and gene therapy breakthroughs for humans. In human subjects research, human genome editing was first reported in China in 2015 followed by a major study in 2017 that reported a successful correction of a defective gene in human embryos (Ma et al., 2017). This was followed by the starkly controversial claims of a Chinese scientist that used CRISPR embryonic genome of twins (Cyranoski, 2019; Science, 2023). The top-down policy approach can also be seen with government implementing legislation and regulation on biotechnology such as the 2022 Issues Guide for Bio-security Measurement of Gene Edited Crops (Reuters, 2022; Zhang et al., 2022) and the backing of 2020 Biosafety Law of the People's Republic of China (Li et al., 2021). Local risk culture holds reservations on human subjects research, although more accepting attitudes towards research on germline gene editing that would reduce or eliminate heritability of debilitating disease (Zhang and Lie, 2018). While less centralized than with China, Japanese biotechnology development has engaged in ample research both upon countering human pathogens as well as research into transgenic food products in a more permissive regulatory environment than the United States or Europe (Fabbri et al., 2023). Other nations have less financially extensive biotechnology research enterprises—such as South Korea or Singapore—though have extensive research in government or university laboratories on furthering pharmaceuticals, medical interventions, or other benefits to human subjects (Mao et al., 2021).

Outside of human subjects research, other nations have established platforms to further production capacity of industrial enzymes, bio-engineered agriculture, and others. In 2019, the Russian Federation approved the "Federal Research Programme for Genetic Technologies Development for 2019–2027," which stated that "the Programme's key objectives are to implement a comprehensive solution to the task of the accelerated development of genetic technologies, including genetic editing; to establish scientific and technological ground for medicine, agriculture and industry; to improve the system of preventing biological emergencies and monitoring in this area." Progress made within this program is expected to be carried out at new laboratories established at research and academic institutions, to increase biosecurity, and to ensure technological independence. It additionally aims to set up at least three world-class level centers for genome research, to design new lines of plants and animals, and to produce *in vitro* and *in vivo* models of human illnesses. Russia's announcement in May of 2019 of a new $1.7 billion dollar program to promote the development of ten new varieties of gene-edited crops and animals by 2020 and twenty more by 2027 for a total of thirty in less than a decade demonstrates their commitment to the program, though lags behind others engaging with research on GMOs or chemical biosynthesis. This announcement suggests governmental exemption on the prohibition of the cultivation of GMOs in Russia (Dobrovidova, 2019).

The United States and European Union are longtime developers of genetic engineering and synthetic biology research, though with diverging regulatory traditions and risk cultures (Fabbri et al., 2023). Focusing on the process of biotechnology development (as opposed to product-focused regulation alone), EU governance has adopted risk averse interpretations of environmental, agricultural, and human subjects research, though more permissive of industrial development. Directives (such as 90/219/EEC on Contained Use of Genetically Modified Materials or 2001/18/EC on Deliberate Release into the Environment of Genetically Modified Materials, later amended by Directive 2018/350 which focused more squarely on environmental risk assessment) have served as a common approach to govern genetically modified organisms, where each member state is required to achieve identified Directive policy goals via their own means. For genetically modified organisms, this often includes the use of existing member state regulatory agencies to cover related research within the respective state's political borders.

This is driven by the sui generis framework for regulating biotechnology and genetically engineered organisms, which is comprised of the collection of Directives and Regulations that explicitly address requirements that govern the process and products of genetic engineering exercises. Specifically, Directives concerning the transfer of genes 2001/18/EC), the deliberate release of genetically modified microorganisms (90/220/EEC), the mutation and potential proliferation of genetically modified microorganisms and biodiversity impacts (2001/18/EC), laboratory and workplace safety with experiments conducting genetic modification (2009/41/EC and 2000/54/EC), general consumer health regulation for products with artificial genetic information (1829/2003), and specific Directives of pharmaceutical products containing artificial genetic material (726/2004) were viewed in literature as capable of covering existing iterations of "semi-synthetic" synthetic biology products, although may be challenged in the future as synthetic biologists are able to foster increasingly artificial synthetic biology -products such as with synthesized vaccines or other therapeutics. European regulation will eventually have to grapple with the question of how to govern fully synthetic cells which lack clear comparisons with products derived from naturally occurring components. Without an alternative to quantitative and comparative risk analysis between such products on a case-by-case basis, European regulatory protocols and requirements may hinder the further development and commercialization of potentially beneficial products as with new pharmaceuticals and vaccine components.

The sheer diversity of synthetic biology research in Europe presents EU regulators with a near impossible problem of trying to assess risk in many differing technological processes and product categories. In some areas, this impasse has spurred some (as with the European Union Court of Justice in a July 2018 ruling) to apply existing EU Directives from earlier generations of genetically modified organisms onto gene editing technologies like CRISPR, which may significantly slow progress on gene editing research in the European Union. In other areas like novel genomic techniques for food production, recent European Commission policy proposals may relax regulations on certain genetic techniques which may garner opportunities to circumvent barriers to market-entry in the future (European Commission, 2023).

Likewise, the United States has engaged in aggressive development in various areas of biotechnology research, though has encountered regulatory and ELSI hurdles in others. The US has been a major developer of engineered agriculture for decades, rising from less than 20% of planted soybean, cotton, and corn seeds in 1996 to over 90% by end-2018 (US Department of Agriculture, 2018). US governance of biotechnology has taken a more product-driven focus than the European Union, China, Japan, Russian Federation, or many other nations, with safety and security process measures captured within product-specific regulation via the Environmental Protection Agency (e.g., Toxic Substances Control Act), the Food and Drug Administration (e.g., Food, Drug, and Cosmetics Act), the US Department of Agriculture-Animal and Plant Health Inspection Service (APHIS) (e.g., Plant Pest Act) (Carter et al., 2014; Wang and Zhang, 2019). Likewise, agencies like APHIS and FDA are compelled to assess broad environmental impacts of products intended for environmental release, influencing permits and approvals, via the National Environmental Policy Act (NEPA). Updates to US hard law pertinent to emerging biotechnologies arise gradually—e.g., the Frank R. Lautenberg Chemical Safety for the 21st Century Act, which amended TSCA to bolster EPA funding for evaluation of existing and future chemical products and institute risk-based assessments of such substances (US Congress, 2016)- though such updates are slower than comparators in Europe and abroad (Trump, 2017). Likewise, US research into stem cells has encountered decades of political resistance and regulatory blocks for the past 2 decades relative to China or Japan, extending to germline editing research on human embryos (Fabbri et al., 2023).

The impact of the SARS-CoV-2 pandemic on international biotechnology funding and development cannot be overstated. Notably, the global crisis prompted a surge in public and private funding towards advancing vaccine research and development, diagnostics, and therapeutics, revealing the extraordinary potential of biotechnologies in addressing emergent health crises. Likewise, "policy windows" of institutional acceptance of certain biotechnologies opened, with a goal to address a rising hazard in the form of a novel human pathogen (Kingdon, 1993). Governments worldwide have recognized the critical role of biotechnology in protecting public health and have accordingly accelerated their investment into this domain. The pandemic has also demonstrated the potency of emerging biotechnologies like mRNA-based vaccines, exemplified by the Pfizer-BioNTech and Moderna COVID-19 vaccines, which were developed at a remarkable pace due to a combination of advanced biotechnological tools and substantial funding.

At the same time, the pandemic has necessitated a drastic shift in global collaborative efforts. Informal international data sharing arrangements were formed, and data was shared at an unprecedented scale to (a) evaluate the hazards and epidemiological trends of the SARS-CoV-2 virus and its variants, and (b) to enable the rapid development and distribution of vaccines (Corey et al., 2020; Cosgriff et al., 2020; Duan et al., 2022). This collective effort underscored the value of open and cooperative approaches to biotechnological advancement, although much of the informal international collaboration around health risk data analytics weakened as the pandemic progressed (Singh et al., 2021). However, the pandemic has also highlighted stark disparities between countries in their biotechnological capacities and their access to biotechnological solutions, such as COVID-19 vaccines (Lucas-Dominguez et al., 2021; Tatar et al., 2021). These disparities underscore the risk of a widening "biotech divide" between nations with robust biotechnology sectors and those without. This is a foretaste of future biotechnology divergence—whether it be a medical breakthrough or a cutting-edge economic capability.

As we consider the future trajectory of international biotechnology, one must consider the possibilities for early actor nations, such as China, to gain significant ground in areas of medical research and human subjects. With their top-down approach to biotechnology modernization and strategic focus on synthetic biology in biomedicine, China has the potential to significantly impact the global landscape in this regard. For instance, China has been notably aggressive in pursuing advancements in gene therapy and genome editing, as demonstrated by the first report of human genome editing in 2015 and the later controversial claims of CRISPR-edited human embryos. The relative permissiveness and adaptiveness of China's regulatory environment, along with

substantial state-backed funding for research, fosters an environment conducive to rapid advances and innovation. Moreover, the country's strategic orientation and commitment to biotechnology as an essential driver of its national development agenda further propels its drive to attain leadership in these areas.

If China becomes a dominant player in the field of biotechnology for medicines and human health, the regulatory and economic implications would be substantial, both for China and the international community. Regulatory implications could include a shift towards the Chinese regulatory model. If China's approach proves successful, it could influence international regulatory standards and norms for biotechnological products and practices. It may also prompt other nations to adjust their policies to remain competitive in the global biotech industry. The Chinese model, which is more product-based and with an emphasis on speed-to-market, could lead to a global acceleration in the development and approval of new treatments, but also raise questions around safety and ethical considerations. Economically, China's dominance in biotechnology could have profound effects on global health markets. As a major producer of biotech products, China could potentially dictate pricing and distribution, influencing global health economics and accessibility to novel treatments. Furthermore, China's dominance could shift the balance of trade, leading to a more East-centric global biotech economy. This may prompt Western companies to increase their investments in biotechnology to keep pace with China, fueling a global "biotech race" towards the longer-term, high-risk applications in human health and gene therapies.

Nations with large public-sector grants, as well as private-sector investment, will continue to excel in biotechnology innovation. The United States retains a dominant role here, including a vast university system and growing bioeconomy to sustain both a mature biotechnology workforce as well as a national economy with demands for biotechnology products. This will ensure competitiveness in most biotechnology development areas but does not guarantee leadership in all product applications. The United States is likely to retain field leadership over engineered agriculture due to a more permissive regulatory and consumer environment in that space, though will face substantial ELSI and regulatory hurdles to keep pace with other nations like China or Japan on human health applications.

Other nations may not achieve overall dominance across multiple biotechnology channels but can achieve leadership in a specific niche or product category. Some, like the Russian Federation or Pakistan, strive for mastery of biotechnology capabilities to facilitate industrial enzyme production and cash crop bioengineering, respectively (ISAAA, 2019). These targeted advances may allow them to keep pace with more well-funded developers like the United States or China and may even grant them some competitive advantage in niche applications of biotechnology for explicit products. Such niche leadership has been observed for other emerging technologies with significant economic and defense benefits—for example, both Estonia and Israel are lauded for their cybersecurity and digital security capabilities, despite having much smaller budgets and research enterprises than the United States, European Union, or Russian Federation (Herzog, 2017; Housen-Couriel, 2017).

Thus, without an international event or accord to align technology governance expectations and best practices, biotechnology divergence will foster an international landscape of clear leaders in specific technology areas, and a larger host of nations that are either (a) dependent upon the early adopter for desirable products, or (b) are locked out of the economic and defense benefits of those technologies. Likewise, early adopters have the privilege not only to set the market for product pricing, but also can exert considerable pressure upon their trade partners to align regulatory requirements around familiar, usually favorable terms (Zhang et al., 2020; Irwin, 2021). Such shifts in regulatory policy might be unpalatable or even impossible for some nations to embrace, depriving them of certain elements of the biotechnology market.

Biotechnology research is not guaranteed to be a fruitful endeavor for early adopters—many experiments will fail or be proven to be too risky to continue. For those that do succeed, however, biotechnology divergence will contribute to greater asymmetry amongst the global commons to understand, prevent, mitigate, govern, and communicate potentially novel hazards that biotechnology may incur to humans or the environment. While early actors incur greater exposure to these unique hazards (e.g., horizontal gene transfer), they also gain critical and usually proprietary or safeguarded knowledge critical to fostering effective safety and security norms and practices. As such, successful early adopters forge a path dependence in their biotechnology research that facilitates compounding improvement in knowledge and operability of biotechnology processes and products in a way that late-adopters will struggle to keep up with. In the coming decades, this gap in knowhow can leave late adopters less capable of governing biotechnology hazards that creep into their political borders, even despite moratoria (e.g., the spreading of animal pathogens or engineered seeds across political borders).

5 Discussion

Looking ahead, if the current divergence remains unmitigated, the implications are multifaceted. Biotechnology divergence has the potential to fundamentally reshape the geopolitical landscape, altering traditional power dynamics based on factors such as economic strength, military prowess, and natural resource availability. Early adopters of biotechnologies are likely to gain not only scientific and technological advantages but also significant diplomatic influence. By leading the development and implementation of new biotechnologies, these nations have the capacity to redefine global norms and standards and shape international policy in ways that protect their own interests and values. Furthermore, they can leverage their advanced capabilities to exert influence over other nations, whether through diplomacy, economic sanctions, or even technological coercion.

In addition, as early adopters establish themselves as central nodes in global biotechnology networks, they gain considerable economic advantages. Their prominence attracts investment, talent, and partnerships from around the world, fueling further innovation and bolstering their competitive position. In contrast, late adopters risk being sidelined in the global biotech industry. They may find themselves dependent on early adopters for access to vital biotech products and services, potentially facing higher costs and reduced availability. Additionally, their lagging capabilities may deter investment and talent, further widening the gap with early adopters.

The divergence in biotechnology capabilities and influence could exacerbate global inequities, fostering a world where access to the benefits of biotechnology—whether in health, agriculture, or industry—is unevenly distributed. This situation could lead to

growing disparities in health outcomes, economic prosperity, and overall quality of life between nations. Furthermore, the concentration of power in a few early adopter nations might stifle global collaboration, hinder knowledge sharing, and create a more fragmented and competitive global biotech landscape.

Twenty years of research and billions of dollars of investment have commenced the process of biotechnology divergence. It is not guaranteed to continue, though absent a focusing event possessing significant harmful consequences to incentivize early adopters to internationally harmonize their technology modernization strategies, there is little incentive for early adopters to change their perceived prospects and sacrifice the potential economic, health, and defense rewards. Moreover, the clues of what that world might look like are unfolding and have considerable ramifications for the biotechnology marketplace of 2030 and beyond.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Abels, G. (2005). The long and winding road from Asilomar to Brussels: science, politics and the public in biotechnology regulation. *Sci. as Cult.* 14, 339–353. doi:10.1080/09505430500369087

Adenle, A. A. (2011). Global capture of crop biotechnology in developing world over a decade. *J. Genet. Eng. Biotechnol.* 9, 83–95. doi:10.1016/j.jgeb.2011.08.003

Akin, H., Rose, K. M., Scheufele, D. A., Simis-Wilkinson, M., Brossard, D., Xenos, M. A., et al. (2017). Mapping the landscape of public attitudes on synthetic biology. *BioScience* 67 (3), biw171–300. doi:10.1093/biosci/biw171

Araz, S. (2020). China adopts biotechnology regulation, amid authoritarianism concerns. Center for strategic and international studies. Available at: https://www.csis.org/blogs/strategic-technologies-blog/china-adopts-biotechnology-regulation-amid-authoritarianism.

Austriaco, R. N. P. G. (2020). "Dignity as a faith-based consideration in the ethics of human genome editing," in *Synthetic biology 2020: Frontiers in risk analysis and governance, risk, systems and decisions*. Editors B. D. Trump, C. L. Cummings, J. Kuzma, and I. Linkov (Cham: Springer International Publishing), 389–397. doi:10.1007/978-3-030-27264-7_17

Barkstrom, J. E. (1985). Recombinant DNA and the regulation of biotechnology: reflections on the asilomar conference, ten years after. *Akron Law Rev.* 19, 81–126.

Benelli, G., Canale, A., and Conti, B. (2014). Eco-friendly control strategies against the asian tiger mosquito, *Aedes albopictus*(Diptera: culicidae): repellency and toxic activity of plant essential oils and extracts. *Pharmacol. online* 1, 44–50.

Berg, P., Baltimore, D., Brenner, S., Roblin, R. O., and Singer, M. F. (1975). Summary statement of the Asilomar conference on recombinant DNA molecules. *Proc. Natl. Acad. Sci. U. S. A.* 72, 1981–1984. doi:10.1073/pnas.72.6.1981

Bier, E. (2022). Gene drives gaining speed. *Nat. Rev. Genet.* 23, 5–22. doi:10.1038/s41576-021-00386-0

Bosley, K. S., Botchan, M., Bredenoord, A. L., Carroll, D., Charo, R. A., Charpentier, E., et al. (2015). CRISPR germline engineering--the community speaks. *Nat. Biotechnol.* 33, 478–486. doi:10.1038/nbt.3227

Brune, K., and Bayer, T. (2012). Engineering microbial consortia to enhance biomining and bioremediation. *Front. Microbiol.* 3, 203. doi:10.3389/fmicb.2012.00203

Cameron, D. E., Bashor, C. J., and Collins, J. J. (2014). A brief history of synthetic biology. *Nat. Rev. Microbiol.* 12, 381–390. doi:10.1038/nrmicro3239

Carter, S. R., Rodemeyer, M., Garfinkel, M. S., and Friedman, R. M. (2014). *Synthetic biology and the U.S. Biotechnology regulatory system: Challenges and options (No. DOE-JCVI-SC0004872)*. Rockville, MD (United States): J. Craig Venter Institute. doi:10.2172/1169537

Corey, L., Mascola, J. R., Fauci, A. S., and Collins, F. S. (2020). A strategic approach to COVID-19 vaccine R&D. *Science* 368, 948–950. doi:10.1126/science.abc5312

Corona, L., Doutriaux, J., and Mian, S. (2006). *Building knowledge regions in north America: Emerging technology innovation Poles*. doi:10.4337/9781845428013

Cosgriff, C. V., Ebner, D. K., and Celi, L. A. (2020). Data sharing in the era of COVID-19. *Lancet Digit. Health* 2, e224. doi:10.1016/S2589-7500(20)30082-0

Cubillos-Ruiz, A., Guo, T., Sokolovska, A., Miller, P. F., Collins, J. J., Lu, T. K., et al. (2021). Engineering living therapeutics with synthetic biology. *Nat. Rev. Drug Discov.* 20, 941–960. doi:10.1038/s41573-021-00285-3

Cyranoski, D. (2019). The CRISPR-baby scandal: what's next for human gene-editing. *Nature* 566, 440–442. doi:10.1038/d41586-019-00673-1

Devos, Y., Mumford, J. D., Bonsall, M. B., Glandorf, D. C. M., and Quemada, H. D. (2022). Risk management recommendations for environmental releases of gene drive modified insects. *Biotechnol. Adv.* 54, 107807. doi:10.1016/j.biotechadv.2021.107807

Dobrovidova, O. (2019). Russia joins in global gene-editing bonanza. *Nature* 569, 319–320. doi:10.1038/d41586-019-01519-6

Duan, Y., Luo, J., Zhao, L., Zhang, X., Miao, J., Moher, D., et al. (2022). Reporting and data sharing level for COVID-19 vaccine trials: a cross-sectional study. *EBioMedicine* 78, 103962. doi:10.1016/j.ebiom.2022.103962

El Karoui, M., Hoyos-Flight, M., and Fletcher, L. (2019). Future trends in synthetic biology—a report. *Front. Bioeng. Biotechnol.* 7, 175. doi:10.3389/fbioe.2019.00175

Epstein, G. L. (2005). *Global evolution of dual-use biotechnology: A report of the project on technology futures and global power, wealth, and conflict*. CSIS.

Eriksson, D., Custers, R., Edvardsson Björnberg, K., Hansson, S. O., Purnhagen, K., Qaim, M., et al. (2020). Options to Reform the European union legislation on GMOs: risk governance. *Trends Biotechnol.* 38, 349–351. doi:10.1016/j.tibtech.2019.12.016

European Commission (2023). *Proposal for a Regulation of the European Parliament and of the Council on plants obtained by certain new genomic techniques and their food and feed, and amending Regulation*. Brussels, Belgium: European Commission. (EU) 2017/625.

Fabbri, M., Ginoza, M., Assen, L., Jongsma, K., and Isasi, R. (2023). Modeling policy development: examining national governance of stem cell-based embryo models. *Regen. Med.* 18, 155–168. doi:10.2217/rme-2022-0136

Fenwick, M., Kaal, W. A., and Vermeulen, E. P. M. (2017). *Regulation tomorrow: What happens when technology is faster than the law?* doi:10.2139/ssrn.2834531

Greer, S. L., and Trump, B. (2019). Regulation and regime: the comparative politics of adaptive regulation in synthetic biology. *Policy Sci.* 52, 505–524. doi:10.1007/s11077-019-09356-0

Herzog, S. (2017). Ten years after the Estonian cyberattacks: defense and adaptation in the age of digital insecurity. *Geo. J. Int'l Aff.* 18, 67–78. doi:10.1353/gia.2017.0038

Housen-Couriel, D. (2017). *National cyber security organisation, Israel*. Washington, DC: NATO Cooperative Cyber Defence Centre of Excellence.

Hurlbut, J. B. (2015). "Remembering the future: science, law, and the legacy of asilomar," in *Dreamscapes of modernity: Sociotechnical imaginaries and the fabrication of power*. Editors S. Jasanoff, and S.-H. Kim (Chicago: University of Chicago Press). doi:10.7208/chicago/9780226276663.003.0006

Irwin, A. (2021). "Risk, science and public communication: third-order thinking about scientific culture," in *Routledge handbook of public communication of science and technology* (Abingdon: Routledge), 147–162. doi:10.4324/9781003039242-9-9

ISAAA (2019). *Pakistan announces national coordinated trials of the 85 Bt cotton varieties*. Ithaca, New York: International Service for the Acquisition of Agri-biotech Applications.

Jasanoff, S. (2015). "Future imperfect: science, technology, and the imaginations of modernity," in *Dreamscapes of modernity: Sociotechnical imaginaries and the fabrication of power*. Editors S. Jasanoff, and S.-H. Kim (Chicago: University of Chicago Press). doi:10.7208/chicago/9780226276663.003.0001

Jasanoff, S. S. (1987). Contested boundaries in policy-relevant science. *Soc. Stud. Sci.* 17, 195–230. doi:10.1177/030631287017002001

Jiang, L. (2020). Commercialization of the gene-edited crop and morality: challenges from the liberal patent law and the strict GMO law in the EU. *New Genet. Soc.* 39 (2), 191–218. doi:10.1080/14636778.2019.1686968

Kahneman, D., and Tversky, A. (2012). "Prospect theory: an analysis of decision under risk," in *Handbook of the fundamentals of financial decision making, world scientific handbook in financial economics series* (Singapore: World Scientific), 99–127. doi:10.1142/9789814417358_0006

Kingdon, J. W. (1993). How do issues get on public policy agendas. *Sociol. public agenda* 8 (1), 40–53.

Klein, T. M., Fromm, M., Weissinger, A., Tomes, D., Schaaf, S., Sletten, M., et al. (1988). Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. *Proc. Natl. Acad. Sci. U. S. A.* 85, 4305–4309. doi:10.1073/pnas.85.12.4305

Kozovska, Z., Rajcaniova, S., Munteanu, P., Dzacovska, S., and Demkova, L. (2021). CRISPR: history and perspectives to the future. *Biomed. Pharmacother.* 141, 111917. doi:10.1016/j.biopha.2021.111917

Kuiken, T., Dana, G., Oye, K., and Rejeski, D. (2014). Shaping ecological risk research for synthetic biology. *J. Environ. Stud. Sci.* 4, 191–199. doi:10.1007/s13412-014-0171-2

Kuzma, J. (2022). *Governance of gene-edited plants: Insights from the history of biotechnology oversight and policy process theory*. Science, Technology, and Human Values. doi:10.1177/01622439221108225

Lee, K., and Malerba, F. (2017). Catch-up cycles and changes in industrial leadership: Windows of opportunity and responses of firms and countries in the evolution of sectoral systems. *Res. Policy* 46, 338–351. doi:10.1016/j.respol.2016.09.006

Levy, J. S. (1996). Loss aversion, framing, and bargaining: the implications of prospect theory for international conflict. *Int. Political Sci. Rev.* 17, 179–195. doi:10.1177/019251296017002004

Li, F., Zhang, P., Huang, X., Sun, J., and Li, Q. (2022). Emergency decision-making for middle route of south-to-north water diversion project using case-based reasoning and prospect theory. *Sustainability* 14, 13707. doi:10.3390/su142113707

Li, J., Zhao, H., Zheng, L., and An, W. (2021). Advances in synthetic biology and biosafety governance. *Front. Bioeng. Biotechnol.* 9, 598087. doi:10.3389/fbioe.2021.598087

Liebowitz, S. J., and Margolis, S. E. (1995). Path dependence, lock-in, and history. *J. Law, Econ. Organ.* 11, 205–226.

Lucas-Dominguez, R., Alonso-Arroyo, A., Vidal-Infer, A., and Aleixandre-Benavent, R. (2021). The sharing of research data facing the COVID-19 pandemic. *Scientometrics* 126, 4975–4990. doi:10.1007/s11192-021-03971-6

Lyall, C., Papaioannou, T., and Smith, M. J. (2012). *The limits to governance: The challenge of policy-making for the new life sciences*. Farnham: Ashgate Publishing, Ltd.

Lyall, C., and Tait, J. (2019). Beyond the limits to governance: new rules of engagement for the tentative governance of the life sciences. *Res. Policy* 48 (5), 1128–1137. doi:10.1016/j.respol.2019.01.009

Marris, C., and Calvert, J. (2020). Science and technology studies in policy: the UK synthetic biology roadmap. *Sci. Technol. Hum. Values* 45 (1), 34–61. doi:10.1177/0162243919828107

Ma, H., Marti-Gutierrez, N., Park, S. W., Wu, J., Lee, Y., Suzuki, K., et al. (2017). Correction of a pathogenic gene mutation in human embryos. *Nature* 548 (7668), 413–419. doi:10.1038/nature23305

Mandel, G. N. (2013). "Emerging technology governance," in *Innovative governance models for emerging technologies* (Cheltenham: Edward Elgar Publishing), 44–62.

Mao, N., Aggarwal, N., Poh, C. L., Cho, B. K., Kondo, A., Liu, C., et al. (2021). Future trends in synthetic biology in Asia. *Adv. Genet. Hob.* 2, e10038. doi:10.1002/ggn2.10038

Marchant, G., Allenby, B., and Herkert, J. (2011). *The growing gap between emerging technologies and legal-ethical oversight: The pacing problem*. doi:10.1007/978-94-007-1356-7

McCauley, D. J., Pinsky, M. L., Palumbi, S. R., Estes, J. A., Joyce, F. H., and Warner, R. R. (2015). Marine defaunation: animal loss in the global ocean. *Science* 347, 1255641. doi:10.1126/science.1255641

Meng, F., and Ellis, T. (2020). The second decade of synthetic biology: 2010–2020. *Nat. Commun.* 11, 5174. doi:10.1038/s41467-020-19092-2

Mercer, J. (2005). Prospect theory and political science. *Annu. Rev. Political Sci.* 8, 1–21. doi:10.1146/annurev.polisci.8.082103.104911

Midgley, M. (2000). Biotechnology and monstrosity: why we should pay attention to the yuk factor. *Hastings Cent. Rep.* 30, 7–15. doi:10.2307/3527881

Mitalipov, S. (2017). Correction of a pathogenic gene mutation in human embryos. *Nature* 548, 413–419. doi:10.1038/nature23305

Moore, S. (2020). China'S role in the global biotechnology sector and implications for U.S. Policy. Brookings institution. Avaialable at: https://www.brookings.edu/wp-content/uploads/2020/04/FP_20200427_china_biotechnology_moore.pdf.

Mueller, S. (2019). Are market GM plants an unrecognized platform for bioterrorism and biocrime? *Front. Bioeng. Biotechnol.* 7, 121. doi:10.3389/fbioe.2019.00121

National Academies of SciencesEngineering, and Medicine (2018). *Biodefense in the age of synthetic biology*. Washington (DC): National Academies Press.

Parthasarathy, S. (2015). Governance lessons for CRISPR/Cas9 from the missed opportunities of asilomar. *EBEM* 6, 305–312. doi:10.1615/EthicsBiologyEngMed.2016016470

Reuters (2022). China to allow gene-edited crops in push for food security-Reuters. Available at: https://www.reuters.com/world/china/china-drafts-new-rules-allow-gene-edited-crops-2022-01-25/ (Accessed May 25, 2023).

Ross, M. (2020). Prospect theory and evolving SOPs: modulating reference points in a deteriorating Afghanistan, soapbox 360. Available at: https://soapbox360.org/2020/03/19/prospect-theory-and-evolving-sops-modulating-reference-points-in-a-deteriorating-afghanistan/ (Accessed June 29, 2023).

Sagar, A., Daemmrich, A., and Ashiya, M. (2000). The tragedy of the commoners: biotechnology and its publics. *Nat. Biotechnol.* 18, 2–4. doi:10.1038/71861

Science (2023). Chinese scientist who produced genetically altered babies sentenced to 3 years in jail. Available at: https://www.science.org/content/article/chinese-scientist-who-produced-genetically-altered-babies-sentenced-3-years-jail (Accessed May 23, 2023).

Singh, S., McNab, C., Olson, R. M., Bristol, N., Nolan, C., Bergstrøm, E., et al. (2021). How an outbreak became a pandemic: a chronological analysis of crucial junctures and international obligations in the early months of the COVID-19 pandemic. *Lancet* 398, 2109–2124. doi:10.1016/S0140-6736(21)01897-3

State Council of China (2019). Regulations of the people's Republic of China on the administration of human genetic resources. State Decree No, 717, Available at: https://www.gov.cn/zhengce/content/2019-06/10/content_5398829.htm.

