THE ENVIRONMENT-ANIMAL-HUMAN WEB: A "ONE HEALTH" VIEW OF TOXICOLOGICAL RISK ANALYSIS

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THE ENVIRONMENT-ANIMAL-HUMAN WEB: A "ONE HEALTH" VIEW OF TOXICOLOGICAL RISK ANALYSIS

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One Health (OH) is the conceptual and operational framework that links environment, food-producing organisms and human health. OH is a developing field, that deals with the multifaceted web of feed-backs and interactions among its components. In order to avoid "drowning into complexity", priority issues should be identified, either for research and for risk analysis. To date OH approaches have frequently pivoted on infectious agents shared among animals and humans and the related problems, such as antibiotic resistance. Nevertheless, the OH scenarios include, and should increasingly include, environment-and-health problems. Food and environment do interact. Environment influences the living organisms that produce human food and, in the meanwhile, food production outputs influence the environmental quality;

as for foods of animal origin, feed materials and practices are driving components of the environment-food interactions. In this book, we aimed at highlighting the importance of environment, chemical exposures and toxicological issues in the field of OH, as well as the need for multidisciplinary integration in order to support OH approaches into diseases prevention and health promotion.

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Editorial: The Environment-Animal-Human Web: A "One Health" View of Toxicological Risk Analysis

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Editorial on the Research Topic

The Environment-Animal-Human Web: A "One Health" View of Toxicological Risk Analysis

One Health (OH) is the conceptual and operational framework that links environment, ecosystems, and human health. Therefore, OH is a developing field, that deals with the multifaceted web of feed-backs and interactions among its components. In order to avoid "drowning into complexity," priority issues should be identified, either for research and for risk analysis. In this book, we aimed at highlighting the importance of environment, chemical exposures and toxicological issues in the field of OH. Indeed OH has been frequently presented as an updated and more comprehensive approach to the infectious agents shared among animals and humans and the related problems, such as antibiotic resistance. Sure, topics like zoonoses feature prominently in the OH scenario which, nevertheless, includes much more environment-and-health problems. Food and environment do interact: environment influences the living organisms that produce human food and, in the meanwhile, food production outputs influence the environmental quality. As for foods of animal origin, feed materials and practices are driving components of the environment-food interactions.

The chapters address the broad spectrum of environmental and toxicological topics linked to OH, from pollution through to feeding stuffs, live animals, safe and sustainable food productions, and human risk assessment.

The dairy farm is a critical topic in the OH web: animal welfare and milk quality can reflect multiple influences, from pesticides used in crops to water quality and farm management. In the meanwhile, milk and dairy products are a major food for a large part of mankind, especially children. Dairy production is also a field for innovation in animal science. Thus, several papers deal with OH approaches in dairy farming. Three papers ("Framework to define structure and boundaries of complex health intervention systems: the ALERT project as example" by Boriani et al.; "From invention to innovation: risk analysis to integrate One Health technology in the dairy farm" by Lombardo et al.; "Understanding seasonal changes to improve good practices in livestock management" by Martelli et al.) directly stem from the results of a national project carried in Italy in order to introduce an innovative, multi-parametric technology for the on-line monitoring of milk quality and safety. Risk analysis within OH brings about the uptake and evaluation of innovations into the agro-farming systems, as well as the need to interact with the requests of stakeholders ("Portable bio/chemosensoristic devices: innovative systems for environmental health and food safety diagnostics" by Dragone et al.).

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Mycotoxins are a selected topic for OH: plant infections by fungi, modulated by agricultural practices, food production chains as well as climate changes, do produce toxins that pose risks to animal and human health. In at least one case, the Aflatoxin M1, there is an important carry-over from fungi-contaminated crops and feeds through to milk for human consumption. Mycotoxins, thus pose a significant challenge to the interdisciplinary framework of OH: approaches to risk analysis depend also from the agricultural and social scenarios ("Engaging One Health for non-communicable diseases in Africa: perspective for mycotoxins" by Ladeira et al.; "The hotspot for (global) One Health in primary food production: Aflatoxin M1 in dairy products" by Frazzoli et al.). One OH challenge is the fight against agents damaging food-producing organisms and environment through the selection of approaches that minimize the concurrent adverse side-effects. Parasytic and ectoparasytic infections, either zoonotic and non-zoonotic, are intertwined with environmental conditions and often require pharmacological treatments, especially in developing Countries. However, antiparasytic drugs can be very toxic. Efficient toxicological screens are required ("A novel strategy to predict carcinogenicity of antiparasitics based on a combination of DNA lesions and bacterial mutagenicity tests" by Liu et al.), as well as protocols that minimize the risks for farmers and the environment ("Experiences in tick control by acaricide in the traditional cattle sector in Zambia and Burkina Faso: possible environmental and public health implications" by De Meneghi et al.).

As for pesticides, they are an unavoidable support for food security. In the meanwhile, all pesticides are potentially toxic; science-based risk assessment evaluates the impact on the health of human beings (farmers and consumers), food-producing animals and on safety of life-supporting environmental goods such as drinking water. Modeling for risk assessment should consider the specific features of the agro-farming systems and allowing the identification of lower-risk options for crop protection ("Pesticides in drinking water—the Brazilian monitoring program" by Barbosa et al.; "Risk factors for non-communicable diseases in Vietnam: a focus on pesticides" by Dang et al.).

Indeed, the OH framework calls for overcoming the boundaries between environmental, biomedical and social sciences; food safety from farm to fork requires to know the "farm" (the agro-farming system) as well as the "fork" (how foods are prepared and consumed). The countries at turning point toward a more industrialized society, like several African countries, can offer scenarios of high interest for the application of OH framework ("Contaminants in Foods of Animal Origin in Cameroon: A One Health Vision for Risk Management" from Farm to Fork by Pouokam et al.). The appraisal of the scenarios is important to protect traditional agrofarming systems and the safety of their products: the development of traditional systems, such as family farming, can be, in fact, the efficient way to extract products valuable for food and nutrition security from low-quality resources. Communication and knowledge sharing with participating communities is essential ("Transdisciplinary project communication and knowledge sharing experiences in Tanzania and Zambia through a One Health lens" by Bagnol et al.; "Safe and sustainable traditional production: the water buffalo in Asia" by Deb et al.).

Finally, this book contains thirteen papers from scientists working in institutions from eighteen Countries in Africa (Burkina Faso, Cameroon, Niger, Nigeria, South Africa, Tanzania, Zambia), Asia (Bangladesh, China, India, Philippines, Vietnam), Europe (Denmark, Italy, Portugal, Switzerland), Latin America (Brazil), and Oceania (Australia). Thus, we can confidently claim that this book, with its multiple scientific voices, provides also a contribution to global health challenges.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Framework to Define Structure and Boundaries of Complex Health Intervention Systems: The ALERT Project

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Health intervention systems are complex and subject to multiple variables in different phases of implementation. This constitutes a concrete challenge for the application of translational science in real life. Complex systems as health-oriented interventions call for interdisciplinary approaches with carefully defined system boundaries. Exploring individual components of such systems from different viewpoints gives a wide overview and helps to understand the elements and the relationships that drive actions and consequences within the system. In this study, we present an application and assessment of a framework with focus on systems and system boundaries of interdisciplinary projects. As an example on how to apply our framework, we analyzed ALERT [an integrated sensors and biosensors' system (BEST) aimed at monitoring the quality, health, and traceability of the chain of the bovine milk], a multidisciplinary and interdisciplinary project based on the application of measurable biomarkers at strategic points of the milk chain for improved food security (including safety), human, and ecosystem health (1). In fact, the European food safety framework calls for science-based support to the primary producers' mandate for legal, scientific, and ethical responsibility in food supply. Because of its multidisciplinary and interdisciplinary approach involving human, animal, and ecosystem health, ALERT can be considered as a One Health project. Within the ALERT context, we identified the need to take into account the main actors, interactions, and relationships of stakeholders to depict a simplified skeleton of the system. The framework can provide elements to highlight how and where to improve the project development when project evaluations are required.

Keywords: food safety, food security, primary production, food chain, dairy chain, interdisciplinary, transdisciplinary, One Health

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INTRODUCTION

Recent financial, economic, social, environmental, and health crises have led to the renewed recognition that collaborative approaches between disciplines are urgently needed to tackle such global challenges (2, 3). Consequently, the approach to emerging pandemics, as well as climate change, drug resistance, food and water security, and safety, has shifted from an interdisciplinary approach of

experts, whereby experts collaborate across disciplinary boundaries, to a transdisciplinary approach that integrates society and science by including all potentially affected or otherwise relevant stakeholders (4–6). This transcends traditional boundaries and integrates knowledge and perspectives from scientific and non-scientific sources (2, 7). Many health communities have proposed transdisciplinary and systemic approaches with different focus points, such as EcoHealth, Global Health, Planetary Health, or Health in scaled Social–Ecological Systems (5, 6). The end goal is to have an additional instrument to improve the effectiveness of health intervention/care projects, thereby ensuring safety for humans, animals, and the environment alike (8).