Sun, T., Song, J., Wang, M., Zhao, C., and Zhang, W. (2022). Challenges and recent progress in the governance of biosecurity risks in the era of synthetic biology. *J. Biosaf. Biosecurity* 4 (1), 59–67. doi:10.1016/j.jobb.2022.02.002

Tatar, M., Shoorekchali, J. M., Faraji, M. R., and Wilson, F. A. (2021). International COVID-19 vaccine inequality amid the pandemic: perpetuating a global crisis? *J. Glob. Health* 11, 03086. doi:10.7189/jogh.11.03086

Titus, A. J., van Opstal, E., and Rozo, M. (2020). Biotechnology in defense of economic and national security. *Health secur.* 18, 310–312. doi:10.1089/hs.2020.0007

Trump, B. D., Foran, C., Rycroft, T., Wood, M. D., Bandolin, N., Cains, M., et al. (2018). Development of community of practice to support quantitative risk assessment for synthetic biology products: contaminant bioremediation and invasive carp control as cases. *Environ. Syst. Decis.* 38, 517–527. doi:10.1007/s10669-018-9710-9

Trump, B. D., Siharulidze, G., and Cummings, C. L. (2020). "Synthetic biology and risk regulation: the case of Singapore," in *Synthetic biology 2020: Frontiers in risk analysis and governance, risk, systems and decisions*. Editors B. D. Trump, C. L. Cummings, J. Kuzma, and I. Linkov (Cham: Springer International Publishing), 297–312. doi:10.1007/978-3-030-27264-7_13

Trump, B. D. (2017). Synthetic biology regulation and governance: lessons from TAPIC for the United States, European Union, and Singapore. *Health Policy, Gov. intersectoral action* 121, 1139–1146. doi:10.1016/j.healthpol.2017.07.010

US Congress (2016). *The Frank R Lautenberg chemical safety for the 21st Century Act pub L.* (June 22, 2016) [2017 March 6].

US Department of Agriculture (2018). Genetically engineered soybean, cotton, and corn seeds have become widely adopted. https://www.ers.usda.gov/data-products/chart-gallery/gallery/chart-detail/?chartId=90362.

Uzogara, S. G. (2000). The impact of genetic modification of human foods in the 21st century: a review. *Biotechnol. Adv.* 18, 179–206. doi:10.1016/s0734-9750(00)00033-1

Wang, F., and Zhang, W. (2019). Synthetic biology: recent progress, biosafety and biosecurity concerns, and possible solutions. *J. Biosaf. Biosecurity* 1, 22–30. doi:10.1016/j.jobb.2018.12.003

Wareham, C., and Nardini, C. (2015). Policy on synthetic biology: deliberation, probability, and the precautionary paradox. *Bioethics* 29, 118–125. doi:10.1111/bioe.12068

Wei, J., Yi, X., Yang, X., and Liu, Y. (2023). Blockchain-based design of a government incentive mechanism for manufacturing supply chain data governance. *Sustainability* 15, 6968. doi:10.3390/su15086968

Wu, X., Ma, R., and Shi, Y. (2010). How do latecomer firms capture value from disruptive technologies? A secondary business-model innovation perspective. *IEEE Trans. Eng. Manag.* 57, 51–62. doi:10.1109/TEM.2009.2033045

Yrjola, S., Ahokangas, P., and Matinmikko-Blue, M. (2022). Value creation and capture from technology innovation in the 6G era. *IEEE Access* 10, 16299–16319. doi:10.1109/ACCESS.2022.3149590

Yuan, L., He, W., Degefu, D. M., Kong, Y., Wu, X., Xu, S., et al. (2022). Elucidating competing strategic behaviors using prospect theory, system dynamics, and evolutionary game: a case of transjurisdictional water pollution problem in China. *Environ. Sci. Pollut. Res. Int.* 29, 20829–20843. doi:10.1007/s11356-021-17034-z

Zhang, D., Hussain, A., Manghwar, H., Xie, K., Xie, S., Zhao, S., et al. (2020). Genome editing with the CRISPR-cas system: an art, ethics and global regulatory perspective. *Plant Biotechnol. J.* 18, 1651–1669. doi:10.1111/pbi.13383

Zhang, D., and Lie, R. K. (2018). Ethical issues in human germline gene editing: a perspective from China. *Monash Bioeth. Rev.* 36, 23–35. doi:10.1007/s40592-018-0091-0

Zhang, K. H. (2020). Industrial policy and technology innovation under the US trade war against China. *Chin. Econ.* 53, 363–373. doi:10.1080/10971475.2020.1730553

Zhang, X., Zhao, C., Shao, M.-W., Chen, Y.-L., Liu, P., and Chen, G.-Q. (2022). The roadmap of bioeconomy in China. *Eng. Biol.* 6, 71–81. doi:10.1049/enb2.12026

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Systematic Review
PUBLISHED 04 September 2023
DOI 10.3389/fbioe.2023.1237561

OPEN ACCESS

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RECEIVED 09 June 2023
ACCEPTED 17 August 2023
PUBLISHED 04 September 2023

CITATION
da Silva RGL and Blasimme A (2023), Organ chip research in Europe: players, initiatives, and policies.
Front. Bioeng. Biotechnol. 11:1237561.
doi: 10.3389/fbioe.2023.1237561

Organ chip research in Europe: players, initiatives, and policies

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Background: Organ chips are microfabricated devices containing living engineered organ substructures in a controlled microenvironment. Research on organ chips has increased considerably over the past two decades.

Aim: This paper offers an overview of the emerging knowledge ecosystem of organ chip research in Europe. Method: This study is based on queries and analyses undertaken through the bibliometric software Dimensions.ai.

Results: Organ chip research has been rapidly growing in Europe in recent years, supported by robust academic science consortia, public-private initiatives, dedicated funding, and science policy instruments. Our data shows that previous investment in basic and fundamental research in centers of excellence in bioengineering science and technology are relevant to future investment in organ chips. Moreover, organ chip research in Europe is characterized by collaborative infrastructures to promote convergence of scientific, technical, and clinical capabilities.

Conclusion: According to our study, the knowledge ecosystem of organ chip research in Europe has been growing sustainably. This growth is due to relevant institutional diversity, public-private initiatives, and ongoing research collaborations supported by robust funding schemes.

KEYWORDS

organ chip, tissue chip, microphysiological systems (MPS), biomedical engineering, bioengineering, knowledge ecosystems, science policy, bibliometrics

1 Introduction

Organ chips are miniature *in vitro* models of human organs created for biomedical research and drug discovery. Their aim is to mimic the functional components and characteristics of human organs and tissues, replicating the dynamic behavior, functionality, and pathophysiological responses of a living organism (Mummery et al., 2016; Sauer and Howard, 2018). They are manufactured at microscale and enable real-time monitoring (Mastrangeli et al., 2019a). The design of organ chips is carefully tailored to recapitulate the physiological characteristics of human organs, including specific cell types, their ratios, and the culture conditions needed to maintain viability (Huh et al., 2010; Kim and Takayama, 2015). Adult stem cells, primary patient cells, or commercially available cell lines can be used to develop organ-specific chips. Beyond offering models to study organ physiology, organ chip research allows scientists to overcome the limitations of using animal models to analyze drug response (van der Meer and van den Berg, 2020; Horejs, 2021). It is relevant to mention that the production of organ chips is a technically complex undertaking, and processes and

technologies in this field continue to evolve at a considerable pace (Strelez et al., 2023). Moreover, organ chip research is a multidisciplinary domain merging biology, physiology, engineering, and microfabrication techniques. Each discipline contributes to the successful creation of microfluidic devices that mimic the structure and function of human organs, allowing researchers to study complex physiological processes and diseases in a controlled laboratory setting. The integration of different forms of expertise is key to the further development of the field. At the same time, organ chip research draws on consolidated advances in the field of tissue engineering, such as scaffold design and the experimental control of cell signaling and biomaterial interaction (Ahmed and Teixeira, 2022; Leung et al., 2022).

Technical questions about the development of organ chips have attracted attention from scientists, bioengineers, and technology developers from academia and industry. A growing number of events and conferences worldwide focus on advancing the state of the art of design and manufacturing of complex microphysiological systems, and connecting organizations in order to foster the introduction of organ chips as suitable animal substitutes for clinical trials. In Europe, the 2nd Annual Microphysiological Systems World Summit (Berlin, June 26–30, 2023) and the 3rd Next Gen Organ-on-Chip and Organoids workshop (Technopark Zurich, August 24–25, 2023) are examples of events that gather the community to discuss ways to accelerate the translation of advanced *in vitro* models in clinical and drug development. Additionally, they aim to expand upon action plans to address barriers associated with the adoption of new methods and technologies in a regulated environment (Cruelty Free Europe, 2023).

This paper explores the social and regulatory aspects of organ chip knowledge ecosystems in Europe. Due to the fast-rising number of global players in this field in the United States and Asia, it is critical to know how European organizations are positioned, and the types of policies and initiatives that could promote this field in the coming years.

2 Methods

This study offers an empirically grounded analysis of the knowledge ecosystem of organ chips in Europe. The concept of "knowledge ecosystems" is employed in science and technology studies to describe how scientific actors, funders, societal stakeholders, and regulators form communities of practice around specific forms of knowledge, with the aim of promoting, channeling, and regulating scientific activities in that area (Järvi et al., 2018). Knowledge ecosystems typically take the form of research networks and scientific infrastructure, composed of public and private research centers, consortia, civil society organizations, science policy actors, regulators, and firms, all collaborating to produce new knowledge and technologies (Järvi et al., 2018; da Silva et al., 2021). Increasingly, knowledge ecosystems are comprised of actors across different geographies and are typically formed in the early stages of research and development, prior to the competitive phases of innovation and commercialization.

Dimensions.ai is a scientific research database facilitating the exploration of research grant repositories, publications, clinical trials, patents, and policy documents. Dimensions.ai aggregates data from a variety of bibliographic repositories that are widely in use in academia, offering a powerful interface to customize and visualize results, thus facilitating data extraction and interpretation. The ability of Dimensions.ai to retrieve information from diverse data sources and explore how data are connected enables a broader and more insightful picture of scientific trends than is available from other scientific databases, making it well-suited to obtain a preliminary overview of an emerging knowledge ecosystem. There are similar tools available to run bibliometric analyses or visualize trends in academic publications, patent filings, and clinical trials registrations, e.g., VOSviewer, CiteSpace, and Netdraw (which extract data from publications in PubMed, Scopus, Web of Science), Orbit Intelligence and PatSeer (patents), and from Clinical Trials.gov and other repositories maintained by the National Institutes of Health of the United States (for information and results from ongoing or concluded clinical trials).

We used Dimensions.ai (Digital Science, 2023) to explore the organ chip knowledge ecosystem taking shape in five European countries (Germany, Netherlands, United Kingdom, Italy, and Switzerland). This selection represents the top five countries in Europe by number of publications about organ chips (Germany: 334; United Kingdom: 225; Netherlands: 155; Switzerland: 115; and Italy: 105). To our knowledge, Dimensions.ai is unique in combining multiple data sources from academic research organizations and commercial entities in the same platform (along with data from policy documents, national and transnational grant repositories, and publications in preprint). It improves analytical capacity by facilitating understanding of the knowledge ecosystem as a multi-disciplinary, multi-sectoral, and interconnected landscape, to an extent not possible with individual data queries. With the implementation of a robust automated system on this platform, which is continuously being tested by the platform's staff to extract reliable data from official sources, the authors are confident that the accessed information maintains a high level of trustworthiness and accuracy.

While Dimensions.ai is an effective tool for analyzing research data in an integrated manner, and providing an overview of key players in a given scientific domain, it has some limitations. While this database provides access to verified research data, coverage, timeliness, and quality of the data may not be complete for all academic domains, sub-fields, or themes. For the time being, the tool provides only partial access to research data from private sector R&D activities. It should also be noted that Dimensions.ai, like similar tools, evolves continually to include more data sources and analytic features.

We explored the knowledge ecosystem of organ chip innovation in Europe along four analytic dimensions: publications trends, research organizations, research funding, and policy trends. In the results section, we illustrate our findings for each dimension.

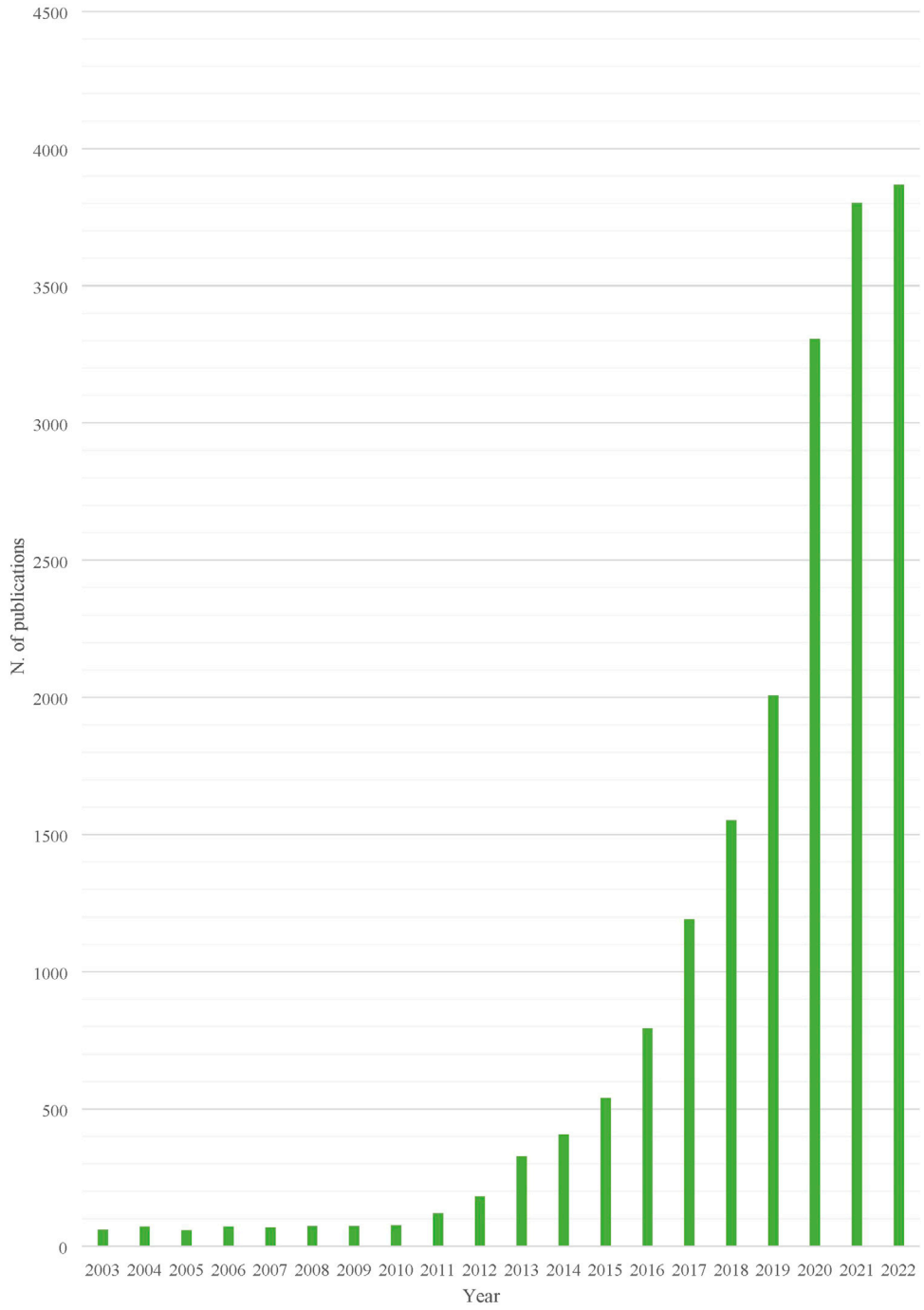


FIGURE 1
Yearly publications on Organ Chips (World, Total), 2003–2022.

We collected data on Dimensions.ai by searching for documents through a thematic query string ("organ-on-a-chip*" OR "organ chip*" OR "tissue chip*" OR "microphysiological systems"); we limited our search to the last 20 years (2003–2022). In total, we retrieved 18,654 publications and 676 grants (search conducted on 20 February 2023). Given the exploratory nature of the present study, we did not use exclusion criteria to screen our results, but subsequently focused our attention on the above-mentioned five countries. With this geographical restriction, we retrieved for the same period 3,991 publications (3,398 articles, 399 book chapters, 146 preprints, 39 conference proceedings, 11 books, and 111 grants). More than two-thirds (67.7%, N = 2,707) of the retrieved publications were published in open access.

We analyzed this data to understand current publication trends in a country-specific manner.

To understand which research organizations are most active in the space of organ chip research, we applied filters to produce a

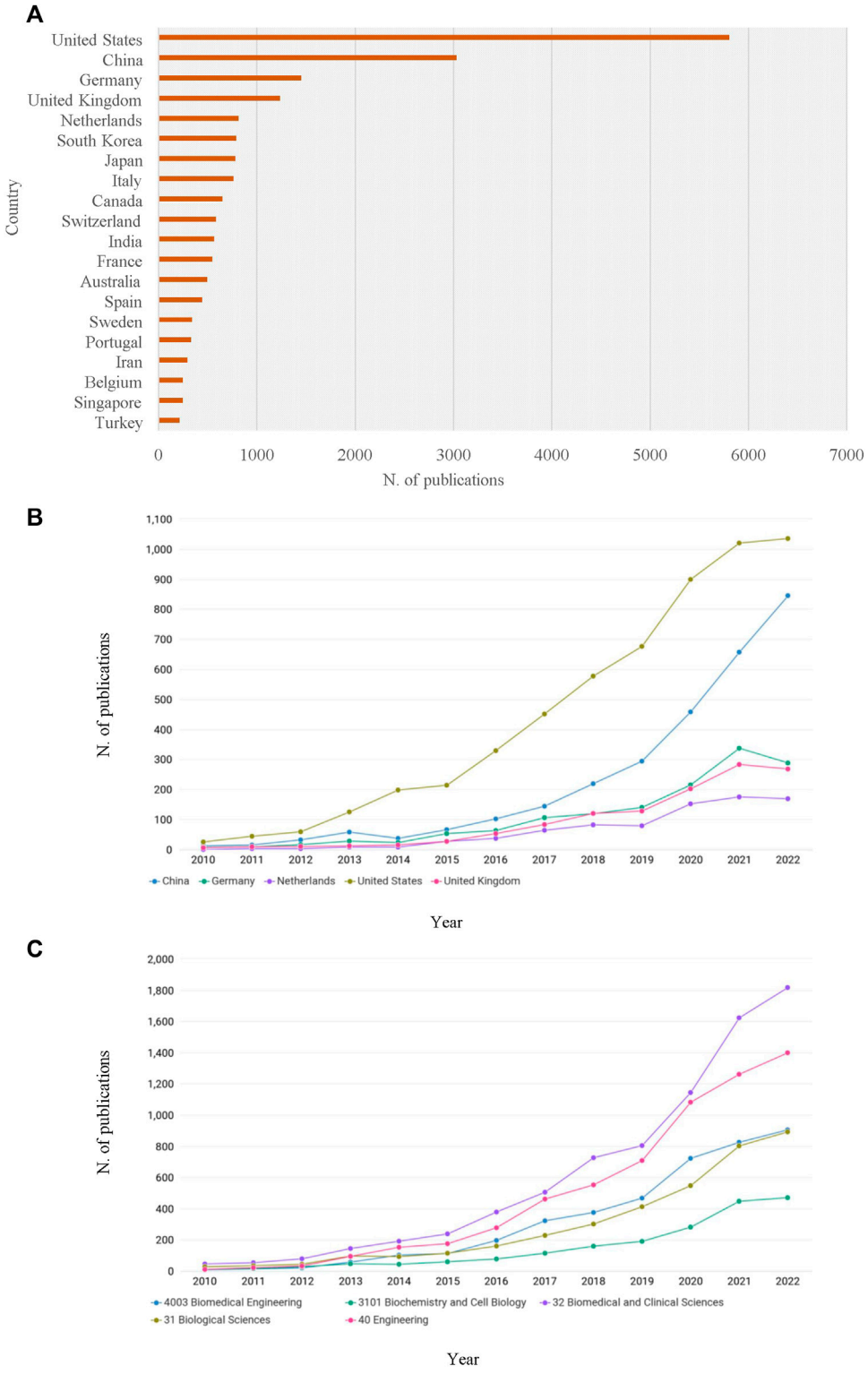
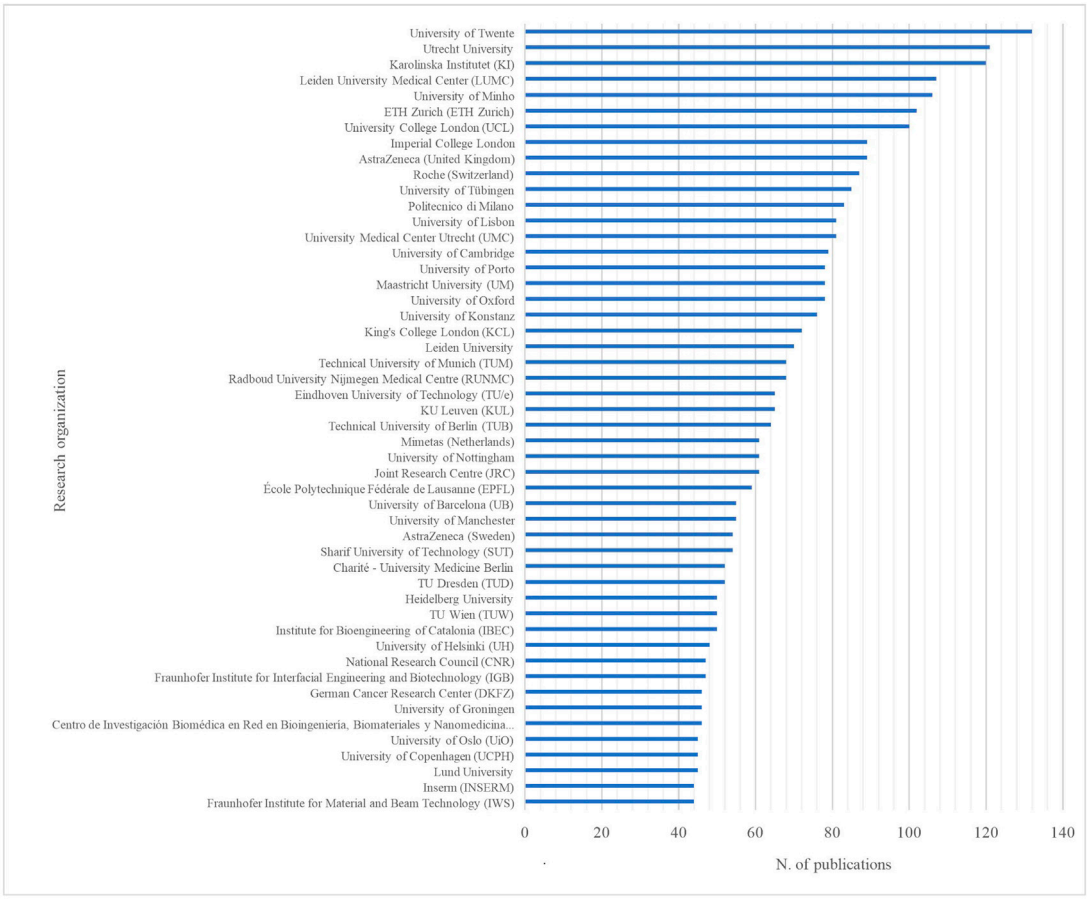


FIGURE 2
Publications, selected countries (Total, Timeline and Research categories) 2010–2022.

ranking of European research institutions with the highest number of publications in the field.

Dimensions.ai also enabled us to extract information about research funding agencies supporting organ chip research, and to collect relevant policy documents. Data on initiatives and regulations were extracted manually between March and May 2023, through literature review, reading of policy documents, and consultation of key funding agency websites, the European



Country	Research organization	n. publications	citations (total)
Netherlands	University of Twente	132	4949
	Utrecht University	121	5158
	Leiden University Medical Center (LUMC)	107	4034
	University Medical Center Utrecht (UMC)	81	3849
	Maastricht University (UM)	78	2962
Germany	University of Tübingen	85	1935
	University of Konstanz	76	3611
	Technical University of Munich (TUM)	68	2764
	Technical University of Berlin (TUB)	64	3406
	Charité - University Medicine Berlin	52	1451
United Kingdom	University College London (UCL)	100	2475
	AstraZeneca (United Kingdom)	89	2396
	Imperial College London	89	3870
	University of Cambridge	79	2471
	University of Oxford	78	3122
Italy	Politecnico di Milano	83	2777
	Joint Research Centre (JRC)	61	2437
	National Research Council (CNR)	47	1075
	Italian Institute of Technology	42	843
	University of Milan (UNIMI)	42	1227
Switzerland	ETH Zurich (ETH Zurich)	102	5439
	Roche (Switzerland)	87	2367
	École Polytechnique Fédérale de Lausanne (EPFL)	59	3026
	University of Basel	43	2151
	University of Bern (UB)	33	1048

FIGURE 3
Number of publications by research organization (Top-50, selected Countries), 2003–2022.

Commission and national medical agencies from each of the five selected countries.

Our method has limitations. As an exploratory study intended to capture a field overview of organ chip research initiatives in Europe, the method targeted collection and analysis of data on the general characteristics of the knowledge ecosystem. As result, initiatives in countries not ranked highest by number of publication, and recent projects and consortia that have not

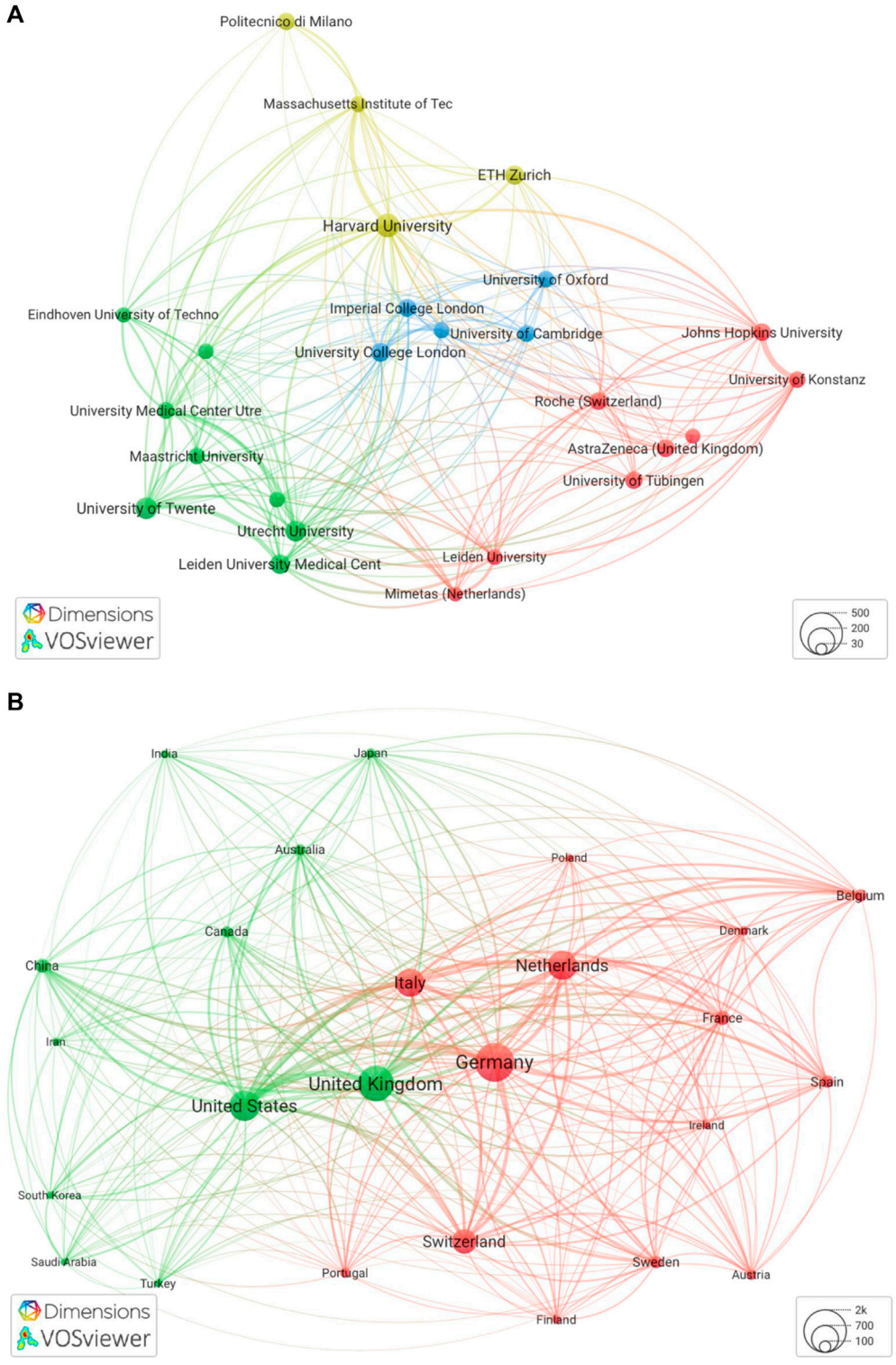


FIGURE 4
Network of research collaborations, selected organizations and countries (2003–2022)

yet produced scientific publications, are not included. Despite such limitations, our study offers valuable insight into the innovation landscape surrounding organ chip research in Europe. This study can thus contribute to a clearer understanding of the knowledge ecosystem of organ chip research and help identify key player in this domain.

TABLE 1 Selected initiatives on organ chips research, selected countries, 2003–2022.

Year	Initiative	Anacronym	Country of headquarters	Project aims
2017 - current (until 2027)	Netherlands Organ-on-Chip Initiative Project	NOCI	Netherlands	Netherlands Organ-on-Chip Initiative (NOCI) has been awarded a prestigious NWO Gravitation subsidy (Zwaartekracht premie) of 18.8 million euros. NOCI aims at creating a new platform, based on a combination of human stem cells and microchips, to learn more about the development of diseases and to better predict the effect of medicines, and will be a decisive step towards personalized healthcare. (Netherlands Organ-on-Chip Initiative, 2023)
2015–current	Institute for human organ and Disease Model Technologies	hDMT		The Institute for human organ and Disease Model Technologies (hDMT) is a consortium consisting of 14 partner organizations, including technical universities, university medical centers and knowledge institutes. It brings together researchers from different disciplines, varying from technologists and biologists to pharmacologists and clinicians (hDMT, 2023)
2017–2019	Organ-on-chip in development	ORCHID		The Organ-on-Chip development project (ORCHID) is an EU initiative, coordinated by Leiden University Medical Center and the Dutch Organ-on-Chip consortium hDMT. ORCHID aims to create a roadmap for organ-on-chip technology and to build a network of all relevant stakeholders in this field (ORCHID, 2023)
2018–current	European Organ-on-chip Society	EURoOCs		The European Organ-on-chip Society (EURoOCs) is an independent not-for-profit organization aimed at encouraging Organ-on-Chip research and development, to share and advance knowledge and expertise in the field (EUROoCS, 2023)
2021–current	SMART Organ-on-chip Project	SMART-OoC		The SMART Organ-on-chip Project aims to "develop and integrate 1) a standardized microfluidic SMART docking plate into which chip modules can be plugged
				2) technical chip modules for microfluidic actuation and sensing
				3) readout technologies for multiparameter monitoring; and 4) prototype tissue chip modules with 3D organ architectures and integrated tissue microenvironment (. . .)
				5) demonstrate functionality of the SMART OoC models by inducing inflammation and testing drugs (SMART OoC, 2023)
2023–current	Organ-on-Chip Centre Twente	OoCCT		The Organ-on-Chip Centre Twente (OoCCT) is a centre of expertise supported by the MESA + Institute and the TechMed Centre of the University of Twente. OoCCT aims to provide services to researchers and companies outside of the University of Twente and give them access to the technology in order to accelerate innovation and real-world application of Organs-on-Chips (University of Twente, 2023b)
2018–2022	Organ-on-a-chip Technology Network	OCTN	United Kingdom	The United Kingdom Organ-on-a-Chip Technologies Network (OCTN) was established in 2018 to represent the United Kingdom community of scientists, industrialists, clinicians, funders and regulators working in the area of organ-on-a-chip technology (University of Twente, 2023a)

(Continued on following page)

TABLE 1 (*Continued*) Selected initiatives on organ chips research, selected countries, 2003–2022.

Year	Initiative	Anacronym	Country of headquarters	Project aims
2020 - current	Centre for Predictive *in Vitro* Models (QM + Emulate Centre) at Queen Mary University of London	OCI/QMU		The CPM brings together academics developing and using predictive *in vitro* models across the faculties of Science and Engineering and Medicine and Dentistry at Queen Mary University of London (QMUL Emulate Centre, 2023)
2021 - current	The Wellcome Leap Health Breakthrough Network - Human Organs, Physiology and Engineering HOPE Program	HOPE		The HOPE Program "aim to leverage the power of bioengineering to advance stem cells, organoids, and whole organ systems and connections that recapitulate human physiology *in vitro* and restore vital functions *in vivo*." According to its official website (HOPE Program, 2023) the program has two key goals: "1. Bioengineer a multiorgan platform that recreates human immunological responses with sufficient fidelity to double the predictive value of a preclinical trial with respect to efficacy, toxicity and immunogenicity for therapeutic interventions targeting cancer, autoimmune and infectious diseases, and 2. Demonstrate the advances necessary to restore organ functions using cultivated organs or biological/synthetic hybrid systems."
(2016) 2021 - current	MicroOrganoLab Tubingen	OC Tubingen	Germany	The initiative µOrganoLab brings basic and translational research and people from different disciplines working together to develop Organ-on-Chip systems as well as enabling technologies to better understand human biology (Micro Organo Lab Tubingen, 2023)
2021–current	Organ-on-a-chip Working Group - Natural and Medical Sciences Institute	NMI Organ-on-a-chip		The Organ-on-a-chip Working Group of the Natural and Medical Sciences Institute of Reutlingen, Germany (NMI Naturwissenschaftliches und Medizinisches Institut) is an interdisciplinary team working at the interface between material and engineering sciences, physics, biology and medicine. The initiative is driven by the principle of reducing use and necessity of animal testing according to the 3R principle (Replace, Reduce, Refine), as well as "to increase the transferability of preclinical results to the clinical phases and thus to make the entire development more cost-effective, safer and faster." (NMI Organ-on-a-chip)
2021 - current	3R-Center Tübingen for *In vitro* Models and Alternatives to Animal Testing	3R Tubingen		The 3R Tubingen Center for *in vitro* Models and Alternatives to Animal Testing (3R Tubingen) aims to create a broad, interdisciplinary awareness of 3R approach with a focus on "Replacement", working on the development of a technology platform for own development or to the 3R-Network Baden-Württemberg partners. (3R Tubingen, 2023)
2020 - current	Organ Chips Group German Cancer Research Center	Orgn Chips DKFZ		The Epithelium Microenvironment Interaction Laboratory is a research group based in the German Cancer Research Center in Heidelberg, and it is specialized in developing and manipulation of human organoid and organ-on-a-chip models to study the roles of bacteria, immune cells and other microenvironmental factors in cancer. The group takes place in the research division Microbiome and Cancer, a bridging division between DKFZ Heidelberg and the Weizmann Institute of Science of Israel

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TABLE 1 (*Continued*) Selected initiatives on organ chips research, selected countries, 2003–2022.

Year	Initiative	Anacronym	Country of headquarters	Project aims
2022 - now	Società Italiana Organ-on-Chip	SIOoC	Italy	The Italian Organ-on-Chip Society (SIOoC) was founded in 2022 aiming to become a meeting point for Italian researchers working in the field of organ-on-chip development. According to its website, "SIOoC promotes advanced training and scientific dissemination on the issue of organ-on-chip. It also promotes dialogue with stakeholders (companies, institutions, regulatory bodies), also through the establishment of thematic tables." (SIOoC, 2022)

3 Results

3.1 Publication trends

For the last decade, publications in the field of tissue or organ chips have burgeoned globally (Figure 1). The United States leads by number of publications (n = 5,797), followed by China (n = 3,028). In the European context, publications are most prevalently produced in Germany (n = 1,453), the United Kingdom (n = 1,233), the Netherlands (n = 814), Italy (n = 762) and Switzerland (n = 582). Information about yearly publications by country is available in Figure 1.

From a disciplinary perspective, publications were most frequently classified as "Biomedical and Clinical Sciences", "Engineering", "Biological sciences", "Biomedical engineering", "Medical Biotechnology", or a combination thereof. (Figure 2C). While the rate of publication in the domain of organ chip research was relatively low up until 2010 (fewer than 20 publications annually), it began to grow steadily over the last decade, from 60 publications in 2014 to 919 in 2021, with a small decrease in 2022 (n = 814). The growth in the number of publications in selected European countries from 2010 is available in Figure 2B.

Globally, Harvard University is the leading research organization with 640 publications, followed by the Massachusetts Institute of Technology (n = 293). These figures may reflect pioneering work at Harvard's Wyss Institute, where the first organ chip was developed in 2010 (Huh et al., 2010). In Europe, the most active research institution in the field is the University of Twente with 132 publications, followed by Utrecht University (n = 121), Karolinska Institute (n = 120), Leiden University Medical Center (n = 107), and the University of Minho (n = 106) (Figure 2A).

European research organizations have made significant contributions to organ chip research in recent years. In Germany, the University of Tübingen, University Konstanz, and the Technical University of Munich are the top-ranked research organizations in terms of the number of publications, with 85, 76, and 68 publications, respectively. The Technical University of Berlin and the Technical University of Dresden follow closely behind with 64 and 52 publications, respectively.

In the United Kingdom, the majority of organ chips publications are housed in research organizations located in the British Golden Triangle, specifically the University College London, AstraZeneca Headquarters, and Imperial College London, with 100, 89, and 89 publications respectively. The University of Cambridge follows with 79 publications.

In the Netherlands, the University of Twente, Utrecht University, and Leiden University Medical Center are the top-ranked research organizations in terms of the number of publications, with 132, 121, and 107 publications, respectively. The University Medical Center Utrecht and the University of Maastricht are also active in the field, with 81 and 78 publications, respectively. Additionally, the company Mimetas, responsible for the development of an early successful organ chip, has 61 publications.

In Italy, Politecnico di Milano, the European Commission's Joint Research Centre, and the National Research Council hold the highest number of publications, with 84, 61, and 48 publications, respectively. The Italian Institute of Technology and the University of Milan follow closely behind with 47 publications each.

In Switzerland, the Institutes of Technology (ETH Zurich and EPFL) and Roche are at the forefront of organ chip research, with 102, 59, and 87 publications, respectively. The University of Basel and the University of Zurich are also central figures in the national knowledge landscape, with 43 and 39 publications, respectively.

Figure 3 presents a list of top-ranked research organizations by number of organ chip publications in the five selected countries.

3.2 Research collaborations

We studied the landscape of research collaborations among scientists active in our subset of European countries. Co-authorship analysis was limited to 25 research organizations, to illuminate key players and allow for visualization of collaborations and clusters (Figure 4). Figure 4A illustrates the four main clusters (see Figure 4A). Two clusters are more geographically homogeneous, in the Netherlands (green) and the United Kingdom (blue); the remaining two clusters are more international, evidencing collaboration with research centers outside a specific geographic area (see yellow cluster featuring Harvard and MIT) as well as the presence of pharmaceutical partners (see red cluster featuring Roche and Astra Zeneca).

TABLE 2 R&D Expenditure by funding agencies (Grants), Selected countries, 2003–2022.

Location of research organization (country)	Funder/Agency	Country	Number of grants	Funding amount (aggregated, in Euros)
Germany	European Commission (EC)	EC	8	28.151.392,00
	Federal Ministry of Education and Research (BMBF)	Germany	16	8.621.578,00
	European Research Council (ERC)	EC	1	1.918.038,00
	Engineering and Physical Sciences Research Council (EPSRC)	United Kingdom	1	1.332.319,00
	Medical Research Council (MRC)	United Kingdom	1	336.586,00
	Deutsche Forschungsgemeinschaft (DFG)	Germany	5	information not available
	Volkswagen Foundation (VolkswagenStiftung)	Germany	2	information not available
	German Association of Joint Industrial Applied Research Institutes (AIF)	Germany	1	information not available
United Kingdom	European Commission (EC)	EC	8	29.924.923,63
	Engineering and Physical Sciences Research Council (EPSRC)	United Kingdom	27	1.963.592,45
	Medical Research Council (MRC)	United Kingdom	7	1.482.581,54
	Biotechnology and Biological Sciences Research Council (BBSRC)	United Kingdom	7	1.188.159,13
	Swiss National Science Foundation (SNF)	Switzerland	1	680.649,29
	Innovate United Kingdom (Innovate United Kingdom)	United Kingdom	2	426.914,41
	Wellcome Trust (WT)	United Kingdom	1	340.075,60
	National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3Rs)	United Kingdom	3	194.618,37
	Deutsche Forschungsgemeinschaft (DFG)	Germany	1	information not available
	National Research Council (CNR)	Italy	1	information not available
Netherlands	European Commission (EC)	EC	8	19.384.162,38
	European Research Council (ERC)	EC	2	2.078.299,69
	Medical Research Council (MRC)	United Kingdom	1	336.586,37
	Dutch Research Council (NWO)	Netherlands	4	information not available
	Netherlands Organisation for Health Research and Development (ZonMw)	Netherlands	6	information not available
Switzerland	European Commission (EC)	EC	4	10.817.940,65
	Swiss National Science Foundation (SNF)	Switzerland	5	3.473.624,54
	Engineering and Physical Sciences Research Council (EPSRC)	United Kingdom	1	information not available
Italy	European Commission (EC)	EC	3	12.253.602,86
	European Research Council (ERC)	EC	1	153.551,26
	Italian Association for Cancer Research (AIRC)	Italy	2	information not available
	National Research Council (CNR)	Italy	1	information not available

[a]Data displayed in this table does not capture the precise values of R&D expenditure on Organ Chips by country and funding agency. The purpose of this table is, then, serve as reference to what agencies and countries can be highlighted based in data automatically extracted and available in dimensions.ai.
[b]Values were changed from US$ to Euros (1 US$ = 0.89 Euros).
Source: Elaborated by the authors with data from Dimensions.ai. (Digital Sciences)

TABLE 3 Selected regulations on organ chips research, EU level and selected countries, 2003–2022.

Country	Year	Regulation	Anacronym	About
European Union	2007	Regulation on Advanced Therapy Medicinal Products	ATMP Regulation 1394/2007	This Regulation introduces additional provisions to those laid down in Directive 2001/83/EC. It regulates advanced therapy medicinal products which are intended to be placed on the market in Member States and either prepared industrially or manufactured by a method involving an industrial process (European Parliament, 2007)
	2009	EU Regulation 1223/2009	EU Regulation 1223/2009	It covers the safety and efficacy of cosmetic products and requires that cosmetic products be tested in animal models before they can be sold in the EU, and tissue chips can be used as an alternative to animal testing for this purpose (European Parliament, 2009)
	2010	DIRECTIVE 2010/63/EU	DIRECTIVE 2010/63/EU of 22 September 2010	Considered the first explicit move towards new microphysiological systems and other bioengineered alternatives to animal research. This directive requires the use alternative methods to animal testing whenever possible; animal testing is only permitted when no other suitable method is available (European Commission, 2010)
	2017	*In vitro* Diagnostic Medical Devices Regulation	IVDR Regulation (EU) 2017/746	The *In Vitro* Diagnostic Medical Devices Regulation (EU) 2017/746 (IVDR) establishes a new regulatory framework for *in vitro* diagnostic medical devices. It is estimated that around 70% of clinical decisions are made using *in vitro* diagnostic medical devices (European Parliament, 2017)
	2021	European Parliament Resolution on plans and actions to accelerate a transition to innovation without the use of animals in research, regulatory testing and education	2021/2784 (RSP)	The resolution recalls the objectives of the Directive on the protection of animals used for scientific purposes (2010/63/EU) and the replacement of procedures on live animals as soon as it is scientifically possible. It requires the European Commission to establish an inter-service taskforce, including Member States and agencies, to develop action plans to accelerate the development of the alternative animal-free methods, technologies and instruments, and to address implementation and enforcement issues. (European Parliament, 2021a)
United Kingdom	1986 (Amended multiple times)	Animals (Scientific Procedures) Act 1986	THE ANIMALS ACT of 1986	The Animals (Scientific Procedures) Act of 1986 regulates the use of animals in scientific research, including the development and testing including disease models and bioengineered tissues. It requires that animal testing be avoided wherever possible and that alternatives to animal testing be used where available
	2003	The Medicines and Healthcare Products Regulatory Agency	MHRA	The Medicines and Healthcare Products Regulatory Agency (MHRA) was created in 2003 and is responsible for regulating the use of medical devices in the United Kingdom, including tissue chips. Tissue chips that are intended for use as medical devices must meet the safety and performance requirements set out by this agency (MHRA, 2023)
	2004	The National Centre for the Replacement, Refinement, and Reduction of Animals in Research	NC3Rs	The National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) was created in 2004 in response to the House of Lords Select Committee report on Animals in Scientific Procedures. It is a UK-based organization that promotes the development and use of alternatives to animal testing. It provides guidance and support to researchers who wish to use tissue chips as an alternative to animal testing

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TABLE 3 (*Continued*) Selected regulations on organ chips research, EU level and selected countries, 2003–2022.

Country	Year	Regulation	Anacronym	About
	2004	Human Tissue Act	HUMAN TISSUE ACT of 2004	This act regulates the removal, storage, use and disposal of human tissue, including tissue chips or other microphysiological systems that may be derived from human tissue (Human Tissue Act, 2004)
Germany	1961 (Amended in 2019*)	German Medicines Act, The Drug Law	Arzneimittelgesetz, AMG	Act to guarantee safety in respect of the trade in medicinal products in the interest of furnishing both human beings and animals, ensuring in particular the quality, efficacy and safety of medicinal products. (German Medicines Act, 2019)
	1989 (Amended in 2018*)	German Medical Devices Act	Medizinproduktegesetz, MPG	This Act is to regulate the trade in medical devices and, by doing so, to guarantee the safety, suitability and performance levels of medical devices as well safe- guard the health and ensure the necessary protection of patients (German Medical Devices Act, 2018)
	2013	Act on the Protection of Animals Used for Scientific Purposes	Animal Welfare Act of 2013	This Act regulates the use of animals in scientific research, including the use of tissue chips or other microphysiological systems in animal testing. (Animal Welfare Act, 2013)
Netherlands	1999	Dutch Medical Research Involving Human Subjects Act	Wet medisch-wetenschappelijk onderzoek met mensen, WMO	The Dutch Animals Act of 1999 regulates the use of animals in scientific research, including to develop and test tissue chips. It requires that animal testing be avoided wherever possible, and that alternatives such as tissue chips be used instead
	2011	Dutch Animals Act	Wet dieren	Wer dieren attests that animal testing is only permissible when there is no suitable alternative and the purpose of the research outweighs any inconvenience to the animal
	2014	Act on the Use of Animals in Scientific Research	Wet op de Dierproeven	This Act regulates the use of animals in scientific research, including the use of tissue chips or other microphysiological systems in animal testing
Switzerland	2000	Federal Act on Medicinal Products and Medical Devices (Therapeutic Products Act)	SR 812.21 - Federal Act of 15/12/2000	This Act aims to protect human and animal health, and to guarantee that only high quality, safe and effective therapeutic products are placed on the market.
	2005	The Swiss Animal Welfare Act	SR 455 - Animal Welfare Act of 16/12/2005, AniWA	The Swiss Animal Welfare Act of 2005 sets out detailed regulations on animal husbandry and research with animals, and includes Tissue Chips as suitable animal substitute in respect to the 3R principle (Swiss Animal Welfare Act, 2005)
	2011	Federal Act on Research involving Human Beings	SR 810.30 Human Research Act, HRA	This act regulates the ethical and legal requirements for research involving human subjects, including the use of tissue chips or other microphysiological systems that may be derived from human tissue (Swiss Human Research Act, 2011)
Italy	2003	Legislative Decree no. 211/2003	Legislative Decree no. 211/2003	This Legislative Decree establishes specific provisions regarding the conduct of clinical trials, including multi-centre trials on human subjects involving medicinal products as defined in section 1 of Legislative Decree no. 178 of 29 May 1991
	2010	Law on Research and Innovation (240/2010)	Law on Research and Innovation (240/2010)	This law provides a framework for the organization and funding of scientific research, which may include the use of tissue chips or other microphysiological systems

When looking at geographical relatedness in co-authored publications, we identified the existence of two clusters (see Figure 4B). One cluster (red) is composed of mostly European countries, with Germany, the Netherlands, Italy, and Switzerland most strongly represented; the other cluster (green) illustrates the United Kingdom as a major node, but includes mostly non-European countries (the US being the other major node).

3.3 Research consortia

Research efforts in Europe are often organized through consortia. Such initiatives focus on bringing together key players and improving harmonization of technical and experimental standards in the field of organ chip research.

Multiple initiatives have launched over the last decade to promote the successful integration of organ chip technologies in the European biomedical research infrastructure. Two examples are the Organ-on-Chip Development Project (ORCHID) and the Europe Organ-on-chip Society (EUROoCS). ORCHID is an EU initiative, coordinated by Leiden University Medical Center and the Dutch Organ-on-Chip consortium, hDMT. This project received funding from the European Parliament, 2021b research and innovation program (grant n. 766884). The initiative (2017–2019) sought to create a roadmap for organ chip technology development, along with a stakeholder network (ORCHID, 2023).

Likewise, EUROoCS, established in 2018 as a not-for-profit organization, continued many of the efforts of ORCHID, bringing together organ chip scientists, industry, and government regulators in support of research and development (European Organ-on-Chip Society, 2022). Similar to the US context, the EUROoCS has prioritized standardization. In creating their priorities, EUROoCS referenced the success of the National Center for Advancing Translational Sciences (NCATS) (2022) at the National Institutes of Health (NIH), in funding the development of multiple organ chip models, and conducting the external testing and standardization requisite for market acceptance and integration.

Indeed, many policy reports published by the European Commission and other European agencies refer to the success of organ chip models in the US as an example to follow in terms of the knowledge ecosystem that has been established there. In December 2022, the "FDA Modernization Act 2.0." was signed into law by the Biden-Harris administration, representing a major shift in the regulatory landscape that paves the way for innovative modeling approaches in early stages of drug discovery and innovation. Following years of advocacy, the bill officially authorizes the use of alternatives to non-human animal models in pre-clinical pharmaceutical testing. The bill points to cell-based assays, predictive computer models, and organ chips as examples of technologies that can be used in place of non-human animal models, which have long been required by the FDA. With the passage of this bill, animal studies are no longer required in pre-clinical testing whenever an alternative suitable method is available to demonstrate drug safety and efficacy of therapeutic candidates and products (Bill S.5002).

Our study accessed data about multiple national initiatives in the field, described in Table 1 (focused on the top five countries by number of scientific publications).

3.4 Funding

National and European funding agencies play a key role in shaping the organ chip landscape in Europe. Our search enabled us to extract information about funders appearing in the acknowledgment section of publications in the field. Figure 4 ranks the top twenty funding agencies in the field by frequency of acknowledgement. The leading funding agencies (ranked by number of publications resulting from grants) are the European Commission (EC) (n = 16 grants; Aggregate funding amount: USD 46.8 M); the European Research Council (ERC) (n = 4 grants; USD 4.8 M); and the German Federal Ministry of Education and Research (BMBF) (n = 16 grants; USD 9.7M). The research organizations that received more grants from EC and ERC (n = 39 grants combined) were three nascent biotechnology companies, Eveflow (France, n = 6, value in US$ 10.4 mi/EUR 9.27 mi), Mimetas (Netherlands, n = 4, US$ 15.4 mi/EUR 13.72 mi), and Cherry Biotech (France, n = 4, US$ 4.1 mi/EUR 3.65 mi). National funding instruments like the German Research Foundation (Federal Ministry of Education, n = 16, US$ 9.7 mi/EUR 8.64 mi) and the "Engineering and Physical Research Council" of the United Kingdom (EPRSC, n = 27, US$ 2.3 mi/EUR 2.05 mi) have funded projects in public institutes, technical universities, and medical centers, such as the Fraunhofer Society (n = 2, US$ 1.5 mi/EUR 1.34 mi) and Technical University of Berlin in Germany, and the University of Southampton, in partnership with international collaborators from the Max Planck Society (n = 2, US$ 1.6 mi/EUR 1.43 mi). Results about R&D expenditures, including data about countries and funding agencies (extracted from "Location of research organization/Grants": n = 111/Analytical views: Funders), are available in Table 2.

4 Discussion

Our study highlights the significance of institutional diversity, research collaborations, and public-private initiatives that promote organ chip research in Europe, as well as the role of public funding in supporting the knowledge ecosystem in this field.

According to a study by da Silva et al. (2020), R&D initiatives are influenced by cultural and political factors. Our study shows the utility of novel bibliometric tools such as Dimensions.ai to reconstruct emerging knowledge ecosystems. This approach can greatly contribute to the understanding of scientific research practices and inform science policy activities to stimulate innovation in many countries - especially in emerging sectors of biotechnology (Karaulova et al., 2016; Au and da Silva, 2021; Heimeriks and Boschma, 2013; Partelow et al., 2020; da Silva et al., 2023).

Regulation is certainly one of the key political factors affecting innovation. Evens and Kaitin (2015) observe that early regulatory reforms and the standardization of national legal frameworks for research involving bioengineered systems and tools have

significantly influenced the evolution and pathways of bioengineering research and innovation in Europe over past decades (Ewart, 2022).

Research on the role of policies and regulations in the context of organ chip research has so far been limited (Kemp et al., 2020). However, it is possible to observe a close association between the development of this field and the legal framework designed to reduce the use of non-human animals in scientific research (Brackenbury, 2017). Similar associations have been identified in the United States, as noted by Heringa et al. (2020) and da Silva and Blasimme (2023).

In Europe, the explicit effort to foster the development of new microphysiological systems and other bioengineered alternatives to animal research began with the enactment of DIRECTIVE 2010/63/EU on 22 September 2010 (European Commission, 2010). This directive endorses the use of alternative methods to animal testing whenever possible, allowing animal testing only as a last resort when no other suitable method is available (Alternatives to Animal Experimentation ALTEX, 2018; National Center for Advancing Translational Sciences, 2022).

Subsequently, the EU has established a regulatory framework for the utilization of alternative methods to animal testing, which encompasses the validation and acceptance of these methods (Moraes et al., 2013; van Meer et al., 2017; Mastrangeli et al., 2019; Politico, 2021).

As mentioned in Table 2, in September 2021, the European Parliament approved a resolution to stimulate EU members to adopt a strategic plan with "ambitious and achievable objectives and timelines for transitioning to a research system that does not rely on animals for research and testing." (European Parliament, 2021a). While not legally binding, the resolution clearly indicates a policy direction for the European Union and invites further legislative activity that is likely to create incentives for organ chip research in Europe (Human Society International, 2021).

Researchers, investors, and regulators share the belief that human organ chips hold great potential for replacing animal models in drug development and serving as living avatars for personalized medicine. According to Nahle (2022), organ chip technology can be seamlessly integrated into the drug development pipeline, from early drug discovery to preclinical stages. This paradigm shift could lead to a post-animal testing era in drug discovery (Wyss Institute, 2014; Herpers, 2022; Ingber, 2022; Zainzinger, 2022).

Key regulations addressing organ chip research and development activities are available in Table 3.

Dimensions.ai is an effective tool for analyzing data in an integrated manner, and contributes significantly to studies aiming to provide an overview of key players of emerging knowledge ecosystems. The tool, however, is still under development, and has relevant limitations in terms of lacking access to precise data about R&D expenditures from national and supranational levels, or from industry. Dimensions.ai, then, should be taken as a complementary tool to support studies on knowledge ecosystems, gaining explanatory power when combined with multiple methods of data collection, analysis, and visualization.

5 Concluding remarks

Organ chip research has gained international recognition as a prominent area of biomedical engineering innovation in recent years. In Europe, the convergence of research efforts, funding, and regulatory incentives has shaped a robust knowledge ecosystem that places many European research institutions as key international players in the field. More research is needed to monitor whether and how, in coming years, present incentives will continue to promote innovation in organ chip research in the European context.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary materials. Further inquiries can be directed to the corresponding author.

Author contributions

Material preparation, data collection and analysis were performed in parallel by Rds and AB. The first draft of the manuscript was written by Rds. AB commented and improved all versions of the manuscript until its final form. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors would like to thank the Swiss National Science Foundation (SNSF) and the National Centre of Competence in Research Molecular Systems Engineering (NCCR MSE), grant no. 51NF40-205608 for their generous support. The authors thank Shannon Hubbs for her proofreading support. This paper was written using data obtained on 20 February 2023 from Digital Science's Dimensions platform, available at https://app.dimensions.ai.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Ahmed, H. M. M. A. M., and Teixeira, L. S. M. (2022). New endeavors of (Micro) Tissue engineering: cells tissues organs on-chip and communication thereof. *Cells Tissues Organs* 211 (6), 721–735. doi:10.1159/000516356

Alternatives to Animal Experimentation ALTEX (2018). The EU directive on the protection of animals used for scientific purposes: its importance for science and society. *ALTEX Proc.* 5 (1), 3–9. doi:10.14573/altexproc.2018.1.003

Animal Welfare Act (2013). Act on the protection of animals used for scientific purposes. Available at: https://www.gesetze-im-internet.de/englisch_amg/.

Au, L., and da Silva, R. G. L. (2021). Globalizing the scientific bandwagon: trajectories of precision medicine in China and Brazil. *Sci. Technol. Hum. Values* 46 (1), 192–225. doi:10.1177/0162243920930282

Brackenbury, L. (2017). "Refinement on the way towards replacement," in *Progress in reducing animal tests*. Editors M. Balls, K. van der Laan, and A. L. Halder (Chennai: Brill), 3–20. doi:10.1163/9789004347853_002

Cruelty Free Europe (2023). *Reducing and replacing animal experiments*. Available at: https://www.crueltyfreeeurope.org/reducing-and-replacing-animal-experiments.

da Silva, R. G. L., and Blasimme, A. (2023). From lab to society: fostering clinical translation of molecular systems engineering. *Bioeng. Transl. Med.* 2023, e10564. doi:10.1002/btm2.10564

da Silva, R. G. L., Fischer, B. B., Schaeffer, P. R., and Novaes, H. M. D. (2023). The industry of therapeutic monoclonal antibodies in Brazil: public policies as instruments of technology upgrading. *Sci. Public Policy* 50 (1), 42–58. doi:10.1093/scipol/scac047

da Silva, R. G. L., Chammas, R., and Novaes, H. M. D. (2021). Rethinking approaches of science, technology, and innovation in healthcare during the COVID-19 pandemic: the challenge of translating knowledge infrastructures to public needs. *Health Res. Policy Sys* 19, 104. doi:10.1186/s12961-021-00760-8

da Silva, R. G. L. D., Chammas, R., Plonski, G. A., Goldbaum, M., Ferreira, L. C. S., and Novaes, H. M. D. (2020). University participation in the production of molecular diagnostic tests for the novel coronavirus in Brazil: the response to health challenges. *Cad. Saude Publica* 36 (6), e00115520. doi:10.1590/0102-311X00115520

Digital Science (2023). *Dimensions.ai*. Available at: https://app.dimensions.ai (Accessed February 20, 2023).