The One Health approach, and ALERT as example, employed in a health intervention project that will be used in our manuscript, aims at simultaneously considering human and ecosystem health (9). Integration of multiple disciplines, sectors, stakeholders, living and inanimate elements yields highly complex constructs with varying dynamics at different scales. Although there is considerable literature describing the integrated approaches to health (10, 11), to the best of our knowledge, there are no recognized guidelines on how to evaluate to what extent the underlying integration as a principle contributes to address especially complex health problems, such as antibiotic resistance, outbreaks of highly infectious or non-communicable diseases, or ecotoxicology (12). There is a clear need for methods to represent and analyze such initiatives for management and evaluation purposes using a systemic and integrated approach (13).

The aim of the Network for Evaluation of One Health,[1] a Transdomain action of the European Cooperation for Science and Technology, is to enable appropriate evaluations of One Health activities and hence comparison of initiatives as well as informed decision-making and resource allocation. Its conceptual framework includes the definition of the (One Health) system in which the initiative is implemented and the definition of the scale and boundaries of the system under evaluation. Human relationships, cultural behaviors, languages expressions, governance organizations, and constructive collaboration within interdisciplinary groups are all elements to be potentially included in a system. A possible way to visualize interactions and connections in and among different systems can be the system network approach.

We propose to employ a complex systems' perspective to overcome the shortcomings of the traditional reductionist approaches (14–16). We consider "system thinking" as the process described by Whitehead et al. (17). These authors describe the "baseline lexicon of systems thinking" as being: descriptive scenarios, system boundaries, system stakeholders, scope of the analysis, type of system (state of system and life cycle of system), metrics, axiological components, observer effects, normative scenarios, indices of performances, and development alternatives (outscoping, evaluating and ranking alternatives, interactions, iterating analysis, and leverage points). Occasionally, more appropriate elements can be added or other lexical components can be used.

In health, systemic techniques have been applied to work on problems such as obesity and epidemic diseases. WHO recommends some main techniques to identify points of interventions (leverage points) in complex health-related systems (3). The guideline differentiates building blocks of a health system such as health services, health workforce, medical technologies, financing, governance, and the main system goals like improved health, responsiveness, and improved efficiency.

The framework advocates a systemic overview of those building blocks to visualize synergies and the dynamic architecture. The main goal of the approach is to consider the effect of an intervention across as many major subsystems of the health system as possible. This process is initiated with a "stakeholders analysis," where the interconnections and perspectives of each stakeholder are inventoried. This is overlaid with an open and transparent network of interventions and their possible consequences.

In a more generic manner, systems' thinking has been applied in project evaluation to inform policy makers and executives for best resource allocation (18). The systemic approach is intended to design programs and policies that are aware of and prepared for possible unintended consequences and that integrate multiple stakeholder perspectives. The resulting framework or model should describe and predict the various ways in which a system might react to change.

For the evaluation of One Health interventions as an example, we have adapted the system thinking techniques to visualize the main elements, stakeholders, constrains, and internal dynamics. Furthermore, defining the system boundaries allows exploring the needs and gaps of a system. Once these elements are established, the system can be studied in a prospective and retrospective way, to optimize it or maximize benefits from interventions on it.

In this study, we propose a framework to describe and delimit One Health initiatives using ALERT as an example project as a first step toward evaluating them as complex adaptive systems.

Our framework will be a further instrument to be used alone or combined and used in synergies with other already existing frameworks. The right choice of a specific framework or a combination of different frameworks for analyzing different types of complex systems will have to be addressed in a separate effort and it will require specific expertise (in particular, socioeconomic background) that will collaborate with us in the future work.

MATERIALS AND METHODS

Preliminary Considerations about Systems and Their Boundaries

Whitehead et al. (17) and Gibson et al. (19) define a system as "a set of elements so interconnected as to aid in driving toward a defined goal." A system might include subsystems or a collection of systems. In other words, abstractions about systems and their constituent components can go to very high and very low levels of detail [intending level as a position in a scale or rank (20)] depending on one's perspective and the purpose of the abstractions. The lack of specificity in defining what is a system vs. a subsystem or system component or element is one reason why all relevant stakeholders should be involved in defining the structure of a system. In public health, "systems are dynamic architectures

[1] http://neoh.onehealthglobal.net/.

of interactions and synergies," where the elements of the system are also coming from social science (3). When working with such complex systems, the meaning of multiple perspectives, interactions, and boundaries must be understood, because each element can be potentially essential for identifying successful interventions. While the perspectives are determined by the stakeholders, these are at the same time players in the system and may become agents of change (3, 21).

Any observation, intervention or evaluation, faces the dilemma between focus and comprehensiveness, and to become operational, the system of interest must have an operational space that defines its limitations (21). Nevertheless, the environment of a system is important and should be well described to generate awareness of the wider context and to avoid missing potential external interactions. Real systems are dynamic and even geophysical boundaries change over time (22). We consider the dynamic of the system adopting an iterative process. If there are modifications in the inner scenario, we consider the modified scenario as new inputs and iteratively integrated it to the new analysis, as described by Gibson et al. (19).

Elements of the System Definition Framework

In the next section, we define the elements contributing directly or indirectly to the system and system boundaries, i.e., the network of connected interactions that temporally close it, represent limits, and contribute to the overall structure of the system. We have then combined these elements to create the framework for defining and analyzing the structure and boundaries of a system and applied this framework on a health intervention project in the Section "Results."

The Aim of a System

The aim should provide an answer to the question "Why are we looking at this system? Which are the problems, questions to solve?" The aim should help to investigate the way a system is used to solve a problem.

In the framework, we differentiate among the declared aim by the system and the observed, enacted, as well as the perceived aims. Each stakeholder may have a different perception of the declared aim and again each of them can have a different way to interpret how the system is performing in relation to its aim (23).

The System Space and Time, and Scale of Analysis

Space

This element identifies how the system extends geospatially, what is the geophysical environment, how large it is, and which ethnopolitical entities are involved (region, state, and nation). It also defines the scale of analysis that is of primary interest, individuals, households, groups or populations, etc., and finally how the different stakeholders are influenced by the spatial conditions.

Time

This element defines at which primary time scale is the system being observed, such as seconds, days, weeks, months, years, etc., and how the stakeholders are influenced by this time scale.

Interactions with Space and Time

This element defines the involvement of iterations and pathways along space and time dimensions.

Stakeholders and Actors

Stakeholders are entities affecting or affected by the system; they can be entities of different size between individuals, families, institutions, government agencies, etc. (24). More specifically, we can define primary actors as stakeholders who *act* on the system and secondary actors that can still partially interact with and modify the system. Examples of stakeholders of a health intervention system are farmers at the beginning of the milk chain, veterinarians checking the animals, dairies, food industry, toxicologists, chemists, veterinarians, biologists, and agronomists part of a research institute or a control institution.

In the framework, the information how the actors and stakeholders influence or are influenced by the system is specifically required.

The Systems Restrictions/Conditions—Boundaries

Which are the restrictions, conditions, and boundaries associated with a system? For example, a limited production due to regulations (e.g., the old milk quotas system in Europe), a closed market for a food due to regulations (e.g., raw milk consumption), or a cultural behavior that will limit a certain system to a group of individuals or animals (e.g., bovine milk consumption in certain Asian communities), relations of control among stakeholders and actors and relevant legal requirements, or constraints imposed by daily food production and market (e.g., quality systems must comply with production time), financial capability of primary food producers (e.g., calling for public incentives) or by sustainability aspects [e.g., the impact of the global milk production on the environment at the global level, i.e., in relation to the planetary boundaries (22)]. Such elements need to be potentially considered when defining the boundaries of a system in line with the system aim and goals. As defined by Senge (25), the description of system boundaries "considers what is improved, affected, or replaced by the system and, conversely, what affects the system under study, as the system changes and is changed by the environment." Being able to well define the system space and its limitations helps in the definition of the system boundaries.

In the framework, constrains and boundaries are also related to the aim of the system. Understand how they interact with the system aims is important information to understand possible leverage points.

The Consequences (of Different Degree)

Consequences are the results of interactions in the system. They follow the path of interactions and stop at the system boundaries. Once the system and the system boundaries are defined, the consequences for that system are determined subsequently inside the system space. The "boundaries," "externalities," and "constraints" to the system should also be considered as surrounding and limiting the consequences to a certain degree (26). As an example, foods and food products are impacting directly human or animal

health and indirectly (e.g., through the food chain or animal or human waste) ecosystem health.