EUROoCS (2023). *About EUROoCS*. Available at: https://euroocs.eu/about/.

Europe Organ-on-Chip Society (2022). *About us Standardization*. Available at: https://www.euroocs.eu/about-us/Europe Organ-on-Chip Society.

European Commission (2010). *DIRECTIVE 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes*. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32010L0063.

European Parliament (2007). *Advanced therapeutic medicinal product - regulation 1394/2007*. EUR-Lex. Available at: https://eur-lex.europa.eu.

European Parliament (2009). *EU regulation 1223/2009*. EUR-Lex. Available at: https://eur-lex.europa.eu.

European Parliament (2017). *In vitro diagnostic medical devices (IVDR) regulation (EU) 2017/746*. EUR-Lex. Available at: https://eur-lex.europa.eu.

European Parliament (2021a). *MEPs demand EU action plan to end the use of animals in research and testing | News*. Available at: https://www.europarl.europa.eu/news/en/press-room/20210909IPR14508/meps-demand-eu-action-plan-to-end-the-use-of-animals-in-research-and-testing.

European Parliament (2021b). *Plans and actions to accelerate a transition to innovation without the use of animals in research, regulatory testing and education*. Available at: https://www.europarl.europa.eu/doceo/document/TA-9-2021-0286_EN.html.

Evens, R., and Kaitin, K. (2015). The evolution of biotechnology and its impact on health care. *Health Aff. (Millwood)* 34 (2), 210–219. doi:10.1377/hlthaff.2014.1023

Ewart, L. (2022). *The coming of age of organ-on-a-chip technology*. Available at: https://www.criver.com/eureka/coming-age-organ-chip-technology hDMT.

German Medical Devices Act (2018). Medizinproduktegesetz, MPG amended by the medical device law implementation act (MPDG). Available at: https://www.bfarm.de/EN/Medical-devices/Overview/Laws-and-ordinances/_node.html.

German Medicines Act (2019). Medicinal products act - arzneimittelgesetz (AMG). Available at: https://www.gesetze-im-internet.de/englisch_amg/.

hDMT (2023). Home, the institute for human organ and disease model technologies. Available at: https://www.hdmt.technology/about/.

Heimeriks, G., and Boschma, R. (2013). The path- and place-dependent nature of scientific knowledge production in biotech 1986–2008. *J. Econ. Geogr.* 14, 339–364. doi:10.1093/jeg/lbs052

Heringa, M. B., Park, M. V. D. Z., Kienhuis, A. S., and Vandebriel, R. J. (2020). The value of organs-on-chip for regulatory safety assessment. *ALTEX* 37 (2), 208–222. doi:10.14573/altex.1910111

Herpers, J., Arsenault, J. T., Vanduffel, W., and Vogels, R. (2022). Stimulation of the ventral tegmental area induces visual cortical plasticity at the neuronal level. *Cell Rep.* 38 (1), 109998–110003. doi:10.1016/j.celrep.2021.109998

HOPE Program (2023). Home, the wellcome leap human organs, physiology and engineering HOPE program. Available at: https://wellcomeleap.org/hope/.

Horejs, C. (2021). Organ chips, organoids and the animal testing conundrum. *Nat. Rev. Mater* 6, 372–373. doi:10.1038/s41578-021-00313-z

Huh, D., Matthews, B. D., Mammoto, A., Montoya-Zavala, M., Hsin, H. Y., and Ingber, D. E. (2010). Reconstituting organ-level lung functions on a chip. *Science* 328 (5986), 1662–1668. doi:10.1126/science.1188302

Human Society International (2021). *Breaking: european parliament votes to phase out animal testing and research*. Available at: https://blog.humanesociety.org/2021/09/breaking-european-parliament-votes-to-phase-out-animal-testing-and-research.html.

Human Tissue Act (2004). The human tissue act of 2004, United Kingdom. Available at: https://www.legislation.gov.uk/ukpga/2004/30/contents.

Ingber, D. E. (2022). Human organs-on-chips for disease modelling, drug development and personalized medicine. *Nat. Rev. Genet.* 23, 467–491. doi:10.1038/s41576-022-00466-9

Järvi, K., Almpanopoulou, A., and Ritala, P. (2018). Organization of knowledge ecosystems: prefigurative and partial forms. *Res. Policy* 47, 1523–1537. doi:10.1016/j.respol.2018.05.007

Karaulova, M., Gök, A., Shackleton, O., and Shapira, P. (2016). Science system path-dependencies and their influences: nanotechnology research in Russia. *Scientometrics* 107 (2), 645–670. doi:10.1007/s11192-016-1916-3

Kemp, L., Adam, L., Boehm, C. R., Breitling, R., Casagrande, R., Dando, M., et al. (2020). Bioengineering horizon scan 2020. *Elife* 9, e54489. doi:10.7554/eLife.54489

Kim, S., and Takayama, S. (2015). Organ-on-a-chip and the kidney. *Kidney Res. Clin. Pract.* 34 (3), 165–169. doi:10.1016/j.krcp.2015.08.001

Leung, C. M., de Haan, P., Ronaldson-Bouchard, K., Kim, G. A., Ko, J., Rho, H. S., et al. (2022). A guide to the organ-on-a-chip. *Nat. Rev. Methods Prim.* 2, 33. doi:10.1038/s43586-022-00118-6

Mastrangeli, M., Millet, S., Mummery, C., Loskill, P., Braeken, D., and Eberle, W. (2019b). Building blocks for a European Organ-on-Chip roadmap. *ALTEX* 36 (4), 481–492. doi:10.14573/altex.1905221

Mastrangeli, M., Millet, S., Orchid Partners, T., and Van den Eijnden-van Raaij, J. (2019a). Organ-on-chip in development: towards a roadmap for organs-on-chip. *ALTEX* 36 (4), 650–668. doi:10.14573/altex.1908271

MHRA (2023). The medicines and healthcare products regulatory agency UK. Available at: https://www.gov.uk/government/organisations/medicines-and-healthcare-products-regulatory-agency.

Micro Organo Lab Tubingen (2023). Home, µOrganoLab. Available at: https://www.organ-on-chip.uni-tuebingen.de.

Moraes, C., Mehta, G., Lesher-Perez, S. C., and Takayama, S. (2013). Organs-on-a-chip: a focus on compartmentalized microdevices. *Ann. Biomed. Eng.* 41 (9), 1866–1883. doi:10.1007/s10439-013-0806-3

Mummery, C., Sarro, L., van den Berg, A., Vulto, P., and van der Meer, A. (2016). Organ-on-chip technology: challenges and prospects. *Curr. Opin. Biotechnol.* 40, 148–156. doi:10.1016/j.copbio.2016.06.010

Nahle, Z. (2022). A proof-of-concept study poised to remodel the drug development process: liver-chip solutions for lead optimization and predictive toxicology. *Front. Med. Technol.* 4, 1053588. doi:10.3389/fmedt.2022.1053588

National Center for Advancing Translational Sciences (2022). *NCATS Tissue chips*. Available at: https://ncats.nih.gov/tissue-chips.

Netherlands Organ-on-Chip Initiative (2017). *Netherlands organ-on-chip initiative*. Available at: https://www.nwo.nl/en/research-and-results/programmes/Gravitation/netherlands-organ-chip-initiative/index.html.

ORCHID (2023). *Organ-on-Chip in development*. Available at: https://www.orchid-project.eu/.

Partelow, S., Hornidge, A. K., Senff, P., Stäbler, M., and Schlüter, A. (2020). Tropical marine sciences: knowledge production in a web of path dependencies. *PLoS One* 15 (2), e0228613. doi:10.1371/journal.pone.0228613

Politico (2021). *Action plan essentials to phase animals out of EU science and regulation*. Available at: https://www.politico.eu/sponsored-content/action-plan-essentials-to-phase-animals-out-of-eu-science-and-regulation/.

QMUL Emulate Centre (2023). *Centre for predictive* in vitro *models (CPM)*. Available at: https://www.qmul.ac.uk/emulate/cpm/.

Sauer, U. G., and Howard, P. C. (2018). One science-driven approach for the regulatory acceptance of non-animal-based approaches in the safety assessment of chemicals. *Comput. Toxicol.* 8, 1–7. doi:10.1016/j.comtox.2018.06.001

SIOoC (2022). *Home, Italian organ-on-chip society*. Available at: https://www.siooc.it/.

Strelez, C., Jiang, H. Y., and Mumenthaler, S. M. (2023). Organs-on-chips: a decade of innovation. *Tends Biotechnol.* 41 (3), 278–280. doi:10.1016/j.tibtech.2023.01.004

Swiss Animal Welfare Act (2005). The Swiss animal welfare act. Available at: https://www.blv.admin.ch/blv/en/home/.

Swiss Human Research Act (2011). The Swiss federal act on research involving human beings. Available at: https://www.blv.admin.ch/blv/en/home/.

University of Twente (2023b). *Organ-on-Chip Centre Twente | MESA+ institute*. Available at: https://www.utwente.nl/en/mesaplus/research/organ-on-chip-centre-twente/.

University of Twente (2023a). *Organs-on-Chip | MESA+ institute for nanotechnology*. Available at: https://www.utwente.nl/en/mesaplus/research/organ-on-chip-centre-twente/organs-on-chip/.

van der Meer, A. D., and van den Berg, A. (2020). Organs-on-chips: breaking the *in vitro* impasse. *Integr. Biol.* 12 (8), 240–257. doi:10.1093/intbio/zyaa008

van Meer, B. J., de Vries, H., Firth, K. S. A., van Weerd, J., Tertoolen, L. G. J., Karperien, H. B. J., et al. (2017). Small molecule absorption by PDMS in the context of drug response bioassays. *Biochem. biophysical Res. Commun.* 482 (3), 323–328. doi:10.1016/j.bbrc.2016.11.062

Wyss Institute (2014). *Human organs-on-chips*. Available at: https://wyss.harvard.edu/technology/human-organs-on-chips/.

Zainzinger, V. (2022). Animal testing in drug discovery: a new era. *Drug Discov. Today* 27 (1), 1–3. doi:10.1016/j.drudis.2021.12.002

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Original Research
PUBLISHED 28 September 2023
DOI 10.3389/fbioe.2023.1256388

OPEN ACCESS

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RECEIVED 10 July 2023
ACCEPTED 15 September 2023
PUBLISHED 28 September 2023

CITATION
Kuzma J, Grieger K, Cimadori I, Cummings CL, Loschin N and Wei W (2023), Parameters, practices, and preferences for regulatory review of emerging biotechnology products in food and agriculture.
Front. Bioeng. Biotechnol. 11:1256388.
doi: 10.3389/fbioe.2023.1256388

Parameters, practices, and preferences for regulatory review of emerging biotechnology products in food and agriculture

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This paper evaluates the U.S. regulatory review of three emerging biotechnology products according to parameters, practices, and endpoints of assessments that are important to stakeholders and publics. First, we present a summary of the literature on variables that are important to non-expert publics in governing biotech products, including ethical, social, policy process, and risk and benefit parameters. Second, we draw from our USDA-funded project results that surveyed stakeholders with subject matter expertise about their attitudes towards important risk, benefit, sustainability, and societal impact parameters for assessing novel agrifood technologies, including biotech. Third, we evaluate the regulatory assessments of three food and agricultural biotechnology case studies that have been reviewed under U.S. regulatory agencies and laws of the Coordinated Framework for the Regulation of Biotechnology, including gene-edited soybeans, beef cattle, and mustard greens. Evaluation of the regulatory review process was based on parameters identified in steps 1 and 2 which were deemed important to both publics and stakeholders. Based on this review, we then propose several policy options for U.S. federal agencies to strengthen their oversight processes to better align with a broader range of parameters to support sustainable agrifood products that rely on novel technologies. These policy options include 1) those that would not require new institutions or legal foundations (such as conducting Environmental Impact Statements and/or requiring a minimal level of safety data), 2) those that would require a novel institutional or cross-institutional framework (such as developing a publicly-available website and/or performing holistic sustainability assessments), and 3) those that would require the agencies to have additional legal authorities (such as requiring agencies to review biotech products according to a minimal set of health, environmental, and socio-economic parameters). Overall, the results of this analysis will be important for guiding policy practice and formulation in the regulatory assessment of emerging biotechnology products that challenge existing legal and institutional frameworks.

KEYWORDS

regulation, risk assessment, governance, biotechnology, gene editing

1 Introduction

Due to recent advancements in biotechnologies, new gene-edited food and agricultural products are now reaching the market. For example, oil from gene-edited soybeans, meat from heat-tolerant gene-edited cattle, and gene-edited mustard greens with lowered pungency have been cleared by regulatory agencies for market release, and many more gene-edited products are in late R&D stages (FDA, 2019a; Splitter, 2019; USDA, 2020b; USDA, 2020c; Erickson, 2022; FDA, 2022; Pixley et al., 2022; Mullins, 2023; USDA, 2023).

Coupled with this growth and innovation, is the evolution of the regulatory landscape of gene-edited products. Among one of the most recent changes has been the implementation of new regulations of genetically engineered organisms in the United State Department of Agriculture's (USDA) SECURE rule (USDA, 2020a). The SECURE rule represents the most comprehensive and substantial set of changes to the oversight of genetically engineered and modified crops in the U.S. in decades. If applied as intended, the vast majority of genetically engineered crops would be exempt from premarket field testing and risk assessment requirements (Kuzma and Grieger, 2020). The SECURE rule and other regulatory oversight mechanisms for biotechnology products often involve assessments that predominantly focus on potential impacts to agriculture (USDA, "plant pest risk" under the Plant Protection Act--PPA), human health (Food and Drug Administration's—FDA's voluntary consultation for food under the Federal Food Drug and Cosmetic Act--FDCA), and non-target species and human health (EPA under the Federal Insecticide, Fungicide, and Rodenticide Act--FIFRA) (NASEM, 2017; OSTP, 2017; Hoffman, 2021).

While this focus on health and environmental assessments is understandable given the limited legal basis of the regulatory system and traditions of risk assessment, a broader focus of oversight may be better suited for the next-generation of agricultural biotechnologies, given the importance of wider ecosystem impacts, sustainability aspects, and associated ethical and societal implications (e.g., Kuzma et al., 2008; Kuzma, 2018; Kuzma, 2021a; Kuzma, 2021b; Kuiken et al., 2021; Rohr et al., 2021; Florin, 2022; Gould et al., 2022; Kjeldaas et al., 2022; Lindberg et al., 2023). Consumers also consider parameters of transparency, trust, choice, equitable distribution of risks and benefits, animal welfare, and longer-term ecosystem consequences to be important for their acceptance of emerging technologies and their products (Kuzma et al., 2009; Frewer et al., 2013; NASEM, 2016; PEW, 2016; Kuzma, 2021a; Cummings and Peters, 2022a; 2022b). Oversight processes and assessments that pay attention to these broader dimensions are likely needed to ensure public confidence and trust in, as well as more robust and holistic analysis of consequences of, emerging biotechnologies in food and agriculture (e.g., Kearnes et al., 2006; Kuzma et al., 2008; Kuzma et al., 2009; Hartley et al., 2016; Kuzma, 2018; Macnaghten and Habets, 2020; Kuzma, 2021b; Kershaw et al., 2021; Kjeldaas et al., 2021; Kokotovich et al., 2022).

Building off this background, this article briefly reviews the regulatory process and assessments for some of the first gene-edited agrifood products cleared for market release in the U.S. and reflects upon how these regulatory processes match up (or not) to the parameters stakeholders and publics indicate that they care about when evaluating novel biotechnologies. In particular, we evaluate the regulatory decision-making processes and assessments for three case studies involving gene-editing (oil-altered soybean, heat tolerant cattle, and less pungent mustard greens), and compare them to the parameters and practices deemed important by a range of stakeholders and consumers when evaluating novel agrifood technologies more broadly. After this review, we provide suggestions for improving the regulatory review under three categories: 1) those that would not require new institutions or legal foundations, 2) those that would require a novel institutional or cross-institutional framework, and 3) those that would require the agencies to have additional legal authorities. Overall, the results of this analysis will be important for guiding policy practice and formulation in the oversight of novel agrifood products that rely on gene-editing in order to ensure safety, consumer confidence, and positive societal impacts.

2 Parameters for governance important to stakeholders and consumers

In this section, we first present a summary of the literature on variables that are important to non-expert publics in governing biotech products, including ethical, cultural, social, policy process, and risk and benefit parameters. Second, we draw from our USDA-funded project results that surveyed U.S. stakeholders with subject matter expertise about their attitudes towards important risk, benefit, sustainability and societal impact parameters for assessing novel agrifood technologies, including biotech.

2.1 Factors important to consumers

Several studies have identified a variety of factors important to consumers regarding gene-edited foods (GEFs) and genetically modified (GM) foods that are crucial in shaping their acceptance and decision-making processes. While it is sensible to believe that people primarily make decisions about food based on cost, appearance, taste, and nutritional content, recent studies by Cummings and Peters (2022a, 2022b) show that other factors influence perceptions and levels of acceptance, including social and ethical values, trust in agricultural biotechnology companies and government, and science and technology beliefs. These factors were found to greatly influence both consumers' willingness to eat GEFs as well as purposeful avoidance of GEF products. In addition, in these studies, individuals who are more willing to eat GEFs generally view science and technology as a primary means to solve society's problems, they place high levels of trust in government food regulators and the agriculture biotechnology industry, and generally do not have strong beliefs about food production. These views were also associated with younger (<30) individuals with higher-than-average education and household incomes. Conversely, individuals who reported they would prefer to purposefully avoid eating GEFs are more skeptical of the value of science and technology, they place greater value on the way their food is produced, and they more readily trust environmental groups rather than government and industry. This group tends to have lower incomes, are more religious, older and female, with

approximately 60% of the women surveyed reporting that they would purposefully avoid eating GEFs. Both groups agree that they would prefer that GEFs be mandated by the federal government to be labelled, with approximately 75% of the entire sampled population desiring labeling regardless of whether they would consume the products. Although the transparency of labeling is preferred by consumers, the effect of providing additional scientific information on consumer acceptance of GM foods in surveys demonstrates mixed findings. In addition, some studies report that information provision increases acceptance (Dolgopolova et al., 2017a; Dolgopolova et al., 2017b; Carrasson, et al., 2021) while others demonstrate that providing new information about GM foods does not improve consumer acceptance (Mcfadden and Wilson, 2018; Scott et al., 2018).

GEFs is also intertwined with the history of genetically modified organisms (GMOs) (Cummings, 2017; Friedrichs et al., 2019; Kuzma, 2022; Cummings et al., 2023; Lindberg et al., 2023). Public trust in GMOs has shown a significant discrepancy between scientific experts and the public. For instance, in 2015, 88% of scientists believed GMO foods were safe for human consumption compared to only 37% of the public (Pew Research Center, 2015). Recent stakeholder studies show that proponents of GEFs are seeking to cultivate public acceptance by focusing on shared values and transparency in their communication while also seeking to define GEFs as heterogeneous to GMOs (Cummings et al., 2023). Critics, on the other hand, view many of the concerns of GEFs as similar to GMOs and often seek to define GEFs as analogous to GMOs so that regulatory oversight and labeling mandates for GEFs are the same as GMOs (Cummings et al., 2023).

In a study comparing GM foods to GEFs, consumers viewed CRISPR and GM food similarly and substantially less positively than conventional food (Shew et al., 2018). Other studies show that cisgenic crops (genetic changes introduced from the same species, such as those produced by some gene-editing technologies) may be more acceptable to consumers than transgenic crops (genetic changes introduced from a different species), but that consumers may be less willing to accept cisgenic crops in comparison with conventionally bred crops (Edenbrandt et al., 2018; De Marchi et al., 2019). In Denmark, Edenbrandt et al. (2018) found a preference for cisgenic over transgenic rye bread production methods, while Marette et al. (2021) observed that French consumers would avoid gene-edited apples if given the choice. However, certain benefits associated with GEFs and GM foods can also outweigh negative perceptions among consumers, such as improved nutrition or safety (Yue et al., 2015a; Yue et al., 2015b). Furthermore, Kato-Nitta et al. (2019) found that Japanese consumers were more concerned about gene-edited livestock (pigs) than they were gene-edited vegetables (tomatoes). This study also found that the public was more willing to accept gene-edited products that provided direct-to-consumer benefits (increased nutritional value in the tomato) than products that benefited farmers (size enlargement of livestock). Only a subset of consumers reject cisgenic and transgenic crops under any circumstance (typically less than 20 percent), and other groups chose them based on health, safety and nutritional benefits, irrespective of whether they were cisgenic or transgenic (Yue et al., 2015b; Siegrist, 2008; Edenbrandt et al., 2018; De Marchi et al., 2019; Busch et al., 2022). For GM foods, benefits of increased health, safety and nutrition, particularly for those with food security needs, tend to be favored by consumers over improved taste and environmental benefits (Yue et al., 2015a). Animal welfare is another benefit from gene-edited agricultural products that can trump negative consumer perceptions. McConnachie et al. (2019) found positive consumer attitudes towards hornless gene-edited cattle, and Kilders and Caputo, (2021) found that animal welfare had the strongest positive impact on consumer willingness to purchase GM or GEF milk. In general however, other surveys show more negative attitudes towards animal gene-editing and genetic engineering than plant-based biotechnology (Frewer et al., 2014).

While ongoing studies investigate potential risks associated with GEFs, including off-target effects, unintended on-target effects, and unintended consequences (Kawall et al., 2020), scholars suggest that trust in emerging technologies for food is influenced by factors beyond technical risks and benefits, including past experiences with technology controversies, transparency and openness on the part of those who manage the technology, and provision of consumer control and choice (Slovic, 1987; Yawson and Kuzma, 2010; Kuzma and Kokotovich, 2011; Brown and Kuzma, 2013; Dietz, 2013; Yue et al., 2015a; Brown et al., 2015; Yue et al., 2015b; Cummings et al., 2023). For example, institutional trust plays a pivotal role in public perceptions and acceptance of both GEFs and GM foods (Frewer et al., 2014; Yue et al., 2015a; Cummings and Peters, 2022a; Cummings and Peters, 2022b). In summary, trust in those who manage the technology which is fostered by openness, transparency, assurance of safety, as well as consumer choice are important to consumers as well as tangible benefits that improve safety, transparency, and animal welfare when it comes to attitudes and acceptance of GEFs and GM foods by consumers.

2.2 Factors important to stakeholders

As a part of a USDA/NIFA-funded research project (Grant number 2022-67023-36730, PI/CoPI = Grieger/Kuzma), our research team conducted an online survey to investigate stakeholder views of parameters that would be important when evaluating novel technologies in food and agriculture, including gene editing. The approach and overview of results are provided below.

2.2.1 Methods

The survey was developed using an online survey platform (Qualtrics) and was conducted anonymously with no identifying information collected. The survey consisted of 8 multiple-choice and open-ended questions to gauge respondents' views of parameters that would be important when evaluating potential benefits and risks of novel technologies in food and agriculture (Table 1). In the multiple choice questions, participants were asked to rate the level of importance of each parameter for inclusion in benefit and risk evaluations of novel agrifoods using a 7-point semantic differential scale (1 = Not important at all, 7 = Extremely important). Participants were asked to rate the level of importance of each parameter as they were relevant for i) human health, ii) the environment, iii) animal health, and iv) ethical, legal, and societal implications (ELSI). Participants were also able to report additional parameters that they considered to be highly important to benefit

TABLE 1 List of parameters included in the stakeholder survey. Participants were asked to rate the level of importance of each parameter as they were relevant for four different categories.

Human health	Environment	Animal health	Ethical, legal, and societal implications
Food quality (e.g., taste, smell, appearance, shelf-life)	Use of environmental resources (e.g., use of environmental resources, such as water, energy, land, fisheries and wildlife resources, natural habitats)	Animal welfare and wellbeing (i.e., an animal's condition or treatment, including physical and emotional wellbeing experienced from living conditions, disease prevalence, and/or management practices)	Food access and/or security (e.g., access to sufficient, affordable, and nutritious foods; Resiliency of food supply)
Food nutrition (e.g., nutritional value, vitamin content)	Use of agrochemicals (e.g., pesticides, herbicides, fertilizers)	Animal growth and production (i.e., an animal's growth, development, and production, including changes in an animal's size or weight over its lifetime)	Social justice and equity (e.g., adequate and equitable access to foods; Equitable distribution of benefits and risks of food supply; Implications for vulnerable individuals and/or communities)
Food safety (e.g., presence of pathogens, contaminants, allergens)	Impacts on climate change (e.g., emissions of greenhouse gasses, ability to sequester carbon)	Animal reproduction (i.e., an animal's ability to reproduce and produce progeny or offspring)	Transparency (e.g., transparency in food supply, including transparency of ingredients in food and use of food labels)
Consumption patterns of nutritious foods (e.g., increased or decreased consumption rates of foods that contain essential nutrients)	Ecotoxicity (i.e., degree to which substance(s) cause harm to the environment, including impacts to living organisms, includes acute and/or chronic ecotoxicity, bioaccumulation persistence, gene transfer, and replicability)	Toxicity to animal health (i.e., degree to which substance(s) cause harm to animal health, including acute and/or chronic toxicity, allergenicity, and other adverse impacts on animal health)	Stakeholder inclusion and engagement (e.g., stakeholder participation and inclusion in development and oversight processes)
Occupational health and safety (e.g., health and safety considerations in production, use, transportation, disposal, and handling of materials and products)	Impacts on ecosystem services (e.g., nutrient cycling, pollination)		Regulations and government oversight (e.g., approval by regulatory agencies, Considered to be Generally Recognized as Safe (GRAS))
Toxicity to human health (i.e., degree to which substance(s) cause harm to human health, including acute and/or chronic toxicity, allergenicity, and other adverse impacts on health)			
Impacts on vulnerable populations (e.g., children, pregnant women, elderly)			

and risk evaluations of novel agrifoods. The parameters included in the survey were parameters or factors included in peer-reviewed publications and based on expert knowledge of benefits and risk of novel food and agricultural technologies. These parameters were included in the survey randomly, and categories of parameters were also shown randomly; meaning the order in which the parameters were included in the survey changed between participants to avoid additional biases based on order rated by participants. The survey also asked respondents about the sector(s) in which they work and area(s) of expertise.

Study participants were identified through reviewing peer-reviewed literature, conferences, and workshops related to novel agrifood technologies. In total, we identified an initial list of 402 potential stakeholder participants from the U.S. across sectors and invited them to participate in the online survey via email. The outreach email included an overview of the survey, approximate time it would take to complete, and how information and results were handled. Before reaching out to participants, the research team submitted the survey protocol to the PI's research institution (NC State, IRB protocol 25434), which was deemed to be IRB exempt. All study participants were able to directly access the survey using a link included in the outreach email. After the study period ended (3 weeks in the fall of 2022), the survey was closed and participants were no longer able to access the survey. Study participants were required to provide consent before responding to survey questions.

A total of 114 participants agreed to participate in the study and completed part of the survey. Out of the 114 initial study participants, only 79 participants completed the entire survey. Using the responses from the 79 participants that completed the survey, we then reviewed and cleaned the data to remove incomplete or invalid responses. This resulted in a dataset consisting of valid and completed responses from 77 participants; therefore 77 participants is considered to be the final sample size for this study. We note here that the 77 participants who completed all survey questions may not be fully representative of all 402 participants that we targeted in the original outreach and recruitment effort. Nonetheless, a final sample size of 77 is a robust sample size for social science research. Out of the 77 participants who completed the survey, more than a third of participants reported to be affiliated with academia (36.9%), followed by industry/private sector (22.62%), non-governmental organization/advocacy group (20.24%), government/public sector (11.9%), and other (8.33%). The participants also reported their areas of expertise within agriculture (23.11%), biotechnology (14.62%), nanotechnology (8.96%), ecology and/or environmental sciences (7.55%), legal or regulatory issues (7.08%), food production or processing (6.60%), life sciences (6.13%), water quality (6.13%), societal issues (5.19%), among other areas.

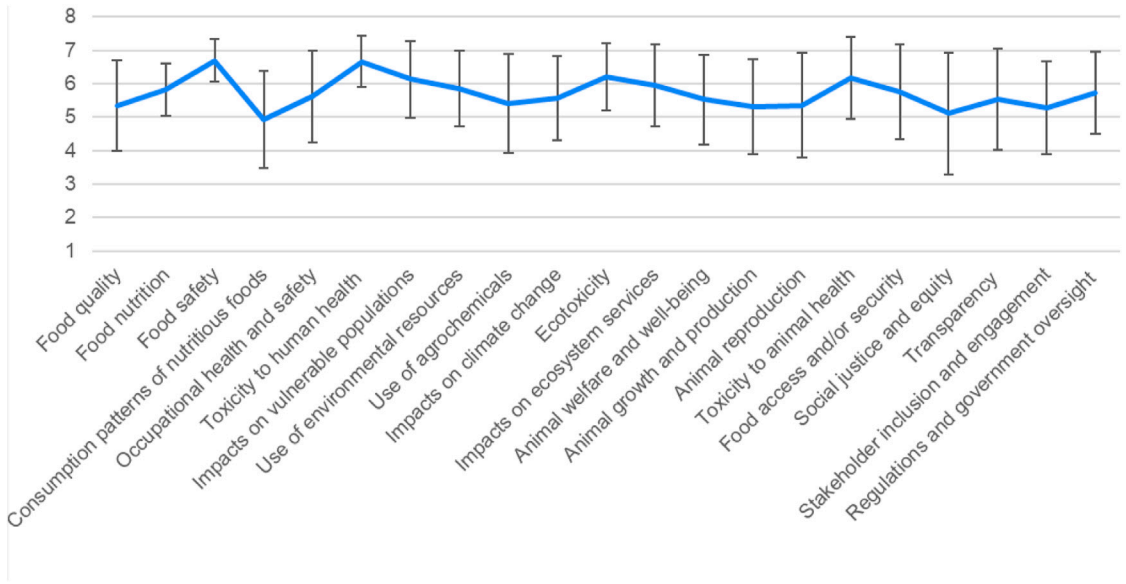

FIGURE 1
Results of stakeholder survey across all parameters, shown in mean scores and standard deviation.