In the framework, the consequences are related to the boundaries/constrains of the system, as described above.

The System Evolution

Following the work of Forrester (27): "there is not a single method, but an approach that uses a set of tools, to understand the behavior of complex systems over time designed to solve the problem of simultaneity (mutual causation)." Every real system is dynamically evolving, and this is why it is important to periodically reevaluate the system (interacting actors and stakeholders, restrictions, and consequences) and redefine it and its boundaries in an iterative way. The various processes of definition of the system and the problems that should be solved, the definition of a way to act/interact with a specific population/culture/disease/habit should be redefined periodically, because the system itself is constantly under change.

The ALERT Project

The ALERT project[2] has been used as an example on how to apply the proposed framework. ALERT is funded by the Italian Ministry for Economic Development and is based on the transfer of technical innovation and technological know-how, which emerged from public research in the field, to the actors in daily food production (primary food producers and food industry). ALERT is coordinated by the Italian National Institute of Health (ISS) and develops a new risk management framework based on recent technological advances to manage the bovine milk chain for improved product safety and quality. It exploits new biomarkers for (i) early detection of production anomalies, (ii) monitoring (and assessment) of effects of corrective actions undertaken as risk management measure, and (iii) for assessment of production improvements (e.g., feed changes).

ALERT involves the human health affected by the environment, animal health and ecosystem health, sectors, as well as the producing farmer community and their web of interactions, and other actors of the food chain up to the food industry and the consumers. It is transdisciplinary and multisectoral to provide space for innovation and harvest the benefits of such integrated approaches and can thus be seen as a One Health project. It further focuses the responsibility of primary producers in the European food safety framework (28). Milk is a food particularly interesting for One Health: it is an animal product, highly susceptible to toxic contaminants (29, 30), highly consumed by vulnerable consumers, and suited as sentinel matrix for environmental monitoring purposes (30). The description of the project system may also be of interest to primary productions in economically developing countries, where environmental conditions and restricted resources amplify both risks of contaminations and challenges for their prevention (29).

In **Table 1,** the ALERT response to the primary producers' mandate for legal, scientific, and ethical responsibility in the EU food safety framework is described.

[2]www.alert2015.it.

ALERT Institutional Framework

ALERT requires the establishment of an institutional setting matching different silos like public vs. private bodies; public health vs. fundamental research; food industry vs. high-tech industry; risk analysis vs. marketable technologies; scientists vs. food producers; and scientists vs. citizens/consumers.

ALERT relies on the integration between three clusters or pool of actors:

(a) A "Risk Management Cluster" including:
- Istituto Superiore di Sanità (ISS), i.e., the Italian National Institute of Health, is the leading technical-scientific body of the Italian National Health Service, with top-level expertise in risk analysis (from risk assessment to formulation of scientific options for risk management) of food chains (Department of Food Safety, Nutrition and Veterinary Public Health), public health (Department of Environment and Health), prevention of non-communicable diseases and relevant technologies development (Department of Cardiovascular, Dysmetabolic and Aging-Associated Diseases). *Disciplines/expertise* deployed: anthropologists, biologists, chemists, engineers, statisticians, toxicologists, and veterinarians.
- Istituto Zooprofilattico Sperimentale of regions Lazio and Toscana, i.e., public institute with top-level expertise and governmental commitment in the protection of food chain wholesomeness and animal health and welfare in the network of IZS regional institutes. *Disciplines/expertise* deployed: agronomists, biologists, chemists, and zootechnicians.
- Lattepiù, i.e., leading enterprises in milk production, including the Pascolini Elio dairy farm, and milk transport and storage system. *Disciplines/expertise* deployed: farmers and livestock staff.
- Centrale del Latte di Roma (CLR), i.e., leading regional milk industry. *Disciplines/expertise* deployed: biologists and chemists.

Lattepiù and CLR are also the final end users of the ALERT products.

(b) A "Technology Cluster" including:
- Consiglio Nazionale delle Ricerche (CNR, Institute on nano-structured materials), i.e., public institute with top-level expertise/commitment in technological innovation, research and technology transfer. *Disciplines/expertise* deployed: biologists, chemists, and biotechnologists.
- Amel, Biosensor, Nutriservice, i.e., enterprises with complementary expertise in the setting, optimization, miniaturization, and robotization of (bio) probes systems as well as the development of management software and electronic systems. *Disciplines/expertise* deployed: biotechnologists, electronic engineers, and software developers.

(c) A "Marketing Cluster" involving different expertise in marketing strategies, strategic partnering at industrial level, as well as dissemination of pre-industrial research deliverables of the Leonardo Business Consulting. *Disciplines/expertise* deployed: economists, marketing managers, and business developers.

TABLE 1 | ALERT project response to develop and translate invention (BEST) into practical innovation for One Health-related needs.

Primary producers' mandate for legal, scientific, and ethical responsibility in the European food safety frame	**ALERT: from invention (BEST) to innovation**
One Health-related needs	**ALERT response**
Filling knowledge gaps Besides the field of physiological, behavioral, and production and reproduction indicators for improving rearing management, strategies and performance of farmed animals (food security), food safety aspects need increasing attention by all food chain stakeholders. New zoonotic threats from foods of animal origin (i.e., from animals as food-producing living organisms) are a scientific topic with many knowledge gaps. Moreover, significant health-relevant know-how in different fields is scarcely integrated due to the different sectoral silos	The Hazard Analysis and Critical Control Points system is the on-enterprise strategy to control and manage the safety of food production process. ALERT develops new knowledge including biomarkers of unmanaged indicators of undesirable substances (chemical and microbial pollutants) and milk quality (milk composition, subclinical mastitis, and metabolomic markers) and updated risk analysis (risk assessment and management) in the supply chain, in different scenarios (e.g., economically developed and developing areas, clean and contaminated sites) through its *multidisciplinary team* (**Figure 1**) integrating *different silos* (**Figure 2**)
Optimization of resources Besides periodic (e.g., annual) controls, to date, self-monitoring plans of dairy enterprises consider only limited systematic activities. Significant resources invested in official control require increased cost-effectiveness through science-based criteria	ALERT designs new strategy to implement the enterprise early risk management system including toxicological risks and based on early warning. ALERT develops control charting of (grids of) *early biomarkers* based on (and feeding) *risk analysis* in food production
Acceptability Based on the end users' perspective, the proactive role of primary food producers in building food safety benefits of tools already in use at farm level (such as control charting) and of standard values taken from the history of the enterprise	ALERT proposes: (i) a *two-lane (top-down and bottom-up) system* for food safety: field biomarkers in sentinel living animals and sentinel food matrices/animal excretion (milk) are suited to integrate the consolidated European system for Official control (fixing maximum residue levels, unacceptable contaminants, and tolerated contaminants at certain maximum levels) with field monitoring of farms environments to reduce vulnerability to unexpected events (ii) to complement the sophisticated and expensive laboratory instruments and techniques of official control with cost-effective probes working daily during farm operations to monitor *invariability* of significant farm quality and wholesomeness parameters as well as deliberate changes/improvements of production components (e.g., effects of feed on milk nutritional quality) (iii) historical trend in quality and safety parameters is relied upon as internal standard. Indeed, instead of burdening producers with closer external control activities and standards, BEST *monitors anomalous variations in historical enterprise's trend* rather than official thresholds (iv) an early risk management system (based on cost-effective technology and self-monitoring plan) eventually allowing timely corrective action and avoiding both *food losses and food waste*
Science in the farm scenario Farm environmental conditions, daily need of food production, as well as farmers limited capacities and resources require highly innovative technology and risk analyses know-how. Animal physiology as well as the complexity of a "living" matrix like milk increases the scientific challenges of the monitoring purpose. On the other side, farmers' expectations from Precision Livestock Farming already proved how farmers welcome the use of technology	ALERT proposes: (i) on-farm *robust technology* (without transferring samples to external laboratory) (ii) a *self-instructed system* (iii) a "*data in/acoustic-luminous signal out*" technology is easily handled and interpreted by unskilled operators (iv) *holistic/metabolomic* approach to monitor animal excretion fluid (milk) of individual animals
Fair value chain Agro-zootechnic enterprises are the most critical and strategic sites for the prevention of environmental adverse effects on health. Indeed, most of the environment-food web of interactions (both beneficial and noxious) occurs here (e.g., relevant to environmental quality, animal health/welfare, and farm management). To date (i) resources invested in official control prevention plans (including traceability) are scarcely focused on early warning in agro-zootechny; (ii) farmers are the most suffering group of food business operators	ALERT proposes: (i) farmers *empowerment*: indeed, farmers are a key building block of public health (ii) monitoring farm's vulnerability to unexpected events (iii) a stable technological platform (BEST) to *interface* dairy enterprises *with scientific research* (iv) tools to increase citizens' trust in milk primary production (v) social innovation [Start Cup prize for social innovation MILKNET (31)]
From farm to fork The *from farm to fork* approach implemented by the EU strategy builds a chain of responsibility (and value thereof) along the different segments of the food chain. It also promotes a common approach by the food chain actors, including innovative and sharable technologies and risk management strategies	ALERT designs: (i) a new strategy for risk management along the entire food chain, i.e., a *centralized system of BEST devices* along the whole chain, including environment/farm interface (watering, milking, and raw milk harvesting) and milk factory (milk exiting tank-lorry, exiting pasteurization, exiting microfiltration, and at packaging) (ii) food chain traceability along the different production segments The food safety system benefits from food operators empowered in their knowledge of the food production chain

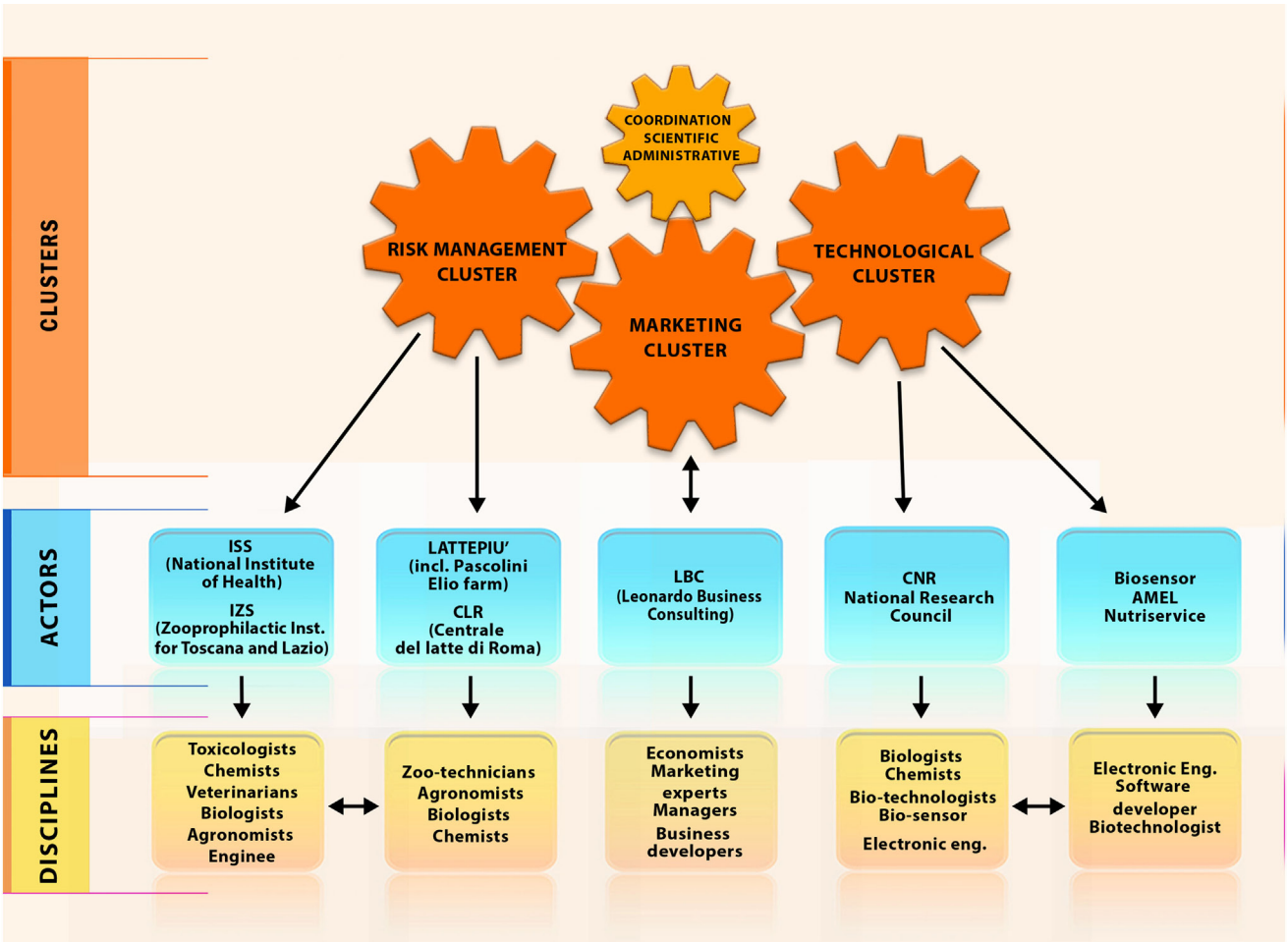

FIGURE 1 | Institutional framework governing the ALERT project.

Indeed, the primary producers' role in the EU food safety frame calls for transdisciplinary work as it is shown in **Figure 2**.

RESULTS

The proposed framework (**Table 2**) provides an overview on elements and relationships of the system in which ALERT operates (**Table 3**). By employing a structured process to define system elements and boundaries, the system representation is developed from the various stakeholders' perspectives and sets the system boundaries. In analyzing the three aspects of contextualization, relationships and evolution, one gains an understanding of static and dynamic properties of the system.

System Identification Framework

A framework of the main definition steps to identify a system and its boundaries is summarized in **Table 2** (below).

The framework is divided into three questions. Main questions are the ones that help to contextualize the system;

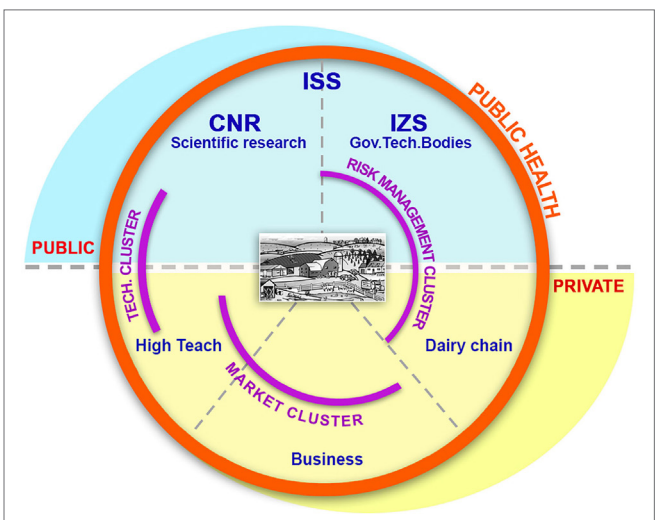

FIGURE 2 | Interdisciplinary team in ALERT.

TABLE 2 | Framework for identifying a system and its boundaries.

	Element	Main question	Secondary question	Tertiary question
	Definition of	**Contextualization**	**Actions/relationships**	**Evolution/dynamics**
1	Aim	Why I am looking at this system? Which are the questions/problems I want to solve by using the system?	What is the declared aim of the system and what is the enacted aim of the system. Is the aim perceived differently by stakeholders?	What are the declared and enacted aims at the onset of the evaluation and do they change as the system evolves?
2	Actors/stakeholders	Which are the main actors/stakeholders? How are they affected by the system and/or how do they affect the systems?	How do actors influence/modify the system to achieve the aim?	Do the actors change their activity and behaviors because of the system evolution (new trade-offs)?
3	System space and time	Which geographical and political space does the system occupy (e.g., geography/area/countries involved)? Which is the most important time scale for observing the system (e.g., months and years), and what is the primary level of analysis for the evaluation of the system (e.g., individuals and family, population)	How are these dimensions connected with the declared aim of the system?	As the system evolves, how do these aspects change?
4	Restrictions/conditions/boundaries	What are the main restrictions/conditions/boundaries of the system? Are there constraints coming from the system's external surroundings?	How do these restrictions/conditions/boundaries interact with the system aims?	Do these restrictions/conditions/boundaries change as the system evolves?
5	Consequences	What are the consequences of the system (outputs/results/products)?	Are these consequences bound by the system boundaries?	Are these consequences change as the system evolves?

secondary questions help to define the relationships among actions and finally the tertiary questions concern the evolution of the system.

These questions are made for each of the element described in the Section "Materials and Methods," namely: (1) aim of the system, (2) system dimension (space and time), (3) actors–stakeholders, (4) restrictions/conditions/boundaries, and (5) consequences.

Application of System and System Boundaries Framework to ALERT

The system and system boundaries framework is applied to the example ALERT to identify its gaps and weaknesses and relevant possible solutions (**Table 3**).