After the study was completed, responses were exported from the Qualtrics platform for analysis in SPSS version 28.0.0.0. For the multiple-choice questions, frequency and percentage of participant responses were calculated from the 77 participants who completed the survey. Cronbach's Alpha reliability testing was conducted to evaluate *a priori* categorization of health and benefit parameters (e.g., human health, environment, ELSI), all categories demonstrated high reliability (alpha >.7). Further exploratory factor analysis was conducted to note possible item dimension reduction using Promax rotation and isolating factors within eigenvalues greater than one–however, these tests demonstrated similar findings to the *a priori* categories which were therefore maintained for subsequent analysis. Tests of difference were conducted using ANOVA to evaluate if there were significant differences between respondent self-reported affiliation groups (e.g., academics, industry, government, etc.). For the open-ended questions, participant responses were coded using descriptive coding processes. In this step, we reviewed participant responses, identified key themes that emerged, and assigned codes and subcodes.

2.2.2 Results

First, survey results show that nearly all the investigated parameters were considered to be important by study participants, as 20 out of 21 were rated above a 5 (with 'impacts on consumption behavior' rated just below 5) (Figure 1). This means that stakeholders thought they were essentially all important when evaluating potential benefits and risks of novel agrifoods products. Study participants also indicated that human health and the environment were more important than animal health and ELSI-based parameters, supported by statistical tests in SPSS.

Moving from most important to least important, the most important parameters indicated by stakeholders were food safety, toxicity to human health, ecotoxicity, toxicity to animal health, and impacts on vulnerable populations, which all had mean scores above 6. The next most important parameters were impacts on ecosystem services, use of environmental resources, food nutrition, food access and/or security, regulations and government oversight, occupational health and safety, impacts on climate change, transparency, and animal welfare and wellbeing, which all had mean scores above 5.5. Following these parameters, use of agrochemicals, animal reproduction, food quality, animal growth and production, stakeholder inclusion, and social justice and equity were important, with mean scores between 5 and 5.5. Consumption patterns was the only parameter that had a mean score less than 5.

Overall, these results indicate that stakeholders consider a wide range of parameters to be important when evaluating novel food and agriculture technologies. These parameters span categories of human health, environment, animal health, as well as ELSI, and go beyond traditional parameters of human health and environmental risk and safety.

2.3 Parameters for evaluating case studies

The parameters in Table 1 are classified into four categories, i.e., environmental, human health, animal health, and ethical, legal and social-economic implications (ELSI). These categories also reflect the pillars of sustainability, which was popularized by the United Nations (2015) through mainstreaming sustainable development goals on a global scale (environment, health, social-economic). Agriculture and food production is one of the most challenging issues for human society regarding sustainability, given the limited natural resource capacities of the planet. Thus, in order to achieve sustainable agriculture through biotechnology, we argue that a more holistic assessment based on these parameters of sustainability should be employed before commercializing gene-edited crops and foods on a large scale in order to ensure the

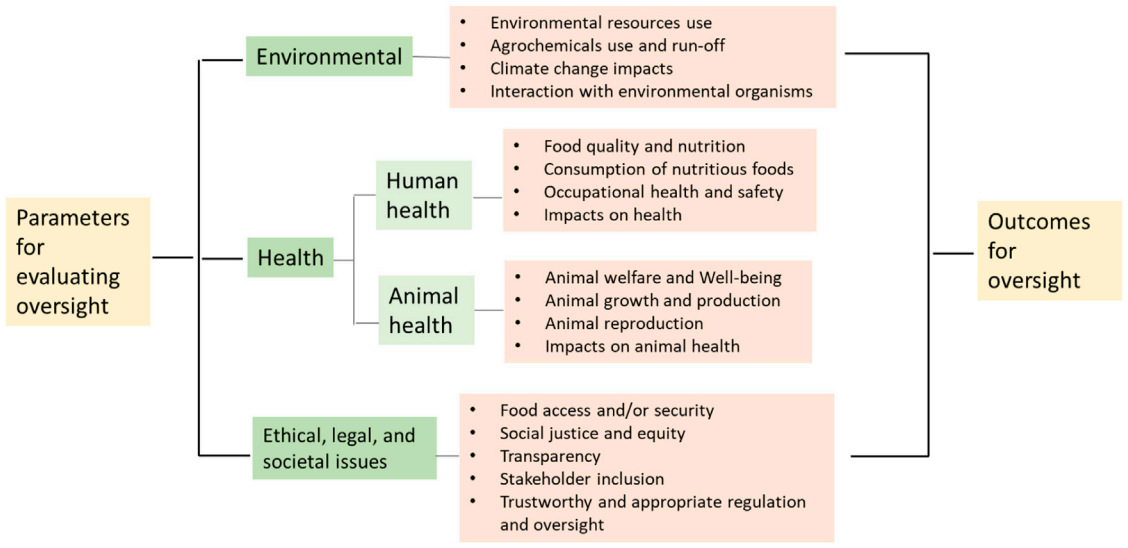

FIGURE 2
Parameters for evaluating oversight.

biotechnology products' contribution to sustainability (see also Wei et al., 2023).

We note here that many of the parameters and their categories may likely overlap with one another and may be difficult to measure (e.g., impacts on climate change). For example, social-economic considerations address the overlapping intersections of ethical, legal, as well as economic issues that may have multiple impacts on society. Similarly, impacts on human health are also known to influence socio-economic issues, etc. In addition to intersections, the perceptions of these key parameters may also interact with one another. For example, consumer views towards human health may directly influence perceptions of food quality as well as ELSI considerations (e.g., transparency).

The parameters in Table 1 also encompass the dimensions that consumers value when it comes to acceptance of gene-edited foods (Section 2.1), including benefits such as improvements in safety and nutrition and process criteria such as transparency and openness for decision-making that creates choice for them. Thus, these parameters may serve as a set of criteria for evaluating the recent oversight of three gene-edited products in the U.S. (Figure 2).

3 Case studies of recent U.S. Oversight involving agricultural biotechnology

We chose three case studies representing the first gene-edited food products cleared for the U.S. market: the first plant-based product designed for improved oil (high-oleic acid soybean); the first animal-based gene-edited food product (heat-tolerant cattle); and the first whole-food vegetable product designed for a less pungent taste (mustard greens). We first collected information about the products from the peer-reviewed literature and other sources, and then analyzed the regulatory process and documents regarding their regulatory clearance. Finally, we looked at the regulatory processes and assessments in light of the parameters stakeholders and consumers identify as important (Table 1; Figure 2). These examples are provided in the subsequent sections to give an indication of the emerging risk and regulatory review processes for gene edited agrifood products in the U.S. to help identify the strengths and shortcomings of oversight and suggest improvements for the future.

3.1 Soybeans with altered oil composition

This case study was chosen because it is the first gene-edited crop available in the market. In 2015 a gene-edited soybean line with increased levels of oleic acid and decreased levels of linoleic acid was cleared by the USDA through its "Am I Regulated" process (USDA, 2015a; b), which was in place from 2010 to 2020 prior to the SECURE rule being implemented (USDA, 2020a). Potential benefits of increasing the levels of oleic acid in soybean include benefits to food manufacturers, as higher oleic soybean oil provides higher heat stability and may extend product shelf lives (Huth et al., 2015). Additional benefits includes serving as a potentially healthier replacement of saturated fats in foods to ones that may reduce risks of coronary heart disease (FDA, 2018). The company that produced this product, Calyxt, consulted with the FDA a few years later under the agency's voluntary notification process for biotechnology-derived novel foods (FDA, 2019a; b, c). The product was generated using Agrobacterium-mediated transformation of TALEN site directed nucleases (gene-editing proteins that were precursors to CRISPR-Cas9) into the host soybean to make deletions in two FAD2 genes (USDA, 2015a; USDA, 2015b; FDA, 2019a; FDA, 2019b; FDA, 2019c). Then the transgenic sequences from Agrobacterium and the TALENs were backcrossed out to leave only the two deletions. As a result,

USDA decided it did not have to go through its Plant Production Act regulations (prior to SECURE) (USDA, 2015a; b) as it did not contain DNA sequences from plant pests. Therefore, the plant did not have to undergo the plant pest risk assessment process or an environmental assessment under the National Environmental Protection Act. The decision document authored by USDA conveys the focus of the USDA determination as to whether the oil-altered gene-edited soybean is a regulated article (USDA, 2015b; Box 1). The focus of USDA's determination is on the presence of plant pest sequences and that soybean plants are not considered plant pests. Weediness of soybeans was also considered, although it should be noted that weediness is not included as a primary risk endpoint in USDA's regulations for genetically engineered plants (USDA, 2020a; Kuzma and Grieger, 2020).

Box 1 Excerpt from determination that gene-edited soybeans are not regulated articles by USDA.

"APHIS regulates the importation, interstate movement and environmental release (field testing) of certain genetically engineered (GE) organisms that are, or have the potential to be, plant pests. Regulations for GE organisms that have the potential to be plant pests, under the Plant Protection Act, are codified at 7 CFR part 340, "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There Is Reason To Believe Are Plant Pests." Under the provisions of these regulations, a GE organism is deemed a regulated article if it has been genetically engineered using a donor organism, recipient organism, or vector or vector agent that is listed in §340.2 and meets the definition of a plant pest, or that is an unclassified organism and/or an organism whose classification is unknown, or if the Administrator determines that the GE organism is a plant pest or has reason to believe is a plant pest. The TALEN and the other genetic sequences important to the development of the soybean were derived from plant pests. . ..

According to your letter, the individual plant cells were genetically engineered to generate nucleotide deletions in two genes and thereby disrupt the function of specific proteins. However, all of the genetic material used to create the deletion was removed from the final soybean plant. Additionally, no genetic material was inserted into the final soybean plant genome. Based on the information cited in your letter, APHIS has determined this FAD2KO soybean variety was developed using [*removed due to Confidential Business Information*] and genetic material from plant pests. However, the final soybean plant does not contain any introduced genetic material and APHIS has no reason to believe that the plants of this soybean variety are plant pests. Therefore, consistent with previous responses to similar letters of inquiry, APHIS does not consider the FAD2KO soybean product as described in your 17 November 2014 letter to be regulated under 7 CFR part 340. Additionally, soybean is not listed as a Federal noxious weed under 7 CFR part 360, and APHIS has no reason to believe that the genetic engineering of your GE soybean would increase the weediness of soybean".

Several parameters from Table 1 and Figure 2 are missing from this assessment including environmental impacts such as ecotoxicity, climate change impacts, resource use, and chemical use. Rather, USDA's authority for GM plants is limited to plant pest risks under its GE plant regulations and the Plant Protection Act, and to a certain extent noxious weed risks under the PPA. This leaves several gaps for environmental toxicity and ecological sustainability that would only be considered under a broader Environmental Impact Statement under the National Environmental Policy Act (NEPA). EIS's have been done for only a handful of decisions for GM plants in their 30 years history (see Kuzma, 2022) and NEPA analyses only take place when GM plants come under USDA's plant pest risk authorities, which the gene edited soybean did not. It should be noted that the EPA has no authority for the gene-edited soybean as it did not introduce a "plant-incorporated protectant" or increase a pesticidal compound in the engineered plant (EPA, 2023). Some ecotoxicity parameters would have been considered under EPA's FIFRA regulations for "plant-incorporated protectants" introduced or altered via genetic engineering (EPA, 2023).

As far as ELSI parameters and important parameters to consumers, transparency and stakeholder inclusion in the USDA decision making process was lacking. The Am I regulated? process under the former USDA plant pest regulations for GM crops involved letters published on the website and some of the information may be considered confidential business information (USDA, 2015a; b; Kuzma and Kokotovich, 2011; Kuzma, 2018; Kuzma, 2022). There was no publication in the Federal Register, no external advisory committee or external scientific input, and little risk or benefit information provided. Furthermore, the gene-edited soybean or oil derived from it would not need to be labeled under the National Bioengineered Food Disclosure Standards as there is no foreign DNA in the final product (Jaffe and Kuzma, 2021). This also means that consumers and other stakeholders will not be able to track where the product is being used in the marketplace and would remain unaware of it being gene-edited (Kuzma and Grieger, 2020).

As far as human health parameters are concerned for the gene-edited soybean, these would come under the FDA's authorities under the FDCA. However, the FDA process is a voluntary consultation process which may decrease trust in consumers. Regardless, the company did take the step to consult with FDA and submitted information about the composition of the product in comparison to conventionally bred soybeans and oil derived from them for consideration by FDA for its suitability for food and feed (FDA, 2019a; b, c). These tests are generally designed to demonstrate nutritional "substantial equivalence" to the conventional counterpart. Endpoints in these documents that were considered include the fatty acid composition and its alteration; moisture, crude protein, crude fat, ash, and carbohydrates by calculation; fiber; amino acids, six fatty acids, three isoflavones (daidzein, genistein, and glycitein), four lecithins, and five anti-nutrients (lectin, phytic acid, raffinose, stachyose, and trypsin inhibitor) in whole seeds; and six fatty acids and lecithins. The FDA notes that "Calyxt states that the genetic modifications (inactivation of the FAD2-1A and FAD2-1B proteins, which are primarily expressed in developing seeds) do not meaningfully affect composition and nutrition of the meal derived from FAD2KO soybeans except for the intended changes in the levels of specific fatty acids" (FDA, 2019b). However, it is important to note that FDA relies on company data and does not make a determination of safety through this process, but states that it has "no further questions" (Box 2). These could reduce consumer trust in the oversight process. Although animal welfare, another important parameter to stakeholders and consumers (Section 2.1, 2.2), was not explicitly considered, impacts on animal health from consumption were according to the review of compositional data by FDA's Center for Veterinary Medicine (CVM) (FDA, 2019b).

Box 2 Excerpt from FDA's consultation letters on gene-edited oil-altered soybean

"Calyxt concludes:

- it has not introduced into food a new protein or other substance that would require premarket approval as a food additive
- food from FAD2KO soybean is comparable to and as safe as human food from other high oleic soybeans
- oil from FAD2KO soybean has a fatty acid profile consistent with criteria for "high oleic soybean oil"
- "high oleic soybean oil" is an appropriate common or usual name for oil from FAD2KO soybean

We evaluated data and information supporting these conclusions and considered whether FAD2KO soybean raises other regulatory issues involving human food under the Federal Food Drug and Cosmetic Act. We have no further questions at this time about the safety, nutrition, and regulatory compliance of food from F AD2KO soybean."

The presence of nontarget edits was considered through Whole Genome Sequencing (WGS) and FDA states that the company found no evidence of new mutations in the seven genes with greatest similarity to the target sites. Although there is a low probability off target edits that would increase or decrease endogenous plant secondary compounds that may be allergenic or toxic to humans and animals, toxicity tests were not required. *A priori,* the product would not be expected to be any less safe for consumption than conventionally bred soybeans, however, unintended biochemical changes due to the change in the oil composition of the product or off-target edits outside of the seven genes with the greatest similarity could lead to a change in the toxicity or allergenicity profile of the product. There would be no way to determine the negligible health risk without whole food testing in animals or comprehensive metabolomic, proteomic, and gene expression testing (as suggested by the National Academies, see NASEM, 2016). The FDA review of gene-edited products and the company's presentation of data are generally based on arguments about "substantial equivalence" yet based on macronutrients. In general, substantial equivalence is ill-defined and not a proxy for equivalent risk to conventional products (Millstone et al., 1999), as the limited nutritional and biochemical analyses done for FDA review may not account for unintended changes in the product (Cellini et al., 2004).

Another important set of parameters missing on the human health side are health benefits to consumers and impacts on food security and improved nutrition. The public has to rely on the company's assessment that high-oleic acid soybean oil may be better for health than regular soybean oil from conventionally bred plants. FDA does not have a mandate to consider health benefits and claims from GM foods.

3.2 Heat tolerant cattle

The PRLR-SLICK cattle is the first gene edited animal to hit the market. In particular, CRISPR-based gene editing has been used in two founder beef calves to alter the prolactin receptor gene (PRLR gene) which shortens the prolactin receptor protein (PRLR protein) in cattle to obtain a short and slick haircoat (FDA, 2022). This intentional genomic alteration (IGA) is heritable and can therefore be passed to their offspring (FDA, 2022). However, the developed cattle are mosaic, therefore first-generation progeny may not all inherit the SLICK phenotype (FDA, 2022). The goal is to make beef cattle more tolerant to heat, similarly to several cattle breeds raised in the tropics which naturally developed this desired mutation as an adaptation response to the environment in which they have been bred (FDA, 2022). As reported in the FDA risk evaluation document, previous studies found that cattle with slick hair are more suitable for hot weather (FDA, 2022). In addition to improving heat tolerance, gene-edited slick hair cattle could also help expand cattle production to new areas as well as better adapting to increased temperatures related to climate change (Karavolias et al., 2021).

Although the slick mutation naturally occurs in some breeds of cattle, the use of gene editing makes the introduction of this mutation in other beef cattle breeds faster compared to traditional breeding, while also avoiding the loss of other desirable traits and potentially minimizing the introduction of undesirable traits (Parliamentary Office of Science and Technology, 2022).

In the U.S., the primary federal agency that regulates gene edited animals is FDA through the new animal drug provision of the Food, Drug and Cosmetic Act (FD&C Act). Section 201(g)(1)(C) of the FD&C Act contains the definition of a "drug", which includes "articles (other than food) intended to affect the structure or any function of the body of man or other animals" (see 21 U.S.C. § 321(g)(1)(C)). Based on the definition of a "drug", the genetic material inserted in the animals' DNA that alters their structure or function falls under the drug definition of the FD&C Act (OSTP, 2017). According to the FD&C Act, any new animal drug needs prior approval from the FDA before being commercialized (OSTP, 2017). Genetically engineered animals with foreign genes, such as the AquaAdvantage Salmon, have been regulated under this act according to the 2009 FDA guidance #187 (revised 2015) to industry on the Regulation of Genetically Engineered Animals Containing Heritable Recombinant DNA Constructs (FDA, 2009; FDA, 2015). Under this guidance, a full Investigative New Animal Drug (INAD) or New Animal Drug (NAD) application was required (e.g., see Kuzma and Williams, 2023 for GE salmon; Kuzma, 2021b for GE mosquitos).

FDA put forth a new draft guidance in 2017 to include gene-edited animals under the FDCA, "GFI #187 Regulation of Intentionally Altered Genomic DNA in Animals" (FDA, 2017). Remarkably, in March 2022, the FDA used its enforcement discretion to review the PRLR-SLICK cattle under a less extensive approval process that did not require a full INAD or NAD, but produced a 8 page risk assessment summary authored by FDA. The agency made this first low-risk determination for enforcement discretion for a gene edited animal concluding in the risk assessment document that "there are no identifiable direct or indirect effects of the truncation of the *PRLR* gene or the IGA on the safety of food derived from the PRLR- SLICK cattle" (FDA, 2022, p 7). FDA also concluded that "the safety of food products made from PRLR- SLICK cattle is no different than the safety of food products made from commercial cattle that do not contain the IGA including those conventionally raised cattle with the naturally occurring slick phenotype" (FDA, 2022, p 7). As a result, the developers are not required to obtain FDA approval for a new animal drug prior to marketing the products derived from the

gene edited cattle (Van Eenennaam and Mueller Maci, 2022). The FDA's decision is limited only to those two founder cattle and their progeny (FDA, 2022).

It is important to note that this determination was made even though both the developer and FDA detected unintended, off-target mutations in the founder calves' genomes (FDA, 2022). This is because the FDA determined that the types of unintended mutations and their positions would not change the protein expression compared to the non-edited cattle, although no data to demonstrate this was included in the risk assessment (FDA, 2022). Therefore, they were not considered as a risk for those that consume the products derived from these cattle (FDA, 2022).

As it relates to Table 1, the parameters considered for this product in the risk assessment include human health parameters such as the quality, nutrition, and safety of the SLICK cattle derived products. However, no data was shown in the risk assessment on the nutritional "substantial equivalence" of the beef from the cattle or toxicity or allergenicity in comparison to conventionally bred cattle, although conclusions of safety were made (FDA, 2022). FDA concluded that "conventionally raised cattle with the slick phenotype are routinely consumed as human food and therefore FDA does not expect a change in the compositional or nutritional content of the edible tissues derived from the PRLR-SLICK cattle because they are similar in genotype, phenotype, and health status of naturally occurring slick cattle. No hazards were identified that required further characterization" (FDA, 2022, p. 6-7).

In terms of food security and access, this product could be beneficial if beef production would be increased and more resilient from rises in global temperature which have already caused thousands of cattle deaths (Bushard, 2022). At the same time, an increased production and consumption of beef may potentially lead to a detrimental increase in environmental resources and land usage, especially if production is expanded to areas previously not suitable for cattle farming. This may also have adverse effects on climate change. Additionally, although there is unclear data on whether the SLICK cattle could lead to increased production and consumption, there is some data on adverse human health effects associated with high consumption of red and processed meat (World Health Organization, 2015). Data on these indirect implications for sustainability (such as land use, climate change, and agrochemical use in Table 1) were not explicitly included in the risk assessment, although a discussion of whether the cattle would escape and become feral was included in the risk assessment under "Environmental Risk" (FDA, 2022, p. 8). We recognize that these land use and consumption patterns may be hard to predict prior to market release of the cattle; however, they could be modeled under different scenarios upstream of market approval to inform post-market monitoring strategies for detecting these landscape changes and subsequent risk mitigation strategies (see Discussion).

Animal welfare and health are also other important parameters that need to be considered for gene editing in animals. Among the three calves with the IGA, one founder animal died unexpectedly due to a heart defect (attributed to bovine congestive heart failure; BCHF), although this was assumed not to be caused by the gene edits but a marker gene also present in the non-edited parents (FDA, 2022). Other aspects of the animals' health were equivalent to non-gene edited comparator cattle (FDA, 2022). In fact, the welfare of cattle could increase because of this mutation, as those animals would tolerate higher temperatures better. At the same time, there is unclear data on the actual welfare of the SLICK cattle, meaning their emotional wellbeing and behavior in industrial living conditions is largely unknown. Although it is reported that the cattle's nutrition, preventive health, and veterinary observation were representative of typical cattle production practices (FDA, 2022), the cattle subject to the evaluation were kept under rigorous physical containment and housing conditions and were not therefore observed in actual industrial farms conditions (FDA, 2022). This is a relevant knowledge gap because to assess whether the DNA changes affected animal welfare and health or to determine whether adjustments to the management, housing or nutrition are required, a wide set of measures as well as multiple indicators and a multi-disciplinary approach should be used (EFSA Panels on GMO and AHAW, 2012). For example, the European Food Safety Authority (EFSA) suggests a three-stage assessment of gene edited animals before commercialization. Stage A requires a laboratory-level monitoring of the intended effects of the edit and potential effects on the animals' welfare through a set of health and welfare measurements, chosen between those established by the Welfare Quality® project, that are tailored to assess the specific gene edit. Stage B requires an experimental farm assessment to assess the effects of the intended and/or any unintended effects of the gene edit on animals' welfare in specified, licensed farms also called experimental farms. This stage would require a higher number of animals in order to observe the behavior of gene edited animals in relation to other animals. Finally, stage C requires a field trial in farms which practices are common across the European Union (EU).

Animal welfare and health are important parameters for stakeholders given that, and as highlighted by recent studies, consumers appear to be generally more supportive of gene editing applications in animals if those lead to increased animal welfare or health, while are generally less supportive of edits that focus on productivity traits (e.g., improved muscle tissue growth) (Yunes et al., 2021). However at the same time, gene editing may be viewed as a misguided substitute for conventional husbandry practices rather than meaningful welfare improvements. In fact, public opinion studies demonstrate that overall, there is less support for gene editing of animals compared to plant species, with ongoing discussions about the ethical and societal implication of gene editing in animals.

3.3 Less pungent mustard greens

This case study was chosen because it is the first whole vegetable product to be marketed for direct human consumption (i.e., without processing). Gene-edited mustard greens are expected to hit retailers and restaurants in late 2023 (Mullins, 2023). Researchers have gene-edited mustard greens (*Brassica juncea*) to remove the pungent and bitter flavors (Karlson et al., 2022; Grinstein, 2023). The potential benefits of developing gene-edited leafy greens include the ability for consumers to have access to nutritious leafy green products that taste better, which in turn, may increase consumption of healthy foods. Developers were able to do this by utilizing CRISPR to target and edit genes in order to reduce the production of oils made from glucosinolates that can cause a pungent taste when chewed or cut (Karlson et al., 2022). The genetic manipulation has significantly

edited multiple genes across seven chromosomes of mustard greens, including the deletion of two whole genes, blocking the conversion of glucosinolates to these pungent oils.

In terms of regulatory oversight, the gene-edited mustard greens fall would conceivably fall under the jurisdiction of the USDA and the FDA according to the Coordinated Framework on Biotechnology Regulation (OSTP, 2017). However, in August 2020, USDA-APHIS determined that the gene-edited mustard greens do not fall under USDA's regulations for genetically engineered crops as they do not contain plant pest DNA and thus do not pose a plant pest risk. This was determined as part of the *Am I Regulated?* process whereby the company sent a letter to USDA inquiring about the regulatory status of the gene-edited mustard greens, and USDA sent a response back as to whether the product would fall under its regulations under the Plant Protection Act (USDA, 2020c; USDA, 2020d; USDA, 2023). In the letter to USDA, the company noted that it "requests formal confirmation from USDA APHIS Biotechnology Regulatory Services (BRS) that *Brassica juncea* (L.) with improved flavor developed using gene-editing plant breeding tools is not a 'regulated article' subject to APHIS oversight under 7 C.F.R. part 340 because it will not contain any inserted genetic material from a plant pest" (USDA, 2020d). The company also described how no species of *Brassica* is listed as a Federal Noxious Weed and that the gene-edit would not be expected to make it into a weed. However, it should be noted that that certain *Brassica* species are considered weeds according to the USDA's own weed risk assessments (e.g., USDA, 2021).

USDA cited the process of the modification and lack of plant pest DNA (and any foreign DNA left in the product) in their decision to exempt the gene-edited mustard from its regulations (USDA, 2020c). Although the USDA considered that the gene-edited mustard was not a plant pest and did not contain plant pest DNA, the assessment did not include investigations into other aspects of plant health such as the environmental consequences of removing genes involved in plant defense and the corresponding potential use of chemicals to control insects in the event of a pest outbreak. The gene-editing process changes glucosinolate metabolism in the plant and may deactivate the plant defense systems by blocking the metabolism of glucosinolate into insect-resistant components (Karlson et al., 2022). These metabolic changes could make the plants more vulnerable to insect pests under certain conditions, although no change was observed in the occurrence of insects in field trials of gene-edited mustard greens in a variety of locations and conditions (Karlson et al., 2022). In addition, environmental gene escape is a potential risk as gene-edited mustard greens may hybridize with other *B. juncea* or *Brassicas* (turnips) or may impact nearby related crops or weedy populations as well as surrounding ecosystems (e.g., such as non-target organisms). Information and data on the increased pest and weediness potential of the use of gene-edited mustard greens was not considered in the brief Am I Regulated letters. Also, toxicity to species in the environment from the biochemical changes in the gene edited mustard was not addressed in the brief *Am I Regulated* letter.

Shortly after USDA's approval of the gene-edited mustard greens, the USDA's Sustainable, Ecological, Consistent, Uniform, Responsible, Efficient (SECURE) rule came into effect at the end of 2020 (Hoffman, 2021). SECURE revised regulations for genetically engineered plants under USDA and the Plant Protection Act under 7 CFR part 340 (USDA, 2020a; Kuzma and Grieger, 2020; Hoffman, 2021). Under the current SECURE rule, the gene-edited mustard greens would also not likely be subject to regulation because the gene editing only deletes genes (USDA, 2020a).

The mustard greens also did not go through the formal, voluntary FDA consultation process[1] and no Biotechnology Notification Files appear for the product on FDA's website, although there are reports that the company consulted with FDA in a private meeting about the product (Mullins, 2023). This negated the investigation of any human health parameters in Table 1, including food safety and toxicity as it relates to the increase in glucosinolates. As it relates to the human health parameters in Table 1, the gene-edited mustard greens were developed to have a change in food quality that could also alter consumption patterns. It is anticipated that the less pungent mustard greens may promote the consumption of nutritious and healthy fresh produce, although no published data are available on this aspect. While pungency may currently prevent some consumers from eating mustard greens, the reduced pungency of their gene-edited counterparts may conceivably lead to unintended elevated exposures to glucosinolates when consumed in large amounts. This could become a health issue for vulnerable individuals who may be more impacted by such exposures.

The product would also not be subject to the National Bioengineered Disclosure Standards which mandate labeling as "bioengineered" or "derived from bioengineering" if a genetically engineered food product has foreign DNA in the final product (Federal Register, 2018; Jaffe and Kuzma, 2021). Given a lack of foreign DNA in the final food product from gene-edited mustard, it would not require labeling (Jaffe and Kuzma, 2021). In addition, much information in the company's letter to USDA was deleted due to confidential business information (USDA, 2020d). Thus, parameters related to consumer transparency and stakeholder inclusion in Table 1 are lacking in the decision making process for this product. However, the developers of the mustard greens have conducted taste tests with consumers to better understand consumer preferences for the gene-edited greens and of gene-editing and CRISPR, and have pushed for transparency in the process of developing and applying this product by making it known publicly that its product is gene-edited. However, attention to many of the ELSI, health and environmental parameters is lacking in the mustard greens case with no FDA review, limited review by the USDA, and a lack of transparency to consumers more broadly.

4 Summary of the case studies

From the case studies above, we demonstrate that there are clear limitations for the federal agencies to consider many of the parameters that are important to consumers and diverse

1 Biotechnology Notification File is available on FDA's website, and in personal communication with the developer's, it was confirmed that the product did not undergo that process. Instead, it was reported in personal communication that the company met with the FDA at some point prior to the press releases that the Conscious Greens would appear on the market. There is no evidence or content of that meeting available to the general public however.

stakeholders and for assessing the sustainability of gene-edited agricultural products. For instance, in-depth environmental assessments were not required for either of the gene-edited plant crops (soybeans, mustard greens) as both were exempt from USDA's plant-pest regulations for genetically engineered crops. Health assessments for the gene-edited soybean oil provided the most data on nutritional "substantial equivalence", although toxicity studies were not conducted. Health assessments for the gene-edited mustard greens were not available and seem not to have been conducted under FDA's voluntary consultation process. For the gene-edited animal product, the health assessment of the beef from gene-edited cattle was primarily qualitative, based on the assumption that the meat would be the same as meat from the non-edited cattle. Animal welfare for the gene-edited cattle was considered, although data was not presented in the assessment. Across all three case studies, broader parameters related to land, water and agrochemical use, and ecotoxicity were not evaluated for any of the products. Further, all agency approval processes were conducted without public or stakeholder input, and only between the product developer and federal agency. None of the products would require labeling under the National Bioengineered Food Disclosure Standards, and limited to no safety data is available to consumers. We also note that even when the assessment documents are available, they are difficult to find on federal agency websites. Overall, we argue that even if there are no obvious health or environmental safety concerns for these gene-edited products based on the available data and information, these aforementioned limitations will likely undermine consumer and public trust in gene editing and the arguments that these products will contribute to greater sustainability. We also note here that potential risks and limitations of these gene-edited agrifood products should be reviewed alongside their potential benefits. Holistic benefit assessments could be conducted in parallel to holistic risk assessments to create a comprehensive and balanced assessment of gene-edited agrifood products, taking into account health, environmental, animal health, and ethical and socio-economic factors. Multi-criteria Decision Analysis (MCDA) is one decision-support tool that may be particularly helpful to consider various benefits and risks of gene-edited agrifoods, and has been used in other food applications decisions when balancing benefits and risks (Ruzante et al., 2017).

5 Conclusions: Policy options

As demonstrated in the preceding sections, U.S. federal agencies that review gene-edited products are limited by their narrow regulatory authorities under current federal laws and the Coordinated Framework for Regulation of Biotechnology. For example, USDA is limited to "plant pest risks" and EPA is limited to "plant pesticide risks." This creates gaps in what sustainability parameters can be assessed for novel agrifood technologies including products of gene-editing. In response, we propose several policy options for U.S. federal agencies to strengthen their oversight processes for agricultural biotechnology.

First, a broader assessment could be required through an Environmental Impact Statement under the National Environmental Policy Act. While no federal agency has exercised such an assessment for a gene-edited crop to date, a few have been done for genetically engineered crops with transgenes and therefore this may serve as a model to follow in future evaluations (see Kuzma, 2022 for details). In addition, federal agencies may have rather narrow regulatory scopes, although they can still require the minimum level of safety data for new gene-edited agrifood products, particularly those that are among the first to come to market. For instance, requiring at least nutritional "substantial equivalence" data or a minimal level of mammalian and non-target animal toxicity testing, and making such results available to consumers, would set the stage for greater consumer safety and trust. This policy recommendation would rely on a more open and comprehensive review process under existing regulatory processes rather than to require new institutions or legal foundations. The most rigorous and transparent process would also include open public advisory committees for decision making about certain gene-edited products and require Environmental Impact Statements under NEPA. At the same time, less rigorous improvements would include requiring more data and analysis for health and environmental safety under the current, closed regulatory processes (e.g., mandating the voluntary consultation process for FDA, assessing nutritional "substantial equivalence", and requiring whole-food toxicity studies).

A second set of policy recommendations stemming from our analysis would require a novel institutional or cross-institutional framework. For example, federal agencies (or a trusted third party) could sponsor the development and ownership of a unified website (or database) for all gene-edited products on the market that are cleared for marketing by the federal agencies, which includes safety information, review documents, and potential market uses. This publicly-available website would also help improve transparency for diverse publics and other stakeholders in terms of better understanding which gene-edited agrifood products are currently on the market. The National Academies of Science, Engineering and Medicine in fact suggested a common portal of entry for biotech products to improve coordination of the federal agencies and avoid potential jurisdictional overlaps or gaps (2017). Further, Kuzma & Grieger (2020) suggested a repository like this for gene edited crops in order to improve public transparency and contribute to greater public choice and trust. In addition to a website or database, another option could be for a trusted third party research agency to do a more holistic sustainability assessment that would accompany each gene-edited product as it reaches the market place. Perhaps a research arm of the federal government or an independently funded think-tank could conduct such assessments and make them publicly available. The importance of this assessment is emphasized by the fact that several biotech developers argue that gene edited products will improve ecosystems, food security, and human health; and hence, it is important to back up these claims with a holistic assessment of the parameters in Table 1. A third party venue for these analyses could also improve public trust by showing that biotech developers' claims are indeed legitimate. One such multi-stakeholder coalition to assess sustainability of gene edited cover crops has already been previously proposed and could serve as an example to move forward (Jordan et al., 2017). We do recognize, however, that upstream assessments for sustainability (e.g., landscape changes, consumption patterns) are likely to come with significant uncertainty and a lack of predictive power. In these cases, modeling can be used to consider impacts on sustainability under different use scenarios to inform decision

making, post-market monitoring, and risk mitigation strategies, rather than as a regulatory checkpoint for initial market release. However, post-market surveillance mechanisms for biotech products in food and agriculture are currently limited under federal agency authorities (e.g., EPA for re-registration of plant-pesticides, FDA recall authorities for adulterated foods).

Finally, a third policy option that could be considered is developing a comprehensive, new biotechnology oversight law that requires the agencies to review each gene-edited product to some extent for a minimal set of health, environmental, and socio-economic variables. This is put forward given that there are several parameters included in Table 1 that are missing in assessments of gene-edited agrifood products including the investigated case studies in this work. For example, important environmental and human health parameters were missing from assessments in each of the case studies investigated, including mandatory food safety reviews (e.g., FDA's process is voluntary, not performed for mustard greens case study) and environmental assessments (e.g., USDA's authority is limited to "plant pest risk," while ecosystem risks are outside the scope, including harm to nontarget organisms or indirect water or land use changes). Requirements for public transparency were lacking in all cases. Such a comprehensive oversight system with new legal authorities for genetically engineered products has in fact been previously considered (e.g., Kuzma, 2016; Kuzma, 2021a). Further, the National Academies of Science Engineering and Medicine also recently suggested a novel governance framework that will enable policymakers to better and anticipate and address the social, legal, ethical, and governance issues associated with emerging technologies as they arise (Mathews et al., 2022), although it is recognized that political will is needed for such approaches (Kuzma, 2023).

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

JK: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Writing–original draft, Writing–review and editing, Formal Analysis, Project administration, Supervision. KG: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing–original draft, Writing–review and editing. IC: Formal Analysis, Investigation, Writing–original draft, Writing–review and editing. CC: Formal Analysis, Investigation, Methodology, Writing–original draft, Writing–review and editing. NL: Formal Analysis, Investigation, Methodology, Writing–original draft, Writing–review and editing. WW: Formal Analysis, Investigation, Methodology, Writing–original draft, Writing–review and editing.

Funding

The author(s) declare financial support was received for the research, authorship, and of this article. The work was supported by USDA/NIFA, Grant No. 2022-67023-36730 (Grieger PI, Kuzma coPI).

Acknowledgments

The authors gratefully acknowledge the support of the GES Center and the suggestions provided by the peer reviewers.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Brown, J., Fatehi, L., and Kuzma, J. (2015). Altruism and skepticism in public attitudes toward food nanotechnologies. *J. Nanoparticle Res.* 17, 122–140. doi:10.1007/s11051-015-2926-4

Brown, J., and Kuzma, J. (2013). Hungry for information: public attitudes toward food nanotechnology and labeling. *Rev. Policy Res.* 30, 512–548. doi:10.1111/ropr.12035

Busch, G., Ryan, E., von Keyserlingk, M. A. G. G., and Weary, D. M. (2022). Citizen views on genome editing: effects of species and purpose. *Agric. Hum. Values* 39 (1), 151–164. doi:10.1007/s10460-021-10235-9

Bushard, B. (2022). Thousands of cattle reportedly dumped into Kansas landfill after dying from extreme heat. Forbes. Available at: https://www.forbes.com/sites/brianbushard/2022/07/26/thousands-of-cattle-reportedly-dumped-into-kansas-landfill-after-dying-from-extreme-heat/?sh=648111eb3254.

Carrasson, M., Soler-Membrives, A., Constenla, M., Escobar, C., Flos, R., Gil, J. M., et al. (2021). Information impact on consumers' perceptions towards aquaculture: dismantling the myth about feeds for farmed fish. *Aquaculture* 544, 737137. doi:10.1016/j.aquaculture.2021.737137

Cellini, F., Chesson, A., Colquhoun, I., Constable, A., Davies, H. V., Engel, K. H., et al. (2004). Unintended effects and their detection in genetically modified crops. *Food Chem. Toxicol.* 42 (7), 1089–1125. doi:10.1016/j.fct.2004.02.003

Cummings, C. L. (2017). "Comprehension of products and messages," in *Consumer perception of product risks and benefits*. Editors G. Emilien, R. Weitkunat, and F. Lüdicke (Cham: Springer), 145–163.

Cummings, C., and Peters, D. (2022a). Gene-edited foods and the public: the first representative survey study of the United States. *Environ. Commun.* 2022, 1–10. doi:10.1080/17524032.2022.2086894

Cummings, C., and Peters, D. (2022b). Who trusts in gene-edited foods? Analysis of a representative survey study predicting willingness to eat- and purposeful avoidance of gene edited foods in the United States. *Front. Food Sci. Technol.* 2. doi:10.3389/frfst.2022.858277

Cummings, C., Selfa, T., Lindberg, S., and Bain, C. (2023). Identifying public trust building priorities of gene editing in agriculture and food. *Agric. Hum. Values* 2023. doi:10.1007/s10460-023-10465-z

De Marchi, E., Cavaliere, A., Bacenetti, J., Milani, F., Pigliafreddo, S., and Banterle, A. (2019). Can consumer food choices contribute to reduce environmental impact? The case of cisgenic apples. *Sci. Total Environ.* 681, 155–162. doi:10.1016/j.scitotenv.2019.05.119

Dietz, T. (2013). Bringing values and deliberation to science communication. *PNAS* 110 (3), 14081–14087. doi:10.1073/pnas.1212740110

Dolgopolova, I., Smith, J., and Johnson, K. (2017a). Gene edited food. *J. Food Sci.* 82 (4), 856–863. doi:10.1111/1750-3841.13657

Dolgopolova, I., Teuber, R., Bruschi, V., Weber, G. W., Danilenko, N., and Galitskiy, E. (2017b). "Modelling consumer preferences for novel foods: random utility and reference point effects approaches," in *Modeling, dynamics, optimization and bioeconomics II: DGS III, porto, Portugal, february 2014, and bioeconomy VII* (Berkeley, USA: Springer International Publishing), 165–182. March 2014-Selected Contributions 3.

Edenbrandt, A. K., Gamborg, C., and Thorsen, B. J. (2018). Consumers' preferences for bread: transgenic, cisgenic, organic or pesticide-free? *J. Agric. Econ.* 69 (1), 121–141. doi:10.1111/1477-9552.12225

EFSA Panels on GMO and AHAW (2012). Guidance on the risk assessment of food and feed from genetically modified animals and on animal health and welfare aspects: guidance on food and feed RA from GM animals and GM animal health and welfare. *EFSA J.* 10 (1), 2501. doi:10.2903/j.efsa.2012.2501

EPA (2023). 40 CFR Part 174 [EPA–HQ–OPP–2019–0508; FRL–7261–04–OCSPP] RIN 2070–AK54 pesticides; exemptions of certain plant-incorporated protectants (PIPs) derived from newer technologies. *Fed. Regist.* 88 (104), 34756–34779.

Erickson, B. (2022). US FDA clears the way for CRISPR beef cows. *Chem. Eng. News* 100 (10).

FDA (2015). Biotechnology consultation - biotechnology notification file No. 132. Available at: http://wayback.archive-it.org/7993/20171031091552/https://www.fda.gov/Food/IngredientsPackagingLabeling/GEPlants/Submissions/ucm436168.html (Accessed March 16, 2023).

FDA (2019c). Biotechnology notification file No. 000164. CFSAN note to the file. Available at https://www.fda.gov/media/120708/download (Accessed June 8, 2023).

FDA (2019b). Biotechnology notification file No. 000164. CVM note to the file: subject: fad2ko, high oleic acid soybean. Available at https://www.fda.gov/media/120660/download (Accessed June 8, 2023).

FDA (2018). FDA completes review of qualified health claim petition for oleic acid and the risk of coronary heart disease. Available at https://www.fda.gov/food/cfsan-constituent-updates/fda-completes-review-qualified-health-claim-petition-oleic-acid-and-risk-coronary-heart-disease (Accessed August 4, 2023).

FDA (2009). Food and drug administration, center for veterinary medicine. Guidance for industry regulation of genetically engineered animals containing heritable recombinant DNA Constructs #187. 2009, revised 2017. Available at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cvm-gfi-187-regulation-intentionally-altered-genomic-dna-animals (Accessed July 1, 2023).

FDA (2017). Guidance for industry regulation of intentionally altered genomic DNA in animals draft guidance. Available at https://www.fda.gov/media/74614/download (Accessed July 1, 2023).

FDA (2019a). RE: biotechnology notification file No. BNF 000164. Available at https://www.fda.gov/media/120707/download (Accessed June 7, 2023).

FDA (2022). Risk assessment summary – V-006378 PRLR-SLICK cattle. Available at https://www.fda.gov/media/155706/download (Accessed June 7, 2023).

Federal Register (2018). National bioengineered food disclosure standard. Available at: https://www.federalregister.gov/documents/2018/12/21/2018-27283/national-bioengineered-food-disclosure-standard (Accessed December 21, 2021).

Florin, M. V. (2022). Risk governance and responsible research and innovation can be mutually supportive. *J. Risk Res.* 25 (8), 976–990. doi:10.1080/13669877.2019.1646311

Frewer, L., Coles, D., Houdebine, L. M., and Kleter, G. (2014). Attitudes towards genetically modified animals in food production. *Br. Food J.* 116 (8), 1291–1313. doi:10.1108/bfj-08-2013-0211

Frewer, L. J., van der Lans, I. A., Fischer, A. R., Reinders, M. J., Menozzi, D., Zhang, X., et al. (2013). Public perceptions of agri-food applications of genetic modification–a systematic review and meta-analysis. *Trends Food Sci. Technol.* 30 (2), 142–152. doi:10.1016/j.tifs.2013.01.003

Friedrichs, S., Takasu, Y., Kearns, P., Dagallier, B., Oshima, R., Schofield, J., et al. (2019). Policy considerations regarding genome editing. *Trends Biotechnol.* 37 (10), 1029–1032. doi:10.1016/j.tibtech.2019.05.005

Gould, F., Amasino, R. M., Brossard, D., Buell, C. R., Dixon, R. A., Falck-Zepeda, J. B., et al. (2022). Toward product-based regulation of crops. *Science* 377 (6610), 1051–1053. doi:10.1126/science.abo3034

Grinstein, J. (2023). Salad days: pairwise gene edits food to topple nutrition barriers. *Gen. Biotechnol.* 2 (1), 5–9. doi:10.1089/genbio.2023.29078.jdg

Hartley, S., Gillund, F., van Hove, L., and Wickson, F. (2016). Essential features of responsible governance of agricultural biotechnology. *PLoS Biol.* 14 (5), e1002453. doi:10.1371/journal.pbio.1002453

Hoffman, N. E. (2021). Revisions to USDA biotechnology regulations: the SECURE rule. *PNAS* 118 (22), e2004841118. doi:10.1073/pnas.2004841118

Huth, P. J., Fulgoni, V. L., and Larson, B. T. (2015). A systematic review of high-oleic vegetable oil substitutions for other fats and oils on cardiovascular disease risk factors; implications for novel high-oleic soybean oils. *Adv. Nutr.* 6 (6), 674–693. doi:10.3945/an.115.008979

Jaffe, G., and Kuzma, J. (2021). New bioengineered (aka GM) food disclosure law: useful information or consumer confusion? *Food Drug and law institute update* 17. Summer. Available at: https://www.fdli.org/2021/04/new-bioengineered-aka-gm-food-disclosure-law-useful-information-or-consumer-confusion/.

Jordan, N. R., Dorn, K. M., Smith, T. M., Wolf, K. E., Ewing, P. M., Fernandez, A. L., et al. (2017). A cooperative governance network for crop genome editing: the success of governance networks in other areas could help to find common ground for applying genome editing in agriculture. *EMBO Rep.* 18 (10), 1683–1687. doi:10.15252/embr.201744394

Karavolias, N. G., Horner, W., Abugu, M. N., and Evanega, S. (2021). Application of gene editing for climate change in agriculture. *Front. Sustain. Food Syst.* 5. doi:10.3389/fsufs.2021.685801

Karlson, D., Mojica, J. P., Poorten, T. J., Lawit, S. J., Jali, S., Chauhan, R. D., et al. (2022). Targeted mutagenesis of the multicopy myrosinase gene family in allotetraploid Brassica juncea reduces pungency in fresh leaves across environments. *Plants* 11, 2494. doi:10.3390/plants11192494

Kato-Nitta, N., Maeda, T., Inagaki, Y., and Tachikawa, M. (2019). Expert and public perceptions of gene-edited crops: attitude changes in relation to scientific knowledge. *Palgrave Commun.* 5 (137), 137–214. doi:10.1057/s41599-019-0328-4

Kearnes, M., Grove-White, R., Macnaghten, P., Wilsdon, J., and Wynne, B. (2006). From bio to nano: learning lessons from the UK agricultural biotechnology controversy. *Sci. as Cult.* 15 (4), 291–307. doi:10.1080/09505430601022619

Kershaw, E. H., Hartley, S., McLeod, C., and Polson, P. (2021). The sustainable path to a circular bioeconomy. *Trends Biotechnol.* 39 (6), 542–545. doi:10.1016/j.tibtech.2020.10.015

Kilders, V., and Caputo, V. (2021). Is animal welfare promoting hornless cattle? Assessing consumer's valuation for milk from gene-edited cows under different information regimes. *J. Agric. Econ.* 72 (3), 735–759. doi:10.1111/1477-9552.12421

Kjeldaas, S., Antonsen, T., Hartley, S., and Myhr, A. I. (2021). Public consultation on proposed revisions to Norway's gene technology act: an analysis of the consultation framing, stakeholder concerns, and the integration of non-safety considerations. *Sustainability* 13 (14), 7643. doi:10.3390/su13147643

Kjeldaas, S., Dassler, T., Antonsen, T., Wikmark, O. G., and Myhr, A. I. (2022). With great power comes great responsibility: why 'safe enough is not good enough in debates on new gene technologies. *Agric. Hum. Values* 40, 533–545. doi:10.1007/s10460-022-10367-6

Kokotovich, A. E., Barnhill-Dilling, S. K., Elsensohn, J. E., Li, R., Delborne, J. A., and Burrack, H. (2022). Stakeholder engagement to inform the risk assessment and governance of gene drive technology to manage spotted-wing drosophila. *J. Environ. Manag.* 307, 114480. doi:10.1016/j.jenvman.2022.114480

Kuiken, T., Barrangou, R., and Grieger, K. (2021). (Broken) promises of sustainable food and agriculture through new biotechnologies: the CRISPR case. *CRISPR J.* 4 (1), 25–31. doi:10.1089/crispr.2020.0098

Kuzma, J. (2016). A missed opportunity for U.S. biotechnology regulation. *Science* 353, 1211–1213. doi:10.1126/science.aai7854

Kuzma, J. (2021a). Deficits of public deliberation in US oversight for gene-edited organisms. *Hastings Cent. Rep.* 51 (6), S25–S33. doi:10.1002/hast.1317

Kuzma, J. (2022). Governance of gene-edited plants: insights from the history of biotechnology oversight and policy process theory. *Sci. Technol. Hum. Values* 2022, 016224392211082–32. doi:10.1177/01622439221108225

Kuzma, J. (2021b). Governance of genetically engineered animals in the wild: A need for procedurally-robust risk analysis. *Regul. Gov.* 15 (4), 1144–1165. doi:10.1111/rego.12245

Kuzma, J., and Grieger, K. (2020). Community-led governance for gene-edited crops. *Science* 370, 916–918. doi:10.1126/science.abd1512

Kuzma, J., and Kokotovich, A. (2011). Renegotiating GM crop regulation: targeted gene-modification technology raises new issues for the oversight of genetically modified crops. *EMBO Rep.* 12 (9), 883–888. doi:10.1038/embor.2011.160

Kuzma, J., Larson, J., and Najmaie, P. (2009). Evaluating oversight systems for emerging technologies: A case study of genetically engineered organisms. *J. Law Med. Ethics* 37 (4), 546–586. doi:10.1111/j.1748-720x.2009.00431.x

Kuzma, J. (2023). Making space for technology governance. *Issues Sci. Technol.* 38 (4).

Kuzma, J., Paradise, J., Kim, J., Kokotovich, A., Ramachandran, G., and Wolf, S. (2008). Integrated oversight assessment: A historical case study and multicriteria approach. *Risk Anal.* 28 (5), 1179–1195. doi:10.1111/j.1539-6924.2008.01086.x

Kuzma, J. (2018). Regulating gene edited crops. *Issues Sci. Technol.* 35 (1), 80–85.

Kuzma, J., and Williams, T. T. (2023). "Public inclusion and responsiveness in governance of genetically engineered animals," in *Democratizing risk governance:*

Bridging science, expertise, deliberation and public values. Editor M. Gattinger (Cham: Springer International Publishing), 191–216.

Lindberg, S. A., Peters, D. J., and Cummings, C. L. (2023). Gene-edited food adoption intentions and institutional trust in the United States: benefits, acceptance, and labeling. *Rural. Sociol.* 88, 392–425. doi:10.1111/ruso.12480

Macnaghten, P., and Habets, M. G. (2020). Breaking the impasse: towards a forward-looking governance framework for gene editing with plants. *Plants, People, Planet* 2 (4), 353–365. doi:10.1002/ppp3.10107

Marette, S. S., Disdier, A.-C., and Beghin, J. C. (2021). A comparison of EU and US consumers willingness to pay for gene-edited food: evidence from apples. *Appetite* 159, 105064. doi:10.1016/j.appet.2020.105064

Mathews, D. J., Fabi, R., and Offodile, I. I. (2022). Imagining governance for emerging technologies. *Issues Sci. Technol.* 38 (3), 40–46.

McConnachie, E., Hötzel, M. J., Robbins, J. A., Shriver, A., Weary, D. M., and Von Keyserlingk, M. A. (2019). Public attitudes towards genetically modified polled cattle. *PLOS One* 14 (5), e0216542. doi:10.1371/journal.pone.0216542

McFadden, B. R., and Wilson, N. (2018). Do consumers care how a genetically engineered food was created or who created it? *Food Policy* 74, 120–129. doi:10.1016/j.foodpol.2018.02.007

Millstone, E., Brunner, E., and Mayer, S. (1999). Beyond 'substantial equivalence'. *Beyond 'substantial equivalence'* Nat. 401 (6753), 525–526. doi:10.1038/44006

Mullins, E. (2023). The first crispr-edited salad is here. *Wired*. Available at https://www.wired.com/story/wired30-crispr-edited-salad-greens/(Accessed June 7, 2023).

NASEM (2016). *Genetically engineered crops: Experiences and prospects*. Washington DC: National Academies Press.

NASEM (2017). *Preparing for future products of biotechnology*. Washington DC: National Academies Press.

OSTP (2017). Modernizing the regulatory system for biotechnology products: final version of the 2017 update to the coordinated framework for the regulation of biotechnology. Available at https://www.epa.gov/sites/default/files/2017-01/documents/2017_coordinated_framework_update.pdf (Accessed June 7, 2023).

Parliamentary Office of Science and Technology (POST) (2022). *POSTbrief 50: Genome-edited animals*. UK Parliament. doi:10.58248/PB50

Pew Research Center (2016). Public opinion about genetically modified foods and trust in scientists connected with these foods. Available at https://www.pewresearch.org/science/2016/12/01/public-opinion-about-genetically-modified-foods-and-trust-in-scientists-connected-with-these-foods/(Accessed June 8, 2023).

Pew Research Center (2015). Public and scientists' views on science and society. Available at: https://www.pewresearch.org/science/2015/01/29/public-and-scientists-views-on-science-and-society/#science (Accessed December 21, 2021).

Pixley, K. V., Falck-Zepeda, J. B., Paarlberg, R. L., Phillips, P. W., Slamet-Loedin, I. H., Dhugga, K. S., et al. (2022). Genome-edited crops for improved food security of smallholder farmers. *Nat. Genet.* 54 (4), 364–367. doi:10.1038/s41588-022-01046-7

Rohr, V., Blakley, J., and Loring, P. (2021). A framework to assess food security in regional strategic environmental assessment. *Environ. Impact Assess. Rev.* 91, 106674. doi:10.1016/j.eiar.2021.106674

Ruzante, J., Grieger, K., Woodward, K., Lambertini, E., and Kowalcyk, B. (2017). *The use of multi-criteria decision analysis in food safety risk-benefit assessment*. Food Protection Trends, 132–139.

Scott, S. E., Inbar, Y., Wirz, C. D., Brossard, D., and Rozin, P. (2018). An overview of attitudes toward genetically engineered food. *Annu. Rev. Nutr.* 38 (1), 459–479. doi:10.1146/annurev-nutr-071715-051223

Shew, A. M., Nalley, L. L., Snell, H. A., Nayga, R. M., and Dixon, B. L. (2018). CRISPR versus GMOs: public acceptance and valuation. *Glob. Food Secur.* 19, 71–80. doi:10.1016/j.gfs.2018.10.005

Slovic, P. (1987). Perception of risk. *Science* 236, 280–285. doi:10.1126/science.3563507

Splitter, J. (2019). The latest gene-edited food is A soybean oil that comes with zero trans fats. *Forbes*, 5 March. Available at https://www.forbes.com/sites/jennysplitter/2019/03/05/trans-fat-free-gene-edited-soybean-oil/?sh=6e1c588e4c91 (Accessed June 7, 2023).

United Nations (UN) (2015). *Transforming our world: the 2030 agenda for sustainable development (resolution adopted by the general assembly on 25 september 2015)*. A/RES/70/1.

USDA (2015a). Cellectis submission Re: confirmation that FAD3KO soybean is not a regulated article. Available at https://www.aphis.usda.gov/biotechnology/downloads/reg_loi/15-071-01air.pdf (Accessed June 8, 2023).

USDA (2020d). Confirmation that Brassica juncea (L.) with improved flavor is not a regulated article. https://www.aphis.usda.gov/biotechnology/downloads/reg_loi/20-168-07_air_inquiry_a3_a2_cbidel.pdf (Accessed July 1, 2023).

USDA (2020a). Movement of certain genetically engineered organisms department of agriculture animal and plant health inspection service, 7 CFR parts 330, 340, and 372. *Fed. Regist.* 85 (96), 29790–29838. Final rule.

USDA (2020b). Re: confirmation of the regulatory status of calyxt's high oleic low linolenic soybean developed using TALEN technology. Available at https://www.aphis.usda.gov/biotechnology/downloads/reg_loi/20-066-01-air-response-signed.pdf (Accessed at June 7, 2023).

USDA (2020c). Re: confirmation of the regulatory status of genome edited Brassica juncea lines with improved flavor. Available at https://www.aphis.usda.gov/biotechnology/downloads/reg_loi/20-168-07_air_response_signed.pdf (Accessed June 7, 2023).

USDA (2023). Regulated articles letters of inquiry. Available at https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/Regulated_Article_Letters_of_Inquiry (Accessed June 7, 2023).

USDA (2015b). USDA response to Re: confirmation that FAD2KO soybean is not a regulated article. Available at Available at https://www.aphis.usda.gov/biotechnology/downloads/reg_loi/15-071-01air_resp.pdf (Accessed June 8, 2023).

USDA (2021). Weed risk assessment for Brassica tournefortii (Brassicaceae) – sahara mustard. Available at https://www.aphis.usda.gov/plant_health/plant_pest_info/weeds/downloads/wra/brassica-tournefortii.pdf (Accessed July 1, 2023).

Van Eenennaam, A., and Mueller Maci, L. (2022). Proceedings, applied reproductive strategies in beef cattle. August 30-31, 2022; san antonio, TX. Accessible at: https://beefrepro.org/wp-content/uploads/2022/08/2022-ARSCB-VanEenennaam-Mueller-Proceedings.pdf (Accessed June 7, 2023).

Wei, W., Grieger, K., Cummings, C. L., Loschin, N., and Kuzma, J. (2023). Identifying sustainability assessment parameters for genetically engineered agrifoods. *Plants, People, Planet* 2023, 10411. doi:10.1002/ppp3.10411

World Health Organization (2015). Cancer: carcinogenicity of the consumption of red meat and processed meat. Available at: https://www.who.int/news-room/questions-and-answers/item/cancer-carcinogenicity-of-the-consumption-of-red-meat-and-processed-meat (Accessed June 15, 2023).