Using the framework, we can evidence some main arguments, highlighted in bold in **Table 3** and summarized in **Box 1**. For clarity, we have associated a letter to each highlighted point in **Table 3** and reported in **Box 1**.

DISCUSSION AND CONCLUSION

Using a structured framework that defines the system, its stakeholders, its boundaries, and its evolution helps in showing the situation as it is conceived, the stakeholder roles, the relationship, and recurrence of the system components. Furthermore, in line with a system thinking approach, we can observe the "leverage" points, described in the Section "Results," that can modify the system evolution, for example, improving its performances, along a time line.

This type of project system and project system boundary analyses helps to have an overview on the project, without missing possible important connections within aim, expertise, business, and development factors (36).

To be able to improve the performance of a project, it is often required to not get "unwanted surprises" about its behavior, so to be able to follow almost predictable results, in line to the aim of the project. Considering that the system properties and behaviors are *per se* unpredictable along the project evolution, it is important to be able to understand why there may be a divergence from the wanted aims and the actual proceeding of the project.

Having such a project system description framework helping to connect the various elements of the project, also the little ramifications of a network of interactions among activities/performances/roles/results, gives the possibility to interpret the aim of the project in its real evolution and dynamic, as shown in **Table 3**. The strategy to defragment a project into such a framework may lead to the possible uses of the framework reassumed in **Box 2**.

Different points are evidenced in the system and system boundaries analyses for ALERT example, in particular from **Table 3** and **Figure 2**.

The aim of the project "to support primary producers" is maintained along the description of the system in the framework but many elements are competing with the main aim (see **Table 3**).

It is worth mentioning how the core aspect of the ALERT project, i.e., the emerging role of toxicological risk in the onset of diseases in the context of One Health (33, 34), is both the key and the "problematic" aspect of ALERT as showed in **Box 1**.

The increasing movement worldwide on the need of primary prevention measures to protect communities from non-communicable diseases as well as of sustainable food safety policies for primary prevention of transgenerational risks in the food chains (33, 35) is expected to modify the system in the medium term.

Indeed, the increasing consumers' awareness of food safety long-term impact on health implies a growing demand of safer and safer products, along with the protection of the environment. In this context, policies facilitating primary food producers in their proactive roles are crucial (37).

The integrated analysis of the system and its boundaries including stakeholders, etc. highlights the importance of

TABLE 3 | The system and system boundaries applied to the ALERT project as an example.

Step	Element	Main questions	Secondary questions	Tertiary questions
		Contextualization	**Actions/relationship**	**Evolution/dynamics**
1	**Aim**	**Why I am looking at this system? Which are the questions/problems I want to solve by using the system?**	**What is the declared aim of the system and what is the enacted aim of the system. Is the aim perceived differently by stakeholders?**	**What are the declared and enacted aims at the onset of the evaluation and do they change as the system evolves?**
		Based on European scientific and policy milestones (32), system was built to support primary producers in their mandate for legal, scientific, and ethical responsibility in the European food safety frame Stakeholders like food industry and bank systems recognize the need of field technologies and approaches for food safety in primary production: START CUP CNR-IlSole24Ore Prize for the best high-tech business idea for Social Innovation coming from public research (2011); MONTANA (meat industry and Cremonini group) Prize for Research in the Food sector (2011)	ALERT answers to identified One Health-related needs (**Table 2**) by combining different silos like public vs. private bodies; public health vs. basic research; food industry vs. high-tech industry; risk analysis vs. marketable technologies; scientists vs. food producers; and scientists vs. citizens/consumers (**Figure 1**). The aim and the stakeholders' role are specified in **Table 1** and **Figures 1** and **2** ALERT points at defining and implementing toxicological risk and non-communicable diseases in One Health: so far the application of One Health has been limited to microbiological risk and infectious diseases	ALERT aims at establishing a frame for long-term bottom-up and top-down collaboration through both an open technological platform (i.e., able to improve its detection capability by hosting new probes made available by the scientific community) and an innovative two-lane system for food safety (**Table 1**) **SMEs, food chain, and institutional/research stakeholders have different vision of risks and benefits, based on different needs, mission and vision (as discussed in the text, Table 1**; **Figures 1** and **2) (a)**
2	**Actors**	**Which are the main actors/stakeholders? How are they affected by the system and/or how do they affect the systems?**	**How do actors influence/modify the system to achieve the aim?**	**Do the actors change their activity and behaviors because of the system evolution (new trade-offs)?**
		Actors (as specified in **Figure 2**) cover the range of public institute with top-level expertise/governmental commitment in food chain protection and technologies certification, public institute with top-level expertise/governmental commitment in the protection of food chain wholesomeness and animal welfare, public institute with top-level expertise/governmental commitment in technological innovation and transfer; leading regional enterprises in milk production, transport and storage; enterprises with complementary expertise in the setting, optimization, miniaturization and automation of (bio)probes systems as well as the development of management software and electronic systems; expertise in marketing strategies, strategic partnering at industrial level, as well as dissemination of pre-industrial research Relevant disciplines and the way they affect the system are detailed in **Figure 2**	Single enterprises of the milk chain **can adopt (b)** new self-monitoring strategies to minimize milk losses and waste as well as to optimize milk safety, nutritional value, and wholesomeness The chain of enterprises **can adopt** (b) new strategies to improve milk chain traceability Public Institutes that have the mission of securing a high level of safety of food products and food productions can update (b) tools and strategies based on modernized primary prevention plans **SMEs, food chain, and institutional/research stakeholders have different vision of risks and benefits, based on different needs, mission and vision. In particular:** – **attitude toward non-traditional approaches to protect food-producing animals and food productions: scientific research approach vs. market-driven food production needs;** – **availability to long-term investment: small-medium enterprises mainly depend on short-term economical benefits do to chronic constraints (d)**	**Awareness of toxicological risks and One Health in the food chain is increasing at both enterprise and scientific community levels (g)**

(*Continued*)

TABLE 3 | Continued

Step	Element	Main questions	Secondary questions	Tertiary questions
		Contextualization	**Actions/relationship**	**Evolution/dynamics**
3	**System dimensions (space and time)**	**Which geographical and political space does the system occupy (e.g., geography/area/countries involved)? Which is the most important time scale for observing the system (e.g., months, years), and what is the primary level of analysis for the evaluation of the system (e.g., individuals, family, and population)**	**How are these dimensions connected with the declared aim of the system?**	**As the system evolves, how do these aspects change?**
		ALERT (2012–2017 project duration) focuses on a relatively large-sized dairy farm of Central Italy (and neighboring farms), and a main bovine milk chain in central Italy (Lazio region) Starting from cost/benefit assessment for all actors in the milk chain, ALERT evaluates the possible impact of the BEST on costs and marketability of milk products. ALERT assesses the value attributed by the consumers to a new brand/logo for the improved food chain control process	**ALERT outcomes can be applied (eventually with appropriate revision in certain milieu such as economically developing areas and contaminated sites) to other regions and nationwide (c)**	Increasing know-how in the chemical/toxicological (emerging) risk assessment Technological solutions, materials and methods change over time: an ALERT web platform collect census data of international probes that could be hosted by the BEST device Increasing consumers' awareness of food safety long-term impact on health Increasing power of rearers' Associations and consortia Through Expo 2015 (Milano, Italy), a unique event of knowledge of the food market and its needs in terms of technologies, ALERT gathers the needs of the national and international markets, and periodically update all relevant possible new stakeholders
4	**Restrictions/ conditions/ boundaries**	**What are the main restrictions/conditions/boundaries of the system? Are there constraints coming from the system external surroundings?**	**How do these restrictions/conditions/ boundaries interact with the system aims?**	**Do these restrictions/conditions/boundaries change as the system evolves?**
		In the specific Italian scenario characterized by a high degree of one health in the institutional setting (veterinary health and food safety both under the Ministry of Health), main constraints are mainly relevant to: **Delayed ripeness worldwide and in different silos** (d) on toxicological risks in One Health (33, 34) **Need to strengthen sustainable food safety policies** (d) worldwide for primary prevention of transgenerational risks in the food chains (33, 35) from the technical viewpoint, ALERT proposes strengthening/modernizing the self-monitoring system to integrate/empower the two-lane system for food safety. This calls for investment (personnel, time, and materials) to set up a new organization flow during routine daily food production	**Limited confidence in the acquisition** (d) of new knowledge through non-traditional approaches vs. market-driven food production needs **Limited policies facilitating** (d) proactiveness toward emerging risks **Perceived different attitude toward the aim, in particular private vs. public institutions (d)**	Awareness on the importance of acquiring new knowledge through non-traditional approaches vs. market-driven food production needs Increasing movement to implement policies for facilitating proactiveness toward emerging (chemical/toxicological) risks in the farm

(*Continued*)

TABLE 3 | Continued

Step	Element	Main questions	Secondary questions	Tertiary questions
		Contextualization	**Actions/relationship**	**Evolution/dynamics**
5	**Consequences**	**What are the consequences of the system (outputs/results/products)?**	**Are these consequences bound by the system boundaries?**	**Are these consequences changing as the system evolves?**
		Development of new field technology, decrease the vulnerability to unexpected events, increase the preparedness to emerging (chemical/toxicological) risks, reduce food losses and waste (risk of product recalls from the market and related costs of food destruction along with damage to the enterprises commercial image). **Turn upside down the responsibility to the primary food producer (and role in the value chain therefore) (e)**	Yes	**Dynamics mainly pivot on different interests in the system, from public health actors pointing at advancements in the protection of human health and the environment (including animals), food chain actors at the balance between needs of food security and food safety, and small-medium enterprises mainly depending on short- term benefits. (f)**

BOX 1 | Main results after the application of system and system boundaries framework to ALERT.