Yawson, R., and Kuzma, J. (2010). Systems mapping of consumer acceptance of agrifood nanotechnology. *J. Consumer Policy* 33 (4), 299–322. doi:10.1007/s10603-010-9134-5

Yue, C., Shuoli, Z., Cummings, C., and Kuzma, J. (2015a). Investigating factors influencing consumer willingness to buy GM food and nano-food: an application of structural equation modeling. *J. Nanoparticle Res.* 17, 283–302. doi:10.1007/s11051-015-3084-4

Yue, C., Shuoli, Z., and Kuzma, J. (2015b). Heterogeneous consumer preferences for nanotechnology and genetic-modification technology in food products. *J. Agric. Econ.* 66 (2), 308–328. doi:10.1111/1477-9552.12090

Yunes, M. C., Osório-Santos, Z., von Keyserlingk, M. A. G., and Hotzel, M. J. (2021). Gene editing for improved animal welfare and production traits in cattle: will this technology be embraced or rejected by the public? *Sustainability* 13, 4966. doi:10.3390/su13094966

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Review
PUBLISHED 27 October 2023
DOI 10.3389/fbioe.2023.1276226

OPEN ACCESS

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RECEIVED 11 August 2023
ACCEPTED 16 October 2023
PUBLISHED 27 October 2023

CITATION
Koller F and Cieslak M (2023), A perspective from the EU: unintended genetic changes in plants caused by NGT—their relevance for a comprehensive molecular characterisation and risk assessment. *Front. Bioeng. Biotechnol.* 11:1276226. doi: 10.3389/fbioe.2023.1276226

A perspective from the EU: unintended genetic changes in plants caused by NGT—their relevance for a comprehensive molecular characterisation and risk assessment

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Several regions in the world are currently holding discussions in regard to the regulation of new genomic techniques (NGTs) and their application in agriculture. The European Commission, for instance, is proposing the introduction of specific regulation for NGT plants. Various questions need to be answered including e.g., the extent to which NGT-induced intended and unintended genetic modifications must be subjected to a mandatory risk assessment as part of an approval procedure. This review mostly focuses on findings in regard to unintended genetic changes that can be caused by the application of NGTs. More specifically, the review deals with the application of the nuclease CRISPR/Cas, which is currently the most important tool for developing NGT plants, and its potential to introduce double strand breaks (DSBs) at a targeted DNA sequence. For this purpose, we identified the differences in comparison to non-targeted mutagenesis methods used in conventional breeding. The review concludes that unintended genetic changes caused by NGT processes are relevant to risk assessment. Due to the technical characteristics of NGTs, the sites of the unintended changes, their genomic context and their frequency (in regard to specific sites) mean that the resulting gene combinations (intended or unintended) may be unlikely to occur with conventional methods. This, in turn, implies that the biological effects (phenotypes) can also be different and may cause risks to health and the environment. Therefore, we conclude that the assessment of intended as well as unintended genetic changes should be part of a mandatory comprehensive molecular characterisation and risk assessment of NGT plants that are meant for environmental releases or for market authorisation.

KEYWORDS

new genomic techniques (NGT), genetically engineered organisms, genome editing, GMO regulation, risk assessment, unintended genetic changes in NGT plants, comprehensive molecular characterisation

1 Introduction

According to EU GMO legislation (European Parliament and Council of the European Union, 2001), genetically modified organisms (GMOs) derived from "recombinant nucleic acid techniques" are to be regulated [Annex 1A, Part 1 of (European Parliament and Council of the European Union, 2001)]. As clarified by the European Court of Justice (Case C-528/16), this also applies to organisms derived from "new genomic techniques" (NGTs). The detailed risk assessment requirements are set out in Annex II of Directive 2001/18/EC (European Parliament and Council of the European Union, 2001) which was last amended in Commission Directive (EU) 2018/350 (European Commission, 2018). As introduced in the Annex (C1) of this in Commission Directive (EU) 2018/350 (European Commission, 2018), risk assessment "shall identify the intended and unintended changes resulting from the genetic modification and shall evaluate their potential to cause adverse effects on human health and on the environment." Furthermore, Annex II of Directive 2001/18/EC (European Parliament and Council of the European Union, 2001) in its "Principles for the environmental risk assessment" also gives weight to cumulative long-term effects.

For the purposes of this review, we use the same specific terminology as Koller et al., 2023 to distinguish between several categories of GMOs belonging to plants (Koller et al., 2023): 1) EU GMO regulation refers to GMOs which have to undergo mandatory approval processes and other GMOs which are exempt from these approval processes, e.g. plants derived from physical and chemical mutagenesis. The term "genetic engineering" (GE) is used throughout the review as a synonym for those GMOs which have to undergo mandatory approval processes; and 2) the term "established genomic techniques" (EGTs) is used to distinguish "old" GE plants (derived from non-targeted insertions of transgenes) from those more recently generated using NGTs (see also (EFSA, 2022a)). It is important to understand that both these categories (EGT and NGT) refer to GMOs which have to undergo mandatory approval processes (GE) according to the current legal situation.

Our review examines whether current EU regulation must in future continue to include the risk assessment of unintended genetic changes in NGT plants. In order to come to a sufficiently reasoned conclusion, our review first provides an overview of published findings related to unintended genetic changes caused by NGT processes in plants. Further, we identify causes for unintended genetic changes to differentiate these changes from non-targeted mutations which occur in conventional breeding. Finally, we discuss the consequences for the risk assessment of single events (individual NGT organisms), and long-term accumulated effects.

2 Differences between genetic changes caused by NGTs and conventional breeding

In short, and as summarized also by Kawall (2019) and Koller et al. (2023), site directed nucleases (SDN), such as CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR associated) (Jinek et al., 2012), are highly relevant in this context: they are designed to target specific DNA sequences in the genome to knock out gene functions (i.e. SDN-1) or to introduce specific changes of particular nucleotides (i.e. SDN-2) or whole genes (i.e. SDN-3). These methods can induce either non-specific changes (SDN-1) via non-homologous end joining (NHEJ) repair mechanisms or specific changes to nucleotide sequences (SDN-2 or SDN-3) via homologous recombination mediated by homology directed repair (HDR). The latter require an additional template. The induced changes at or around the target site can be substitutions, deletions or insertions of one or more base pairs. Depending on the specific SDN-1 or SDN-2 application, more extensive overall alterations are possible. For example, using multiplexing it is possible to target several genes simultaneously in a single application (Raitskin and Patron, 2016; Wang et al., 2016; Zetsche et al., 2017). Repeated applications of SDN-1 or SDN-2 can also be combined (Kawall et al., 2020). Changes involving the insertion of whole (cis- or trans-) genes (including gene-stacking) are also possible (SDN-3) and are mediated by the use of specific donor DNA (Sander and Joung, 2014; Eckerstorfer et al., 2019). For this review, we mostly focus on applications using CRISPR/Cas and its potential to introduce DSBs at targeted DNA sequences which is currently the most important tool for developing NGT plants (Parisi and Rodriguez, 2021). Other nucleases, such as TALENs (transcription activator-like effector nucleases) or variations of CRISPR nucleases (Parisi and Rodriguez, 2021), are also relevant, but so far of less importance for NGT in plants.

As has been shown many times [see for example (Morineau et al., 2017; Nonaka et al., 2017; Sánchez-León et al., 2018; Raffan et al., 2021)], NGTs enable the emergence of new genotypes and phenotypes to be generated in different ways and with different outcomes compared to previously used genetic engineering methods or conventional breeding (including non-targeted mutagenesis) (Eckerstorfer et al., 2019; Kawall, 2019; EFSA et al., 2021a; Kawall, 2021a; Kawall, 2021b).

In comparison to methods of conventional breeding (including non-targeted mutagenesis), NGTs can overcome the boundaries of natural genome organization: Relevant factors include repair mechanisms, gene duplications, genetic linkages and other epigenetic mechanisms [see, for example, (Lin et al., 2014; Wendel et al., 2016; Filler Hayut et al., 2017; Frigola et al., 2017; Roldan et al., 2017; Belfield et al., 2018; Huang and Li, 2018; Jones et al., 2018; Halstead et al., 2020; Monroe et al., 2022)]. By overcoming these boundaries, NGTs can make the genome much more extensively available for genetic changes (Kawall, 2019; Kawall et al., 2020).

In comparison to conventional plant breeding using non-targeted mutagenesis, the overall number of mutations is typically lower in NGT plants (Modrzejewski et al., 2020). However, due to the technical characteristics of NGTs, the sites of the mutations, their genomic context and their frequency (in regard to specific sites) can differ if compared to plants derived from conventional breeding methods. Such a non-random occurrence of mutations along the genome can therefore also be expected for the unintended genetic changes. This, in turn, means that the biological effects (phenotypes) can also be different and may cause specific risks for health and the environment.

Furthermore, it has to be considered that the processes of NGTs involve several technical steps that, in the case of plants, very often

include transformation processes which are also used in EGTs. These non-targeted methods are used to introduce the nucleases into the cells [see for example (Morineau et al., 2017; Nonaka et al., 2017; Sánchez-León et al., 2018; Raffan et al., 2021)] and may lead to unintended effects in off-target regions [for example (Braatz et al., 2017), see also below].

3 Five categories of unintended genetic changes resulting from NGT processes with relevance to risk assessment

Unintended genetic changes resulting from NGT processes can be differentiated as those with or without the insertion of transgenes, off-target changes or on-target changes, and those which are likely to be associated with or without the production of new gene products. Furthermore, this includes the identification of smaller genetic changes versus those that involve larger parts of the genome or even complex patterns of genetic changes. While some of the "types" of genetic alteration might also be observed in conventional breeding, there may also be some differences in regard to the probability of these changes occurring at specific sites in the genome (see above). In order to differentiate between unintended genetic changes resulting from NGTs and those resulting from conventional breeding, we suggest aligning them with the following five categories.

3.1 Unintended genetic changes resulting from the insertion of transgenes via EGTs (off-target)

At present, NGT applications in plants are in most cases a multi-step process. For example, NGTs, such as CRISPR/Cas applications in plants, typically make use of EGT techniques, i.e. non-targeted methods, to deliver the DNA coding for the nuclease into the cells [for overview, see (Kawall et al., 2020)]. Thus, in most cases, the result of the first step of the CRISPR/Cas application is a transgenic plant which may show a broad range of unintended genetic changes, which may be different to those emerging from conventional breeding, as for example discussed by Latham et al., 2006 and more recently confirmed by Yue et al. (2022). As recently summarized by Koller et al. (2023), such effects may be linked to epigenetic regulation, the disruption of genes, position effects, open reading frames, the unintended introduction of additional genes, changes in gene expression, genomic interactions which can involve plant constituents, or plant composition and agronomic characteristics (Forsbach et al., 2003; Makarevitch et al., 2003; Windels et al., 2003; Rang et al., 2005; Gelvin, 2017; Jupe et al., 2019; Liu et al., 2019; Chu and Agapito-Tenfen, 2022; Yue et al., 2022). There are several studies showing that the problem of unintended insertion of transgenes is relevant to NGT applications in plants (Li et al., 2015; Braatz et al., 2017; Biswas et al., 2020; Michno et al., 2020) or also animals (Norris et al., 2020). Even if segregation breeding is used in plant species with sexual reproduction at the end of the multistep process, to remove the functional transgenic elements from the plant genome, unintended genetic changes may still remain in the genome unnoticed.

3.2 Unintended insertion of transgenes with NGT processes

As several publications show, DSBs caused by CRISPR/Cas interventions are associated with the insertion of transgenes, especially at the target site or elsewhere in the genome. These on-target and off-target effects often include the integration of DNA from vector DNA derived from transformation processes, where, for example, fragments of the transgenes were unexpectedly integrated (Li et al., 2015; Andersson et al., 2017; Braatz et al., 2017; Sánchez-León et al., 2018; Zhang et al., 2018; Biswas et al., 2020).

Also in animal cells, it was found that unintentionally inserted foreign DNA fragments may originate from the vector construct (Norris et al., 2020). In some cases, in mammalian cells, inserted additional DNA taken up from the growth medium were also found (Ono et al., 2019). Overall, the CRISPR/Cas9 system has been confirmed to have a high frequency of unintended integration of additional DNA into the target sites (Lee et al., 2019; Yang et al., 2022).

Research is underway to develop transgene free delivery of the CRISPR/Cas molecules into the plant cells [see for example (Banakar et al., 2019; Kocsisova and Coneva, 2023)]. However, questions remain upon their application in practice [see for example (Kawall et al., 2020)]. Therefore, we assume that unintended insertion of transgenes will remain a challenge in future.

3.3 Unintended genetic changes without the insertion of transgenes (on-target and off-target)

Various unintended genetic changes resulting from CRISPR/Cas applications have been described for plants. These include off-target DNA cleavage, repetitive unit deletion, indels of various sizes, larger structural changes in the targeted genomic region (with and without the insertion of transgenes) (Zhang et al., 2014; Kapahnke et al., 2016; Wolt et al., 2016; Braatz et al., 2017; Kapusi et al., 2017; Lalonde et al., 2017; Sharpe and Cooper, 2017; Kosicki et al., 2018; Chakrabarti et al., 2019; Biswas et al., 2020; Burgio and Teboul, 2020; Kawall et al., 2020; Manghwar et al., 2020; Michno et al., 2020; Molla and Yang, 2020; Skryabin et al., 2020; Liu et al., 2021; Yang et al., 2022; Samach et al., 2023a).

Although some of these "types" of genetic alteration might also be observed in conventional breeding (EFSA, 2020), they differ in terms of their likelihood of occurring at specific sites in the genome. Therefore, these effects can not be generally equated to those emerging from conventional breeding.

For example, larger structural genomic changes, such as translocations, deletions, duplications, inversions and scrambling of chromosomal sequences, can occur in or near the targeted genomic region which would otherwise be unlikely to occur [see e.g., (Hahn and Nekrasov, 2019)]. It should be considered that especially so-called bystander deletions and complex rearrangements in neighboring on-target sequences (EFSA et al., 2021a) may be difficult to detect (Simeonov et al., 2019).

It is known that the nucleases rather recognize and target specific DNA sequences of a particular length rather than functional genetic elements at specific genomic sites (Ahloowalia and Maluszynski,

2001; Höijer et al., 2022). Therefore, the CRISPR/Cas machinery has a potential to bind not only to the targeted regions, but also to additional off-target regions that share similarity–within a given mismatch tolerance–to the target DNA sequences. Accordingly, research is underway that tries to improve to increase the on-target efficiency and mitigate the off-target impact on intended genome-editing outcomes [such as (Wolt et al., 2016; Manghwar et al., 2020)]. However, previous studies focussing on these unintended genetic changes (Modrzejewski et al., 2019; 2020) identified gaps in the methodology such as studies being very heterogeneous in their structure and design, as well as the number of published data. Therefore, it looks like off-target effects will remain a challenge at least for the near future.

Since many of these undesirable effects as described above are often caused by DSBs introduced by the nuclease, other methods are under development that are purposed to introduce genetic changes without DSBs, especially in the area of human medicine such as base editing (Anzalone et al., 2020). These methods are also known to cause unintended genetic changes throughout the genome which requires in depth molecular characterisation and risk assessment (Rao et al., 2023). However, since these methods, so far, do not play a major role in NGT plants, they are not discussed in this review.

3.4 Chromothripsis-like effects

Chromothripsis is a genetic phenomenon where possibly hundreds of clustered chromosomal rearrangements can happen in a single catastrophic event. In mammals (including humans), the phenomenon is associated with cancer and congenital diseases. Available publications (Leibowitz et al., 2021; Samach et al., 2023a; de Groot et al., 2023) show that biotechnological mutagens, such as nucleases that cause a DSB in the DNA, are a likely cause of chromothripsis-like effects. According to de Groot et al. (2023), in cases where DSBs are not quickly resolved, they can be involved in rearrangements with other parts of the genome involving one or a few chromosomes. The process can be associated with deletions, insertions, inversions, duplications and double-minute formation.

It has been known that CRISPR/Cas applications strongly increase the likelihood of chromothripsis occurring in mammalian cells (Leibowitz et al., 2021; Amendola et al., 2022). Just recently, these effects were also reported in plants by Samach et al. (2023a). They identified whole chromosome losses as well as major chromosomal rearrangements, including the loss of large fragments, inversions, translocations and somatic crossovers associated with CRISPR/Cas-induced DSBs.

DSBs also may occur if, for example, plant cells are exposed to high dosage of radiation (non-targeted mutagenesis) (EFSA et al., 2021b). However, NGTs may impact the probability of chromothripsis occurring at specific genomic sites with a higher likelihood and therefore, its biological effects may depend on the genomic regions that are targeted by the processes of NGTs. For example, in plants with many copies of the targeted genes [see, for example, (Sánchez-León et al., 2018)], CRISPR/Cas is likely to cause several DSBs simultaneously in a specific pattern. Similarly, many DSBs can be caused by targeting several genes in parallel ["multiplexing", see (Zsögön et al., 2018)]. Furthermore, the CRISPR/Cas machinery can interfere with the repair mechanisms in the cells, preventing them from restoring the original gene functions and stopping the cells from rapidly resolving the DSB [see (Kawall, 2019)].

These findings make it plausible that DSBs and chromothripsis-like effects caused by biotechnological mutagens (nucleases) should not generally be equated with those of non-targeted physical-chemical mutagens.

3.5 Unintended genetic changes that may cause the formation of new gene products (without insertion of transgenes)

The use of CRISPR/Cas gene scissors can induce various changes at the target sites. The targeted site (or also off-target sites) can be altered in such a way that no more mRNAs are formed, thus preventing the formation of the corresponding protein. However, new mRNAs can also be unintentionally formed, and thus cause new proteins to emerge.

For example, the changes introduced by the nucleases can lead to an effect called exon skipping. In exon skipping, mRNAs can be assembled differently than planned even if the intended changes are induced at the target site. This can lead to the formation of shortened mRNAs. The resulting proteins are then also shorter, but can still carry out functions in the cell. The effects of exon skipping were described in mammalian cells (Kapahnke et al., 2016; Mou et al., 2017) as well as in plant cells (Ramírez-Sánchez et al., 2016). In this context, also frameshift mutations are described. They cause a shift in the reading frame of a DNA sequence which may go along with change in the gene function (Lalonde et al., 2017).

As a result of exon skipping and frameshift mutations, new mRNAs and proteins, or also non-coding RNAs (ncRNA) with effects on gene regulation, can be formed and fulfill new functions in cell metabolism (Kapahnke et al., 2016; Lalonde et al., 2017; Mou et al., 2017; Tuladhar et al., 2019; Jia et al., 2022). For example, effects caused by knocking out of 35 gene copies in wheat (Sánchez-León et al., 2018) were discussed by EFSA et al. (2021a) as a potential cause for the occurrence of peptide fragments that could play a role in the inflammatory cascade (see also below). Frameshift mutations may play a significant role in the emergence of such fragmented peptides.

Again, since these unintended genetic changes may not occur randomly across the genome, its biological effects may depend on the genomic regions that are targeted by the processes of NGT. These effects can not be generally equated to those emerging from conventional breeding.

4 Consequences for a comprehensive molecular characterisation and risk assessment of single events

According to EU regulation as cited above (European Parliament and Council of the European Union, 2001; European Commission, 2018), it has to be taken into account that unintended genetic changes "can have either direct or indirect, and either immediate or delayed effects on human health and on the

environment." Therefore, the risk assessment "shall identify the intended and unintended changes resulting from the genetic modification and shall evaluate their potential to cause adverse effects on human health and on the environment."

Based on the various findings regarding unintended genetic effects that NGTs can cause, it does not appear possible to predict or control their occurrence and associated effects for specific events. As shown, unintended genetic changes can affect large sections of chromosomes and result in the emergence of unintended gene products. Since these unintended genetic changes may not occur randomly across the genome, its biological effects may depend on the genomic regions that are targeted by the processes of NGTs and therefore are also relevant for risk assessment.

It is only afterwards through applying methods, e.g. whole genome sequencing (WGS) and other methods to identify long and short DNA sequence alterations [see, for example, (Kawall et al., 2020; Chu and Agapito-Tenfen, 2022; Park et al., 2023)] that the unintended changes can be detected in the cell. By comparing the "wild type" with the one resulting from NGT applications, the unintended genetic alterations can become detectable and be made comparable to those that are likely to occur with conventional methods. As especially large deletions and chromosomal rearrangements are hardly detectable by standard short-range PCR based assays, it is important to combine multiple approaches to assess all types of gene alterations (Park et al., 2023). Park et al. (2023) state, no single tool can detect all types of large gene modifications accurately that can be caused by CRISPR/Cas9. Therefore, it is important to combine multiple approaches to comprehensively identify and assess the unintended changes throughout the genome [see also (Mou et al., 2017; Hahn and Nekrasov, 2019; Yasumoto and Muranaka, 2023)].

As DNA sequencing will not always allow the identification of the associated unintended biological effects, additional methods, such as transcriptomics and metabolomics, should be used to draw reliable conclusions [see (Kawall et al., 2020; EFSA et al., 2022c)]. If no unintended genetic alterations are detected that are specific to NGT processes, risk assessment may focus on the intended changes.

After comprehensive molecular characterisation has been concluded, further steps in risk assessment should follow, such as the analysis of plant composition, agronomic and other phenotypical characteristics, that also may include further investigations in regard to human health and the environment [see (EFSA, 2010; European Commission, 2013;)]. Data from the molecular assessment can be used to inform and guide these further steps in risk assessment and the development of a specific risk hypothesis.

5 Consequences of a comprehensive molecular characterisation and risk assessment regarding long-term cumulative effects

As cited above, Directive 2001/18/EC (European Parliament and Council of the European Union, 2001) also gives weight to cumulative long-term effects: "A general principle for environmental risk assessment is also that an analysis of the cumulative long-term effects relevant to the release and the placing on the market is to be carried out. "Cumulative long-term effects" refers to the accumulated effects of consents on human health and the environment, including *inter alia* flora and fauna, soil fertility, soil degradation of organic material, the feed/food chain, biological diversity, animal health and resistance problems in relation to antibiotics." Furthermore, similarly to Commission Directive (EU) 2018/350 (European Commission, 2018), Commission Implementing Regulation (EU) No 503/2013 (European Commission, 2013) also requires the assessment of stacked events in regard to their "potential additive, synergistic or antagonistic effects resulting from the combination of the transformation events."

It should not be overlooked that several databases show that there are dozens of current NGT projects using species such as oilseed rape (*Brassica napus*), tomato (*Solanum lycopersicum*) or wheat (*Triticum aestivum*) [for example see (Koller et al., 2023)]. In this respect, it is necessary to consider the overall gene pool of the species concerned. As Koller et al. (2023) show, if NGTs are used to generate different traits in one species, the resulting intended and/or unintended genetic changes may lead to interactions between the individual NGT organisms, and are thus relevant to risk assessment (Koller et al., 2023). There is also the need to take into account simultaneous spatial cultivation, further crossings and technical stacking of the various events. The resulting effects may be dependent on specific combinations of intended or unintended genetic variants, or the intended traits. In addition, the exposure to stress conditions in the receiving environment may have an influence. Even if all the individual events were considered to be "safe", uncertainties or unknowns will still remain because of possible interactions of the intended and unintended genetic changes and associated effects in each event. The environmental risk assessment of individual events may, therefore, not be sufficient to predict and assess all these interactions. Special caution will be needed if the plants have the potential to persist, propagate and spontaneously cross in the environmental and/or perform gene flow to related species (Bauer-Panskus et al., 2020).

When developing relevant risk scenarios [see (Koller et al., 2023)], it also has to be considered that unintended genetic changes might be passed to offspring and introgress various genetic backgrounds that, for example, can cause changes in gene expression. Furthermore, the unintended genetic changes caused by NGT processes may also accumulate through subsequent crossings in following generations. This can result in phenotypes that differ significantly from those of their precursor plants (Bauer-Panskus et al., 2020).

As also mentioned by Koller et al. (2023), unpredictable genomic interactions may, for example, be caused by cryptic gene variants depending on the genetic background. Cryptic variations are considered to be mutations that, regardless of whether they occur naturally or are introduced by technical processes, have little or no phenotypic consequences unless exposed to additional genetic or environmental interactions, as for example discussed in the context of tomatoes (Rodríguez-Leal et al., 2017; Soyk et al., 2019; Alonge et al., 2020). Therefore, the genomic interactions emerging from spontaneous crossings or intended stacking may also become relevant to the assessment of unintended (as well as intended) genetic changes caused by NGT processes.

In some cases, too many uncertainties may remain due to the potential interactions and cumulative effects. Therefore, cut-off criteria will be needed to identify applications that will not allow robust conclusions on safety (Bauer-Panskus et al., 2020).

6 Discussion

As shown in this review, NGTs can cause different intended and unintended genetic changes in comparison to conventional breeding (including random mutagenesis). Relevant differences concern the site of the genetic alterations and their resulting pattern in the genome, the insertion of transgenes and the probability of chromothripsis-like events occurring at specific genomic sites.

It is conceivable that in some cases, the unintended genetic changes may have a higher relevance for risk assessment than the intended changes. Therefore, requirements regarding a mandatory investigation of intended and unintended genetic alterations, e.g. in the context of the EU GMO regulation, seem to be a scientifically justified necessity as also confirmed by Eckerstorfer et al. (2023).

There is an ongoing debate within the EU about the future regulation of NGT plants. Therefore, the European Food Safety Authority (EFSA), as mandated by the European Commission, has published several opinions dealing with aspects of risk assessment in relation to NGT plants (EFSA, 2012; EFSA, 2020; EFSA E. et al., 2021; EFSA, 2022c). As EFSA is a main source of science-based decision-making in the EU, we think it is important to compare our findings with the EFSA opinions.

EFSA concluded that in some cases, intended and unintended effects caused by NGT processes may require in-depth risk assessment. For example, EFSA (EFSA et al., 2021a) discusses an NGT wheat with a reduction of alpha-gliadin proteins (Sánchez-León et al., 2018). In this wheat, 35 out of 45 targeted alpha-gliadin genes were altered with CRISPR/Cas (SDN-1) to reduce the gluten content in food products. Many insertions and/or deletions at the targeted DNA sequences were described. EFSA came to the conclusion that the intended and unintended changes at the target sites pose in this case new challenges for risk assessment: "While plants with a small number of mutations have already reached the market, the large number of mutations required to achieve gluten-free wheat is far beyond any plant previously assessed. This is likely to require SynBio approaches to correctly identify all gliadins and glutenins in the hexaploid genome of bread wheat and to identify an engineering strategy that introduced mutations of the correct nature and positions in each gene to prevent the accumulation of any peptide fragments associated with initiation of the inflammatory cascade" (EFSA et al., 2021a).

From the findings of EFSA it seems that at least each targeted genetic site would undergo a detailed examination to determine whether the alpha-gliadin proteins are still produced, or if new proteins are being unintentionally produced, or if there are any other unintended effects.

Furthermore, EFSA (2020) also believes that the unintended insertions of transgenes in NGT plants need to be risk assessed: "When plant transformation is used to introduce the SDN module, the unintended insertion of plasmid DNA or other exogenous DNA into the plant genome can happen. Furthermore, the application of some methods (e.g. transient expression and DNA-free methods) to achieve SDN-1 and SDN-2 modifications can result in the unintended integration of exogenous DNA whose sequence may be known *a priori* [examples of unintended on-target insertion of exogenous DNA can be found in Clasen et al. (2015), Andersson et al., 2017, Norris et al. (2020), Solomon (2020)]. If the final product is not intended to retain any exogenous DNA, the applicant should assess the potential presence of a DNA sequence derived from the methods used to generate the SDN modification (e.g. plasmids or vectors). It should be noted that the assessment of the unintentional integration of exogenous DNA is already part of the molecular characterisation in the risk assessment of GM plants, under EU Regulations. Therefore, this is not to be considered a new requirement for risk-assessing genome-edited plants." (EFSA, 2020).

However, in regard to other off-target effects, EFSA indicates that these would not require mandatory risk assessment, as they would be the same type of mutations caused by conventional breeding and/or random mutagenesis. A lot of emphasis is placed on the number of mutations–these are generally considered to be lower for NGTs in comparison to non-targeted methods. It appears to have escaped the notice of EFSA that these criteria may not be sufficient to draw reliable conclusions on health and environmental safety.

EFSA already dealt with the issue of unintended genetic changes in its opinion published in 2012. In its opinion, EFSA only addressed the type of mutations (such as indels) and the frequency of mutations. EFSA (2012) concluded at that time: "Whilst the SDN-3 technique can induce off-target changes in the genome of the recipient plant these would be fewer than those occurring with most mutagenesis techniques used in conventional breeding. Furthermore, where such changes occur they would be of the same types as those produced by conventional breeding techniques."

EFSA in its 2020 opinion again deals with the frequency and type of mutations and does not consider other criteria, e.g. the site of the mutation, the genomic context, the resulting genetic combinations or any associated unintended phenotypical effects (EFSA, 2020). As EFSA (2020) states in its summary: "The EFSA Opinion on SDN-3 concluded that the application of SDN-3 can induce off-target mutations but these would be fewer than those occurring with most mutagenesis techniques (EFSA, 2020). Where they do occur, these changes would be the same types as those derived by conventional breeding techniques (EFSA, 2012). As SDN-1 and SDN-2 techniques use the same molecular mechanisms to generate DSB as SDN-3, the conclusions for SDN-3 are also applicable to SDN-1 and SDN-2."

Once more, in its updated opinion on cisgenic plants, EFSA deals with the frequency and type of mutations and states that the frequency of mutations might be lower in the case of SDN-plants in comparison to previously used breeding methods (EFSA, 2022c). Again, EFSA did not consider the site of the mutation, the resulting gene combinations and specific unintended effects that may by caused by NGT processes. It appears that EFSA also became aware of some gaps in research, stating that: "Moreover, the GMO Panel was not mandated to provide a comprehensive literature review on the SDN-based technology and its unintended effects." (EFSA, 2022b).

We conclude that the differences in the EFSA findings and our review are to a certain extent due to methodology: in regard to off-target unintended genetic changes resulting from NGT processes, EFSA mainly considered the overall frequency of mutations and the types of mutation that can be observed. However, EFSA did not take into account that unintended genetic changes caused by the processes of NGTs may not occur randomly across the genome and its biological effects may depend on the genomic regions that are targeted by the NGT processes. Therefore, EFSA did not consider the likelihood of unintended changes occurring at specific sites. It also

did not consider resulting specific gene combinations, the frequency of chromothripsis-like events or the emergence of unintended gene products. Unintended effects in regard to the phenotypes and the environment were also not taken into consideration, although they may be associated with these unintended genetic changes.