The aim of the system is not interpreted in the same way by different stakeholders (a, d)
Stakeholders from different backgrounds and disciplines miss a harmonized collaboration to fulfill the aims of the project in the most productive way (b, d)
It is shown from the space and dimension elements that the system has potential to be applied at a larger scale (e.g., other regions and nationwide) (c)
Along the restrictions/constrictions/system boundaries, two main aspects (market-driven or daily tasks vs. importance of acquiring new knowledge through non-traditional approaches, and missing policies facilitating proactiveness at the farm) lead to the evolution scenario of weaknesses in synergizing activities between partners (d)
The consequences follow and detail the fact that the project aims to give more responsibility to the primary food producer and the different stakeholders approach this task with limited synergies (e)
The evolution of the system depicts needs and constrains in the future: namely, short-term/long-term effects along the awareness of toxicological risk/One Health approach in the food chain (g)

BOX 2 | Summary of possible use of the framework.

To find possible representative information from the network of connections among aims, causes, consequences, and results of a project.
To prove the role of the different actors along the time line of the project, causes and consequences of their behaviors, and their points of view/background. This will help in getting a meta-perspective to evaluate a project. A further development of this basic framework can be to ask different stakeholders to apply the framework to the same project. Because their perspectives differ, such an analysis would further highlight synergies and antagonisms allowing for improvement.
To monitor progress along the project implementation phase (e.g., first and second year).
To compare and evaluate projects' impacts and to measure their progress and compliance with the aim.

the collaboration within different disciplines, stakeholders, methodologies, expertise, etc. To allow the smooth project implementation taking into consideration the evidenced needed for interaction/collaboration/links among stakeholders and disciplines, governance mechanisms should be defined. However, integrating health aspects of humans, animals, and the environment is often not sufficient to identify relevant trade-offs, potential burden shifting, and undesired consequences in a system change, e.g., *via* an intervention. To address this issue, a more comprehensive approach is required where in addition to health- and risk management-related aspects also sustainability related aspects are considered along the entire system life cycle. Combining risk and sustainability aspects in a consistent manner to provide a more reliable decision support of health intervention and various other systems is proposed by the Global Decision Support Initiative (GDSI[3]).

In conclusion, a system view of a complex project and the definition of its boundaries help in understanding the way how to structure and optimize a system and actions (e.g., health interventions) within that system: how to integrate different expertise,

[3]http://gdsi.dtu.dk.

meet different needs, mission and vision, and improve communications. This framework is a good way to have a concise, common overview of the most important elements that run a system now and in the future, considering the several externalities and impacts generated, but also to identify current system limitations.

AUTHOR CONTRIBUTIONS

All the authors contributed to the design of the work, to the acquisition and interpretation of data for the work, and to revise it critically and all the authors approved the final version to be published.

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From Invention to Innovation: Risk Analysis to Integrate One Health Technology in the Dairy Farm

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Current Hazard Analysis Critical Control Points (HACCP) approaches mainly fit for food industry, while their application in primary food production is still rudimentary. The European food safety framework calls for science-based support to the primary producers' mandate for legal, scientific, and ethical responsibility in food supply. The multidisciplinary and interdisciplinary project ALERT pivots on the development of the technological invention (BEST platform) and application of its measurable (bio)markers—as well as scientific advances in risk analysis—at strategic points of the milk chain for time and cost-effective early identification of unwanted and/or unexpected events of both microbiological and toxicological nature. Health-oriented innovation is complex and subject to multiple variables. Through field activities in a dairy farm in central Italy, we explored individual components of the dairy farm system to overcome concrete challenges for the application of translational science in real life and (veterinary) public health. Based on an HACCP-like approach in animal production, the farm characterization focused on points of particular attention (POPAs) and critical control points to draw a farm management decision tree under the One Health view (environment, animal health, food safety). The analysis was based on the integrated use of checklists (environment; agricultural and zootechnical practices; animal health and welfare) and laboratory analyses of well water, feed and silage, individual fecal samples, and bulk milk. The understanding of complex systems is a condition to accomplish true innovation through new technologies. BEST is a detection and monitoring system in support of production security, quality and safety: a grid of its (bio)markers can find direct application in critical points for early identification of potential hazards or anomalies. The HACCP-like self-monitoring in primary production is feasible, as well as the biomonitoring of live food producing animals as sentinel population for One Health.

Keywords: dairy chain, cow milk, biosensoristic devices, risk management, risk assessment, food safety, environmental health, Hazard Analysis and Critical Control Point

PRIMARY PRODUCERS' MANDATE FOR LEGAL, SCIENTIFIC, AND ETHICAL RESPONSIBILITY IN THE EUROPEAN FOOD SAFETY FRAME: THE ROLE OF RISK ANALYSIS AND SCIENTIFIC RESEARCH

The European White Book for food safety points out the ethic, scientific, and legal responsibility of all food operators, including food primary producers, in guaranteeing the safety of their products. Food safety and traceability have to be ensured at every stage of the food chain, and the primary production is the first critical step (1). In fact, several main food safety alarms in past decades and years (e.g., BSE, VTEC, dioxin contamination of animal feedstuffs) took place in the primary production sector; in the meanwhile, the understanding of the web of interactions among humans, animals, and the environment (One Health) determines the increasing importance of prevention and safety in the primary livestock production.

Dairy farming is among the most complex, and potentially vulnerable, components of farm animal production: the maintenance of good qualitative standards of milk and dairy products still represents a challenge for farmers and manufacturers who, in their turn, ask the scientific community to furnish them with proper tools for hazard identification and risk management. The best strategy to ensure safety calls for implementing preventive approaches, such as good breeding and manufacturing practices or the application of procedures based on the Hazard Analysis and Critical Control Point (HACCP).

HACCP was firstly used in food production in the 1970s, providing precise process control measures for each step of the entire food manufacturing process. The Codex Alimentarius Commission has recognized HACCP as an effective tool to improve safety standards; HACCP identifies priority hazards and allows establishing targeted control systems, thus putting focus mainly on preventive measures rather than on end-product testing (2). HACCP is a food safety system, and ISO 22000:2005 is a food safety management system standard. As described in the Codex Alimentarius, ISO 22000:2005 mainly fits postprimary production/transformation (pasteurization/microfiltration and cheese factories) and, more than HACCP, focuses on policy, standards, targets, communication, and planning.

The application of HACCP-like systems to animal health and primary production still represent the best approach (3). The European Union forced the implementation of HACCP after the revision of the hygiene directives (4–6) and the general food law (7). Currently, HACCP focuses on microbiological hazards and risks, as can be found in public and animal health state instituted plans. HACCP should focus also on hazards of different nature, such as chemical and physical contamination of products and even on animal welfare disorders.

Currently, the European Union recommends primary producers, such as dairy farmers, to apply a HACCP-like program to prevent milk-borne zoonoses; noticeably, the modern concept of zoonoses does include toxicological risks carried over in foods of animal origin (8). However, the application of such programs on dairy farms is still not developed: indeed, implementing new strategies and technologies for the application of HACCP in primary production represents a point of utmost importance. ALERT[1] is a project funded by the Italian Ministry for Economic Development, and based on the BEST technological integrated bioelectronic system and relevant control charting for early intervention on food chain and the environment (9). Along with a new field and self-instructed technology working in the farm environment, ALERT aims at developing and making available to dairy farmers a modernized risk management framework based on scientific evidence and recommendations by international agencies (9).

In this paper, we define the framework for technology transfer. Indeed, true innovation needs translational activities to make inventions (in this case, the BEST system) be sustainably integrated in complex and dynamics real systems. Through field activities in a selected dairy farm in central Italy, we explored individual components of the dairy farm system to define both opportunities and challenges of the BEST technology transfer. The farm-specific scenario is then considered at a broader spatial scale, together with neighboring farms, in order to highlight possible significant aspects associated to managerial or environmental factors.

The multidisciplinary and interdisciplinary One Health profile (environment, animal health, food safety) of the ALERT project is further amplified by the involvements of technological innovation. Farm characterization and risk analysis are basic inputs to establish a targeted grid of probes of the BEST platform in order to monitor a farm-tailored panel of analytical parameters. Indeed, as all health-oriented innovation initiatives the ALERT framework is complex and subject to multiple variables (10).

MATERIALS AND METHODS

The dairy farm (Lazio region, 41°54′47.94′′N, 12°15′48 ′25′′E) object of the present study was selected as representative of a well-conducted, relatively large-sized dairy farm of Central Italy.

The characterization of the farm both as an environment, an animal rearing facility and a segment of the food chain was carried out following the seven HACCP principles (11) during 12 onsite monthly visits to the selected farm, from January to December 2012.

The farm characterization made avail of the checklists elaborated by the Agricultural Agency of the Tuscany region and by National and European Authorities [(12), Welfare Quality-Cattle protocol,[2] (4)] and currently in force in the official control system. The main topics covered the following:

(1) Farm position and territorial analysis of the macro-area around the farm. The dataset comprises farm position and area, geo-climatic factors, possible pollution sources (e.g., waste disposal sites), presence of neighboring protected

[1] http://www.alert2015.it.

[2] http://www.welfarequality.net.

TABLE 1 | Analysis performed at the identified points of particular attention (POPAs) and critical control points (CCPs).

Element	CCP or POPA	Indicators/analysis performed	Technique	Reference
Water quality (beverage and cleaning)	CCP	Total bacterial count, coliforms, *Escherichia coli*	Cultural	UNI EN ISO 6222:2001 UNI EN ISO 9308-1:2014
		Heavy metals (cadmium, lead) and pesticides residues (florasulam, 2,4-dichlorophenoxyacetic acid, mesotrione, terbuthylazine, desethyl-terbuthylazine, and S-metolachlor)	GC MS, ICP MS	Internal certified method (POS CHI 051 INT rev 0 2011, POS CHI 028 INT rev 4, 2013)
Feed and silage quality	CCP	Heavy metals (cadmium, lead), pesticides residues (florasulam, 2,4-dichlorophenoxyacetic acid, mesotrione, terbuthylazine, desethyl-terbuthylazine and S-metolachlor), and mycotoxins residues (aflatoxin B_1)	GC MS, ICP MS, ELISA	Internal certified methods (POS CHI 051 INT rev 0 2011, POS CHI 028 INT rev 4, 2013, POS 037 INT rev 0, 2009)
Animal health/ zoonoses	POPA	Gastrointestinal pathogens (*Salmonella*, *Campylobacter*, *E. coli*, *Cryptosporidium*)	Cultural, microscopic analysis	OIE Manual for terrestrial animals 2010 cap 2.9
Bulk milk quality	CCP	Total bacterial count, somatic cell count Fat, protein and lactose content Mycotoxins (aflatoxin M_1) Antimicrobials residues (lincomycin, spectinomycin, marbofloxacin, ciprofloxacin, amoxicillin, flunixin, and 5-hydroxy flunixin)	Opto-fluorometric ELISA Microbiological	Internal certified methods (POS CIP 021 INT rev5 2015). AFNOR DSM 28/02–02/12 Delvotest® and internal certified methods (POS CIP 018 INT rev11 2015, POS CHI 038 INT rev5 2015)

areas, presence of endangered species, land usage, main crops, agricultural techniques, previous mycotoxins alerts, hydro-geographic network, and presence of farms and/or factories within a 20-km buffer around the farm. In order to identify possible health risks from zootechnical activities within the buffer area, among the 40 small size (<300 heads) dairy farms and 1 larger farm (>500 heads) identified, three farms were selected based on structural homogeneity, productive capacity, and lower distance from the chosen farm. Milk quality analysis data of these three farms from 2010 to 2013 were collected (Source: Istituto Zooprofilattico Sperimentale del Lazio e Toscana (IZSLT) Laboratory Information System) and statistically analyzed (MedCalc version 12© 1993–2012 MedCalc Software bvba).

(2) General farming conditions. The dataset comprises animal identification, number of heads for each category, structures, conditions of animal barns (ventilation, illuminations, etc.), dry period management, biosecurity, and prevention tools.
(3) Agricultural, fertilizing, and weeding practices, with particular attention to main crops, pesticides management including the risk of groundwater pollution.
(4) Animal nutrition, with particular attention to feed quality, safety, and origin.
(5) Animal health and welfare (anti-microbials and anti-parasitic drugs usage and management, udder health).
(6) Milking techniques and milking parlor hygiene.

Critical points were monitored through routine laboratory analysis with instruments and methods currently used by the Official Control System. Routine laboratory analyses were performed at the "Istituto Zooprofilattico Sperimentale del Lazio e Toscana M. Aleandri" laboratories under a total quality assurance system and were certified by the Italian Bureau for Laboratory Accreditation "Accredia,"[3] Rome, Italy (number of accreditation 0201). Laboratory analysis covered the following: well water (total bacterial count, coliforms and *Escherichia coli*, heavy metals, pesticides), feed and silage (pesticides, heavy metals, mycotoxins) (13), individual fecal samples (parasitological analysis, *Salmonella* spp., *Campylobacter* spp.), and bulk milk (total bacterial count, somatic cell count, fat, protein, lactose, aflatoxin M_1, antimicrobial residues) (**Table 1**) for comparison with the site-specific set of (bio)markers in the BEST Platform (**Table 2**).

TABLE 2 | Set of (bio)markers selected for the site-specific BEST platform.

Non-targeted (indicators of safety/quality)	Targeted (specific analytes, including milk components and residues/contaminants)
Temperature	Calcium ions
pH	Sodium ions
Redox potential	Potassium ions
Total milk quantity/milk flow	Iodide ions
Conductivity	Fluoride ions
Aerobic cellular respiration[a]	Chloride ions
Oxygen	Nitrate ions
Carbon dioxide	Ammonium ions
Chlorophyll a fluorescence[b]	**Heavy metals**
Tyrosinase[c]	**Antibiotic residues**
Laccase[d]	**Fat**
Urease	**Protein**
Lactate dehydrogenase	**Lactose**
Glucose oxidase	Blood
	Somatic cell count
	Total bacterial count/mastitis-causing bacteria (*Streptococcus uberis* and *Escherichia coli*)
	Pesticides
	Aflatoxin M_1

Parameters in bold were monitored through laboratory analysis in this study.
[a]General toxicity/wholesomeness.
[b]Exposure to pesticides inhibitor of photosystem-II complex, including phenyl-carbamate, pyridazinone, triazine, uracils, ureas, benzothiadiazinones, and phenyl-pyridazines pesticide.
[c]Exposure to phenolic, organophosphate, and carbamate pesticides.
[d]Exposure to phenolic and carbamate pesticides.

Farm owners and farmers have been formally enrolled in the Consortium of the project ALERT and thus they consented

[3]http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7&dipartimento=L%2CS&.

to the collection and use of data. According to EC (14) of the European Parliament and of The Council of 22 September 2010 on the protection of animal used for scientific purposes and the Italian law "Decreto Legislativo 26/2016," (15) the authors can assert that all the animals involved in the study were exclusively submitted to practices respecting animal welfare and undertaken for the purposes of recognized animal husbandry, in accordance with good veterinary practice. Thus, the study does not require any further specification regarding ethics approval by authors.

RESULTS

Farm Characterization: Checklists

Farm Position and Territorial Analysis of the Macroarea around the Farm

The selected farm is located in Central Italy and rears high-production Italian Holstein cows (average 9.5 tons milk/cow/year). The farm covers an area of over 350 ha of cultivated land, ranging from a 400 to 500 m high hilly zone to the plain along the Tyrrenian coast. According to Mayr-Pavari definition for phytoclimatic zones, the area lays in the Lauretum zone (warmer subzone, with summer drought). The broader area including the dairy farm is involved in agricultural production (grasslands, woods, cereals and herbaceous crops, olive groves and vineyards).

Concerning nitrogen pollution, the farm lays in a nitrate non-prone zone (Council Directive 75/440/EEC and Italian National Regulations: D.lgs. 152/99 and D.lgs. 258/2000) (16). This area does not include any chemical factories or other potential sources of water and environmental pollution. The analysis highlights the presence of simple cropping systems (dry and irrigated), permanent herbaceous crops (lawns, meadows pastures, and alfalfa *Medicago sativa*), and uncultivated areas with natural vegetation (wild trees and shrubs, and uncultivated fields). The quality of crop does not require specialized use of chemicals in their growing cycle. The absence of specialized fruit orchards, vineyards, and vegetable crops reduces the possible direct contamination by agrochemicals (fungicides, insecticides, herbicides, etc.).