7 Conclusion

As required in current EU regulation, unintended genetic changes and their potential effects have to be taken into account in the mandatory molecular characterisation and risk assessment of NGT plants. This requirement is relevant to the single event as well as all events within the gene pool of the species.

Since the unintended genetic changes as categorized above can neither be predicted nor excluded *a priori*, comprehensive molecular characterisation and risk assessment has to be performed for each single event. In many cases, if unintended genetic changes are caused by the processes of NGT, they may not occur randomly across the genome and its biological effects may depend on the genomic regions that are targeted by the NGT processes. Therefore, risk assessment should aim to identify those unintended genetic changes which (for example, in regard to the site, the frequency, its potential gene products or its origin) are unlikely to occur with conventional (non-regulated) methods. The methodology to identify these changes should include WGS by using long read sequencings, also in combination with other methods for gene analysis (Park et al., 2023). Comparison should be performed to the genome of the "wild type" plants that were used as starting point. In addition, comparisons with genome databases may be performed.

Furthermore, the comprehensive molecular characterisation and risk assessment should also comprise "Omics" (such as transcriptomics, proteomics and metabolomics) as discussed by EFSA (EFSA et al., 2022c).

It will depend on the findings of this molecular characterisation what further data for risk assessment will be required, i.a. for the analysis of plant composition and other phenotypical characteristics [as, for example, outlined in (EFSA, 2010; European Commission, 2013)].

Even if specific unintended effects arising from molecular changes due to NGTs cannot be identified in a specific event, the regulator still has to consider cumulative effects and potential interactions which could result from future crossings within the same species or wild relative species.

The resulting unintended effects may be dependent on specific combinations of intended or unintended genetic variants, which may become obvious only after exposure to stress conditions in the receiving environment (Koller et al., 2023).

If unintended genetic changes, potentially causing adverse effects are overlooked, these may endanger health, the environment and also agricultural production. Therefore, unintended genetic changes caused by the processes of NGTs has to be included in mandatory risk assessment before the plants are released into the environment or placed onto the market.

Author contributions

FK: Writing–original draft, Writing–review and editing. MC: Writing–original draft, Writing–review and editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. Funding was received by the Bundesamt für Naturschutz (BfN), FKZ 3522840500.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Ahloowalia, B. S., and Maluszynski, M. (2001). Induced mutations – a new paradigm in plant breeding. *Euphytica* 118, 167–173. doi:10.1023/A:1004162323428

Alonge, M., Wang, X., Benoit, M., Soyk, S., Pereira, L., Zhang, L., et al. (2020). Major impacts of widespread structural variation on gene expression and crop improvement in tomato. *Cell* 182, 145–161.e23. doi:10.1016/j.cell.2020.05.021

Amendola, M., Brusson, M., and Miccio, A. (2022). CRISPRthripsis: the risk of CRISPR/Cas9-induced chromothripsis in gene therapy. *Stem Cells Transl. Med.* 11, 1003–1009. doi:10.1093/stcltm/szac064

Andersson, M., Turesson, H., Nicolia, A., Fält, A.-S., Samuelsson, M., and Hofvander, P. (2017). Efficient targeted multiallelic mutagenesis in tetraploid potato (Solanum tuberosum) by transient CRISPR-Cas9 expression in protoplasts. *Plant Cell Rep.* 36, 117–128. doi:10.1007/s00299-016-2062-3

Anzalone, A. V., Koblan, L. W., and Liu, D. R. (2020). Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. *Nat. Biotechnol.* 38, 824–844. doi:10.1038/s41587-020-0561-9

Banakar, R., Eggenberger, A. L., Lee, K., Wright, D. A., Murugan, K., Zarecor, S., et al. (2019). High-frequency random DNA insertions upon co-delivery of CRISPR-Cas9 ribonucleoprotein and selectable marker plasmid in rice. *Sci. Rep.* 9, 19902. doi:10.1038/s41598-019-55681-y

Bauer-Panskus, A., Miyazaki, J., Kawall, K., and Then, C. (2020). Risk assessment of genetically engineered plants that can persist and propagate in the environment. *Environ. Sci. Eur.* 32, 32. doi:10.1186/s12302-020-00301-0

Belfield, E. J., Ding, Z. J., Jamieson, F. J. C., Visscher, A. M., Zheng, S. J., Mithani, A., et al. (2018). DNA mismatch repair preferentially protects genes from mutation. *Genome Res.* 28, 66–74. doi:10.1101/gr.219303.116

Biswas, S., Tian, J., Li, R., Chen, X., Luo, Z., Chen, M., et al. (2020). Investigation of CRISPR/Cas9-induced SD1 rice mutants highlights the importance of molecular characterization in plant molecular breeding. *J. Genet. Genomics* 47, 273–280. doi:10.1016/j.jgg.2020.04.004

Braatz, J., Harloff, H.-J., Mascher, M., Stein, N., Himmelbach, A., and Jung, C. (2017). CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different

homoeologous gene copies in polyploid oilseed rape (*Brassica napus*). *Plant Physiol.* 174, 935–942. doi:10.1104/pp.17.00426

Burgio, G., and Teboul, L. (2020). Anticipating and identifying collateral damage in genome editing. *Trends Genet.* 36, 905–914. doi:10.1016/j.tig.2020.09.011

Chakrabarti, A. M., Henser-Brownhill, T., Monserrat, J., Poetsch, A. R., Luscombe, N. M., and Scaffidi, P. (2019). Target-specific precision of CRISPR-mediated genome editing. *Mol. Cell* 73, 699–713.e6. doi:10.1016/j.molcel.2018.11.031

Chu, P., and Agapito-Tenfen, S. Z. (2022). Unintended genomic outcomes in current and next generation GM techniques: a systematic review. *Plants* 11, 2997. doi:10.3390/plants11212997

Clasen, B. M., Stoddard, T. J., Luo, S., Demorest, Z. L., Li, J., Cedrone, F., et al. (2015). Improving cold storage and processing traits in potato through targeted gene knockout. *Plant Biotechnol J.* 14, 169–76. doi:10.1111/pbi.12370

de Groot, D., Spanjaard, A., Hogenbirk, M. A., and Jacobs, H. (2023). Chromosomal rearrangements and chromothripsis: the alternative end generation model. *Int. J. Mol. Sci.* 24, 794. doi:10.3390/ijms24010794

Eckerstorfer, M. F., Dolezel, M., Engelhard, M., Giovannelli, V., Grabowski, M., Heissenberger, A., et al. (2023). Recommendations for the assessment of potential environmental effects of genome-editing applications in plants in the EU. *Plants (Basel)* 12, 1764. doi:10.3390/plants12091764

Eckerstorfer, M. F., Dolezel, M., Heissenberger, A., Miklau, M., Reichenbecher, W., Steinbrecher, R. A., et al. (2019). An EU perspective on biosafety considerations for plants developed by genome editing and other new genetic modification techniques (nGMs). *Front. Bioeng. Biotechnol.* 7, 31–13. doi:10.3389/fbioe.2019.00031

EFSA (2010). Guidance on the environmental risk assessment of genetically modified plants: EFSA Guidance Document on the ERA of GM plants. *EFSA J.* 8, 1879. doi:10.2903/j.efsa.2010.1879

EFSA (2012). Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function | EFSA. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/2943 (Accessed August 8, 2023).

EFSA (2020). Applicability of the EFSA Opinion on site-directed nucleases type 3 for the safety assessment of plants developed using site-directed nucleases type 1 and 2 and oligonucleotide-directed mutagenesis | EFSA. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/6299 (Accessed August 8, 2023).

EFSA, Naegeli, H., Bresson, J., Dalmay, T., Dewhurst, I. C., Epstein, M. M., et al. (2021a). Evaluation of existing guidelines for their adequacy for the molecular characterisation and environmental risk assessment of genetically modified plants obtained through synthetic biology. *EFSA J.* 19, e06301. doi:10.2903/j.efsa.2021.6301

EFSA, Mullins, E., Bresson, J., Dalmay, T., Dewhurst, I. C., Epstein, M. M., et al. (2021b). *In vivo* and *in vitro* random mutagenesis techniques in plants. *EFSA J.* 19, e06611. doi:10.2903/j.efsa.2021.6611

EFSA (2022a). Criteria for risk assessment of plants produced by targeted mutagenesis, cisgenesis and intragenesis | EFSA. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/7618 (Accessed August 8, 2023).

EFSA, Iacono, G., Guerra, B., Kass, G., Paraskevopoulos, K., Kleiner, J., et al. (2022c). Theme (concept) paper - application of OMICS and BIOINFORMATICS approaches: towards next generation risk assessment. *EFSA Support. Publ.* 19, E200506E. doi:10.2903/sp.efsa.2022.e200506

EFSA (2022b). Public consultation on the updated scientific opinion on plants developed through cisgenesis and intragenesis. Available at: https://connect.efsa.europa.eu/RM/s/publicconsultation2/a0l7U0000011Zb2/pc0176 (Accessed August 8, 2023).

EFSA (2022c). Updated scientific opinion on plants developed through cisgenesis and intragenesis | EFSA. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/7621 (Accessed August 8, 2023).

European Commission (2013). Commission implementing regulation (EU) No 503/2013 of 3 april 2013 on applications for authorisation of genetically modified food and feed in accordance with regulation (EC) No 1829/2003 of the European parliament and of the Council and amending commission regulations (EC) No 641/2004 and (EC) No 1981/2006 text with EEA relevance. Available at: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32013R0503 (Accessed June 7, 2022).

European Commission (2018). Commission Directive (EU) 2018/350 of 8 March 2018 amending Directive 2001/18/EC of the European Parliament and of the Council as regards the environmental risk assessment of genetically modified organisms. Available at: http://data.europa.eu/eli/dir/2018/350/oj/eng (Accessed June 7, 2022).

European Parliament, and Council of the European Union (2001). Directive 2001/18/EC of the European parliament and of the Council of 12 march 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council directive 90/220/EEC - commission declaration. Available at: https://eur-lex.europa.eu/eli/dir/2001/18/oj/eng (Accessed June 7, 2022).

Filler Hayut, S., Melamed Bessudo, C., and Levy, A. A. (2017). Targeted recombination between homologous chromosomes for precise breeding in tomato. *Nat. Commun.* 8, 15605. doi:10.1038/ncomms15605

Forsbach, A., Schubert, D., Lechtenberg, B., Gils, M., and Schmidt, R. (2003). A comprehensive characterization of single-copy T-DNA insertions in the *Arabidopsis thaliana* genome. *Plant Mol. Biol.* 52, 161–176. doi:10.1023/A:1023929630687

Frigola, J., Sabarinathan, R., Mularoni, L., Muiños, F., Gonzalez-Perez, A., and López-Bigas, N. (2017). Reduced mutation rate in exons due to differential mismatch repair. *Nat. Genet.* 49, 1684–1692. doi:10.1038/ng.3991

Gelvin, S. B. (2017). Integration of *agrobacterium* T-DNA into the plant genome. *Annu. Rev. Genet.* 51, 195–217. doi:10.1146/annurev-genet-120215-035320

Hahn, F., and Nekrasov, V. (2019). CRISPR/Cas precision: do we need to worry about off-targeting in plants? *Plant Cell Rep.* 38, 437–441. doi:10.1007/s00299-018-2355-9

Halstead, M. M., Kern, C., Saelao, P., Wang, Y., Chanthavixay, G., Medrano, J. F., et al. (2020). A comparative analysis of chromatin accessibility in cattle, pig, and mouse tissues. *BMC Genomics* 21, 698. doi:10.1186/s12864-020-07078-9

Höijer, I., Emmanouilidou, A., Östlund, R., van Schendel, R., Bozorgpana, S., Tijsterman, M., et al. (2022). CRISPR-Cas9 induces large structural variants at on-target and off-target sites *in vivo* that segregate across generations. *Nat. Commun.* 13, 627. doi:10.1038/s41467-022-28244-5

Huang, Y., and Li, G.-M. (2018). DNA mismatch repair preferentially safeguards actively transcribed genes. *DNA Repair (Amst)* 71, 82–86. doi:10.1016/j.dnarep.2018.08.010

Jia, Y., Qin, C., Traw, M. B., Chen, X., He, Y., Kai, J., et al. (2022). In rice splice variants that restore the reading frame after frameshifting indel introduction are common, often induced by the indels and sometimes lead to organism-level rescue. *PLOS Genet.* 18, e1010071. doi:10.1371/journal.pgen.1010071

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821. doi:10.1126/science.1225829

Jones, D. M., Wells, R., Pullen, N., Trick, M., Irwin, J. A., and Morris, R. J. (2018). Spatio-temporal expression dynamics differ between homologues of flowering time genes in the allopolyploid *Brassica napus*. *Plant J.* 96, 103–118. doi:10.1111/tpj.14020

Jupe, F., Rivkin, A. C., Michael, T. P., Zander, M., Motley, S. T., Sandoval, J. P., et al. (2019). The complex architecture and epigenomic impact of plant T-DNA insertions. *PLoS Genet.* 15, e1007819. doi:10.1371/journal.pgen.1007819

Kapahnke, M., Banning, A., and Tikkanen, R. (2016). Random splicing of several exons caused by a single base change in the target exon of CRISPR/Cas9 mediated gene knockout. *Cells* 5, 45. doi:10.3390/cells5040045

Kapusi, E., Corcuera-Gómez, M., Melnik, S., and Stoger, E. (2017). Heritable genomic fragment deletions and small indels in the putative ENGase gene induced by CRISPR/Cas9 in barley. *Front. Plant Sci.* 8, 540. doi:10.3389/fpls.2017.00540

Kawall, K. (2019). New possibilities on the horizon: genome editing makes the whole genome accessible for changes. *Front. Plant Sci.* 10, 525. doi:10.3389/fpls.2019.00525

Kawall, K. (2021a). Genome-edited *Camelina sativa* with a unique fatty acid content and its potential impact on ecosystems. *Environ. Sci. Eur.* 33, 38. doi:10.1186/s12302-021-00482-2

Kawall, K. (2021b). The generic risks and the potential of SDN-1 applications in crop plants. *Plants* 10, 2259. doi:10.3390/plants10112259

Kawall, K., Cotter, J., and Then, C. (2020). Broadening the GMO risk assessment in the EU for genome editing technologies in agriculture. *Environ. Sci. Eur.* 32, 106. doi:10.1186/s12302-020-00361-2

Kocsisova, Z., and Coneva, V. (2023). Strategies for delivery of CRISPR/Cas-mediated genome editing to obtain edited plants directly without transgene integration. *Front. Genome Ed.* 5, 1209586. doi:10.3389/fgeed.2023.1209586

Koller, F., Schulz, M., Juhas, M., Bauer-Panskus, A., and Then, C. (2023). The need for assessment of risks arising from interactions between NGT organisms from an EU perspective. *Environ. Sci. Eur.* 35, 27. doi:10.1186/s12302-023-00734-3

Kosicki, M., Tomberg, K., and Bradley, A. (2018). Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nat. Biotechnol.* 36, 765–771. doi:10.1038/nbt.4192

Lalonde, S., Stone, O. A., Lessard, S., Lavertu, A., Desjardins, J., Beaudoin, M., et al. (2017). Frameshift indels introduced by genome editing can lead to in-frame exon skipping. *PLOS ONE* 12, e0178700. doi:10.1371/journal.pone.0178700

Latham, J. R., Wilson, A. K., and Steinbrecher, R. A. (2006). The mutational consequences of plant transformation. *J. Biomed. Biotechnol.* 2006, 1–7. doi:10.1155/JBB/2006/25376

Lee, K., Eggenberger, A. L., Banakar, R., McCaw, M. E., Zhu, H., Main, M., et al. (2019). CRISPR/Cas9-mediated targeted T-DNA integration in rice. *Plant Mol. Biol.* 99, 317–328. doi:10.1007/s11103-018-00819-1

Leibowitz, M. L., Papathanasiou, S., Doerfler, P. A., Blaine, L. J., Sun, L., Yao, Y., et al. (2021). Chromothripsis as an on-target consequence of CRISPR-Cas9 genome editing. *Nat. Genet.* 53, 895–905. doi:10.1038/s41588-021-00838-7

Li, Z., Liu, Z.-B., Xing, A., Moon, B. P., Koellhoffer, J. P., Huang, L., et al. (2015). Cas9-Guide RNA directed genome editing in soybean. *Plant Physiol.* 169, 960–970. doi:10.1104/pp.15.00783

Lin, T., Zhu, G., Zhang, J., Xu, X., Yu, Q., Zheng, Z., et al. (2014). Genomic analyses provide insights into the history of tomato breeding. *Nat. Genet.* 46, 1220–1226. doi:10.1038/ng.3117

Liu, J., Nannas, N. J., Fu, F.-F., Shi, J., Aspinwall, B., Parrott, W. A., et al. (2019). Genome-scale sequence disruption following biolistic transformation in rice and maize. *Plant Cell* 31, 368–383. doi:10.1105/tpc.18.00613

Liu, M., Zhang, W., Xin, C., Yin, J., Shang, Y., Ai, C., et al. (2021). Global detection of DNA repair outcomes induced by CRISPR-Cas9. *Nucleic Acids Res.* 49, 8732–8742. doi:10.1093/nar/gkab686

Makarevitch, I., Svitashev, S. K., and Somers, D. A. (2003). Complete sequence analysis of transgene loci from plants transformed via microprojectile bombardment. *Plant Mol. Biol.* 52, 421–432. doi:10.1023/A:1023968920830

Manghwar, H., Li, B., Ding, X., Hussain, A., Lindsey, K., Zhang, X., et al. (2020). CRISPR/Cas systems in genome editing: methodologies and tools for sgRNA design, off-target evaluation, and strategies to mitigate off-target effects. *Adv. Sci. (Weinh)* 7, 1902312. doi:10.1002/advs.201902312

Michno, J.-M., Virdi, K., Stec, A. O., Liu, J., Wang, X., Xiong, Y., et al. (2020). Integration, abundance, and transmission of mutations and transgenes in a series of CRISPR/Cas9 soybean lines. *BMC Biotechnol.* 20, 10. doi:10.1186/s12896-020-00604-3

Modrzejewski, D., Hartung, F., Lehnert, H., Sprink, T., Kohl, C., Keilwagen, J., et al. (2020). Which factors affect the occurrence of off-target effects caused by the use of CRISPR/cas: a systematic review in plants. *Front. Plant Sci.* 11, 574959. doi:10.3389/fpls.2020.574959

Modrzejewski, D., Hartung, F., Sprink, T., Krause, D., Kohl, C., and Wilhelm, R. (2019). What is the available evidence for the range of applications of genome-editing as a new tool for plant trait modification and the potential occurrence of associated off-target effects: a systematic map. *Environ. Evid.* 8, 27. doi:10.1186/s13750-019-0171-5

Molla, K. A., and Yang, Y. (2020). Predicting CRISPR/Cas9-Induced mutations for precise genome editing. *Trends Biotechnol.* 38, 136–141. doi:10.1016/j.tibtech.2019.08.002

Monroe, J. G., Srikant, T., Carbonell-Bejerano, P., Becker, C., Lensink, M., Exposito-Alonso, M., et al. (2022). Mutation bias reflects natural selection in *Arabidopsis thaliana*. *Nature* 602, 101–105. doi:10.1038/s41586-021-04269-6

Morineau, C., Bellec, Y., Tellier, F., Gissot, L., Kelemen, Z., Nogué, F., et al. (2017). Selective gene dosage by CRISPR-Cas9 genome editing in hexaploid *Camelina sativa*. *Plant Biotechnol. J.* 15, 729–739. doi:10.1111/pbi.12671

Mou, H., Smith, J. L., Peng, L., Yin, H., Moore, J., Zhang, X.-O., et al. (2017). CRISPR/Cas9-mediated genome editing induces exon skipping by alternative splicing or exon deletion. *Genome Biol.* 18, 108. doi:10.1186/s13059-017-1237-8

Nonaka, S., Arai, C., Takayama, M., Matsukura, C., and Ezura, H. (2017). Efficient increase of ɣ-aminobutyric acid (GABA) content in tomato fruits by targeted mutagenesis. *Sci. Rep.* 7, 7057. doi:10.1038/s41598-017-06400-y

Norris, A. L., Lee, S. S., Greenlees, K. J., Tadesse, D. A., Miller, M. F., and Lombardi, H. A. (2020). Template plasmid integration in germline genome-edited cattle. *Nat. Biotechnol.* 38, 163–164. doi:10.1038/s41587-019-0394-6

Ono, R., Yasuhiko, Y., Aisaki, K.-I., Kitajima, S., Kanno, J., and Hirabayashi, Y. (2019). Exosome-mediated horizontal gene transfer occurs in double-strand break repair during genome editing. *Commun. Biol.* 2, 57. doi:10.1038/s42003-019-0300-2

Parisi, C., and Rodriguez, C. E. (2021). *Current and future market applications of new genomic techniques*. Luxembourg: Publications Office of the European Union. doi:10.2760/02472

Park, S. H., Cao, M., and Bao, G. (2023). Detection and quantification of unintended large on-target gene modifications due to CRISPR/Cas9 editing. *Curr. Opin. Biomed. Eng.* 28, 100478. doi:10.1016/j.cobme.2023.100478

Raffan, S., Sparks, C., Huttly, A., Hyde, L., Martignago, D., Mead, A., et al. (2021). Wheat with greatly reduced accumulation of free asparagine in the grain, produced by CRISPR/Cas9 editing of asparagine synthetase gene *TaASN2*. *Plant Biotechnol. J.* 19, 1602–1613. doi:10.1111/pbi.13573

Raitskin, O., and Patron, N. J. (2016). Multi-gene engineering in plants with RNA-guided Cas9 nuclease. *Curr. Opin. Biotechnol.* 37, 69–75. doi:10.1016/j.copbio.2015.11.008

Ramírez-Sánchez, O., Pérez-Rodríguez, P., Delaye, L., and Tiessen, A. (2016). Plant proteins are smaller because they are encoded by fewer exons than animal proteins. *Genomics Proteomics Bioinforma.* 14, 357–370. doi:10.1016/j.gpb.2016.06.003

Rang, A., Linke, B., and Jansen, B. (2005). Detection of RNA variants transcribed from the transgene in Roundup Ready soybean. *Eur. Food Res. Technol.* 220, 438–443. doi:10.1007/s00217-004-1064-5

Rao, X., Zhao, H., Shao, C., and Yi, C. (2023). Characterizing off-target effects of genome editors. *Curr. Opin. Biomed. Eng.* 28, 100480. doi:10.1016/j.cobme.2023.100480

Rodríguez-Leal, D., Lemmon, Z. H., Man, J., Bartlett, M. E., and Lippman, Z. B. (2017). Engineering quantitative trait variation for crop improvement by genome editing. *Cell* 171, 470–480.e8. doi:10.1016/j.cell.2017.08.030

Roldan, M. V. G., Périlleux, C., Morin, H., Huerga-Fernandez, S., Latrasse, D., Benhamed, M., et al. (2017). Natural and induced loss of function mutations in SlMBP21 MADS-box gene led to jointless-2 phenotype in tomato. *Sci. Rep.* 7, 4402. doi:10.1038/s41598-017-04556-1

Samach, A., Mafessoni, F., Gross, O., Melamed-Bessudo, C., Filler-Hayut, S., Dahan-Meir, T., et al. (2023a). CRISPR/Cas9-induced DNA breaks trigger crossover, chromosomal loss, and chromothripsis-like rearrangements. *Plant Cell*, koad209. doi:10.1093/plcell/koad209

Sánchez-León, S., Gil-Humanes, J., Ozuna, C. V., Giménez, M. J., Sousa, C., Voytas, D. F., et al. (2018). Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. *Plant Biotechnol. J.* 16, 902–910. doi:10.1111/pbi.12837

Sander, J. D., and Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat. Biotechnol.* 32, 347–355. doi:10.1038/nbt.2842

Sharpe, J. J., and Cooper, T. A. (2017). Unexpected consequences: exon skipping caused by CRISPR-generated mutations. *Genome Biol.* 18, 109. doi:10.1186/s13059-017-1240-0

Simeonov, D. R., Brandt, A. J., Chan, A. Y., Cortez, J. T., Li, Z., Woo, J. M., et al. (2019). A large CRISPR-induced bystander mutation causes immune dysregulation. *Commun. Biol.* 2, 70. doi:10.1038/s42003-019-0321-x

Skryabin, B. V., Kummerfeld, D.-M., Gubar, L., Seeger, B., Kaiser, H., Stegemann, A., et al. (2020). Pervasive head-to-tail insertions of DNA templates mask desired CRISPR-Cas9-mediated genome editing events. *Sci. Adv.* 6, eaax2941. doi:10.1126/sciadv.aax2941

Solomon, S. M. (2020). Genome editing in animals: why FDA regulation matters. *Nat Biotechnol.* 38, 142–143. doi:10.1038/s41587-020-0413-7

Soyk, S., Lemmon, Z. H., Sedlazeck, F. J., Jiménez-Gómez, J. M., Alonge, M., Hutton, S. F., et al. (2019). Duplication of a domestication locus neutralized a cryptic variant that caused a breeding barrier in tomato. *Nat. Plants* 5, 471–479. doi:10.1038/s41477-019-0422-z

Tuladhar, R., Yeu, Y., Tyler Piazza, J., Tan, Z., Rene Clemenceau, J., Wu, X., et al. (2019). CRISPR-Cas9-based mutagenesis frequently provokes on-target mRNA misregulation. *Nat. Commun.* 10, 4056. doi:10.1038/s41467-019-12028-5

Wang, H., La Russa, M., and Qi, L. S. (2016). CRISPR/Cas9 in genome editing and beyond. *Annu. Rev. Biochem.* 85, 227–264. doi:10.1146/annurev-biochem-060815-014607

Wendel, J. F., Jackson, S. A., Meyers, B. C., and Wing, R. A. (2016). Evolution of plant genome architecture. *Genome Biol.* 17, 37. doi:10.1186/s13059-016-0908-1

Windels, P., De Buck, S., Van Bockstaele, E., De Loose, M., and Depicker, A. (2003). T-DNA integration in Arabidopsis chromosomes. presence and origin of filler DNA sequences. *Plant Physiol.* 133, 2061–2068. doi:10.1104/pp.103.027532

Wolt, J. D., Wang, K., Sashital, D., and Lawrence-Dill, C. J. (2016). Achieving plant CRISPR targeting that limits off-target effects. *Plant Genome* 9. doi:10.3835/plantgenome2016.05.0047

Yang, Q., Tae-Sung, P., Bumkyu, L., and Myung-Ho, L. (2022). Unusual removal of T-DNA in T1 progenies of rice after agrobacterium-mediated CRISPR/Cas9 editin. doi:10.21203/rs.3.rs-1066224/v1

Yasumoto, S., and Muranaka, T. (2023). Foreign DNA detection in genome-edited potatoes by high-throughput sequencing. *Sci. Rep.* 13, 12246. doi:10.1038/s41598-023-38897-x

Yue, J., VanBuren, R., Liu, J., Fang, J., Zhang, X., Liao, Z., et al. (2022). SunUp and Sunset genomes revealed impact of particle bombardment mediated transformation and domestication history in papaya. *Nat. Genet.* 54, 715–724. doi:10.1038/s41588-022-01068-1

Zetsche, B., Heidenreich, M., Mohanraju, P., Fedorova, I., Kneppers, J., DeGennaro, E. M., et al. (2017). Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. *Nat. Biotechnol.* 35, 31–34. doi:10.1038/nbt.3737

Zhang, H., Zhang, J., Wei, P., Zhang, B., Gou, F., Feng, Z., et al. (2014). The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnol. J.* 12, 797–807. doi:10.1111/pbi.12200

Zhang, Q., Xing, H.-L., Wang, Z.-P., Zhang, H.-Y., Yang, F., Wang, X.-C., et al. (2018). Potential high-frequency off-target mutagenesis induced by CRISPR/Cas9 in Arabidopsis and its prevention. *Plant Mol. Biol.* 96, 445–456. doi:10.1007/s11103-018-0709-x

Zsögön, A., Čermák, T., Naves, E. R., Notini, M. M., Edel, K. H., Weinl, S., et al. (2018). *De novo* domestication of wild tomato using genome editing. *Nat. Biotechnol.* 36, 1211–1216. doi:10.1038/nbt.4272

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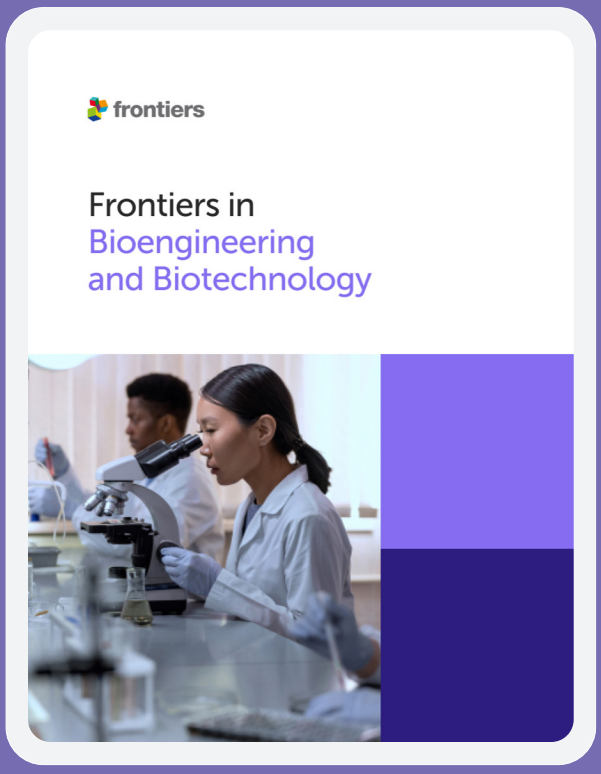

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