Mycotoxins contamination is considered the most important toxicological risk of the macroarea; nevertheless, contamination of milk can be considered a rare event. From 2009 to 2013, aggregated data of Official Controls for Aflatoxin M1 in bovine raw milk in Tuscany and Lazio Regions reveals 324 (3.3%) samples above the legal thresholds on 9,723 total analyzed samples, with a peak prevalence (9.6%) in September; data about the occurrence of Aflatoxin B1 in feed and silage in the same years showed a prevalence of 100 (12.7%) samples above the legal thresholds out of total 570 analyzed samples (Source: IZSLT Laboratory Information System). These prevalence rates of Aflatoxin B1 contamination events could be overrated by the introduction of feed from other parts of Italy or from abroad.

Milk quality analysis data from 2010 to 2013 are shown in **Tables 3–6**. Data show a good health status and a substantial similarity among the three farms.

General Farming Conditions and Animal Housing

The farm is registered due to EC Regulation 852/2004 and authorized for the production of high-quality milk due to the Italian law DM 185/91. The whole milk produced is destined to pasteurization and direct consumption, without transformation. The farm owns 420 total heads (160 lactating cows, 30 primiparous). The animals are correctly identified due to EC Regulation 1760/2001. The farm is composed by six different areas for animal housing: (1) Lactating Cows, (2) Dry Cows, (3) Heifers, (4) Calves (paddock and individual cages), (5) Infirmary, and (6) Grazing land. All the animals (except for calves up to 40 days reared in single boxes) are reared in multiple boxes with an indoor section with permanent hay litter (density 6.5 mq/head) and an outdoor paddock. Bedding is renewed daily (5–6 kg hay/head in autumn and winter and 2–3 kg hay/head in spring and summer) and the hygienic condition is very good. Ventilation and illumination are natural; air flowing is guaranteed by mean of large windows and there is no fecal or ammonia smell in the animal premises.

TABLE 3 | Average values of fat and protein content, total bacterial count, and somatic cells of bulk milk of the three nearer farms from 2010 to 2013.

Farms	Fat (%)	Protein (%)	Total bacterial count (CFU*1,000/mL)	Somatic cell count (cells*1,000/mL)
1	3.68	3.27	14	239
2	3.79	3.36	53	289
3	3.77	3.40	28	285

TABLE 4 | Antimicrobials residues and aflatoxin M_1 of the three nearer farms from 2010 to 2013.

Farms	Antimicrobials residues (positive samples)	Aflatoxin M_1 (ng/kg)
1	0	<30
2	0	<30
3	0	<30

TABLE 5 | Average values of fat and protein content, total bacterial count, and somatic cells of bulk milk of the three nearer farms per year.

Year	Fat (%)	Protein (%)	Total bacterial count (CFU*1,000/mL)	Somatic cell count (cells*1,000/mL)
2010	3.69	3.32	35	292
2011	3.76	3.35	32	273
2012	3.77	3.37	35	240
2013	3.81	3.36	20	294

TABLE 6 | Antimicrobials residues and aflatoxin M_1 of the three nearer farms per year.

Year	Antimicrobials residues (positive samples)	Aflatoxin M_1 (ng/kg)
2010	0	<30
2011	0	<30
2012	0	<30
2013	0	<30

Agricultural Management

The total agricultural area is about 360 ha, while the utilized agricultural area (UAA) is about 350 ha. Such area is involved in the phytosanitary measures that the Lazio Region has issued for the control of the Western corn rootworm (*Diabrotica virgifera virgifera*).

The currently employed crops are listed in **Table 7**. The final use of the crops is entirely dedicated to animal supply. The main cultivation operations such as tillage, seeding, fertilizing, weeding, herbicide and pesticide treatments, irrigation, hay, and silage are performed without external intervention.

Fertilization is performed either with farm's manure and synthetic fertilizers, such as ammonium nitrate (NH_4NO_3) and urea [$CO(NH_2)_2$]. Herbicides and pesticides treatments are carried out with specific products [mesotrion 3.39% (37.5 g/L), S-metolachlor 28.23% (312.5 g/L), terbutilazine 16.94% (187.5 g/L), and florasulam (6.25 g/L)]. Even though treatments are carried out respecting the relevant legal limits, there is the need to monitor the possible pollution of groundwater or crops by the parent molecules or their main by-products, considering also the possible accumulation and mixture effect.

Animal Nutrition

100% of forage and silage are produced within the farm, while a varying proportion of grain, protein nucleus, and flour (corn, barley, faba beans, and wheat bran) are purchased outside. There are no different feeding groups for the different production levels; feedstuffs are administered twice a day as unifeed. The unifeed present in the manger is in good condition and particle size is homogeneous. Dry cows are fed only with hay herbage and mineral supplement. The mangers are clean and dry and feed residues are modest. The documents relating to purchased feed and the records of loading and unloading are properly managed and are analyzed once a year. The core and flour are guaranteed as genetically modified organism and aflatoxin-free by the manufacturer.

Animal Welfare and Health Management

The farm is officially free from tuberculosis, brucellosis, and enzootic bovine leukosis. Vaccination against clostridial infections is regularly practiced. The parasitic load is evaluated yearly by coprological evaluation, and on rare occasions ivermectin treatments are required. The main health problems are represented by (i) placental retention (8%), (ii) mastitis (5–6%) caused by *Streptococcus uberis* and *E. coli* (17), (iii) lameness and claw disorders (5%), (iv) cutaneous papillomatosis (1%), and (v) neonatal diarrhea reported as a very rare event.

The most used veterinary drugs in the farm are antimicrobials: lincomycin and spectinomycin, marbofloxacin, flunixin meglumine, and amoxicillin. Treated animals are identified on the mantle to ensure the isolation of milk at milking time. The farm is not authorized to hold stocks of drugs; veterinary prescriptions are properly recorded. Nutritional, health, and hygienic status has been assessed for all dry cows, about 10% of lactating cows and 10% of heifers.

TABLE 7 | Main culture and crop production.

Crop	UAA (ha)	Production
Corn	55	Silage
Grass (wheat, barley, triticale)	35	Silage
Alfalfa	55	Silage, hay
Grass (oats, Lolium, clover)	165	Hay
Wheat	40	Grain

Milking Techniques and Hygiene

Cows are milked immediately after calving and from 1 week after calving milk is collected in a buklet (during the first week colostrums is collected separately) up to 305 days. Cows are dried through drastic reduction of the feed (straw, hay, little, herbage, and water only) and use of intramammary antibiotics; milking is interrupted abruptly. The whole farm produces an average of 30–35 L/head/day, for a total of 8.5–9.0 tons/head/year. Cows are milked twice a day by two operators.

The parlor consists of two herringbone lines, originally 5 + 5, then extended to 7 + 7, with Afimilk® automatic milking machine adopted in the frame of the ALERT activities and integrated in the BEST platform (42-kPa vacuum level, 60 cycle per minute, pulsation ratio 1:1) with electronic recognition of cows through the use of pedometers. The whole milking process lasts about 3 h (mean time of attack-detachment for each cow is 7–8 min). The operators do not wear gloves during milking and pre-milking teat dipping is not performed. There is no use of oxytocin, even in primiparous cows.

Pre-Milking Routine

Udder is washed with drinking water (from municipal aqueduct) and disinfected with chlorhexidine and finally dried with disposable paper. The first streams of milk are usually discarded.

Mechanical Milking

Operators attach the milking clusters ensuring a well-balanced contact with teats. Milk is firstly collected in a small collector tank, filling and emptying every 20 s, which conveys the milk into the main cooling tank.

Post-Milking Routine

In order to remove/reduce the risk of cross-contamination with contagious mastitis pathogens, a post-milking teat dipping is performed using a filming iodophor disinfectant (IODO PVP FILM) as a barrier preventing bacteria from colonizing teat's surface and orifice.

Milking Machine and Tank Disinfection

Disinfection of the milking machine is performed with an acid–alkaline treatment after each milking. Collection time, temperature, and quantity of the milk are properly recorded.

Farm Characterization: Flow Diagrams and CCPs and POPAs

The flow diagrams of the production process were drawn. Based on the flow diagrams, critical steps and risk factors for risk management in the farm were identified based on risk assessment.

Critical points associated with a potentially occurring hazard impacting on production were identified and classified as control points [critical control points (CCPs)] or points of particular attention (POPAs) (**Figures 1** and **2**). In particular, according to the principles and methodology of Noordhuizen et al. (3), CCPs are measurable or observable and have standard external values possibly subject to official regulations (e.g., governing production stoppage) as well as available corrective actions to restore control.

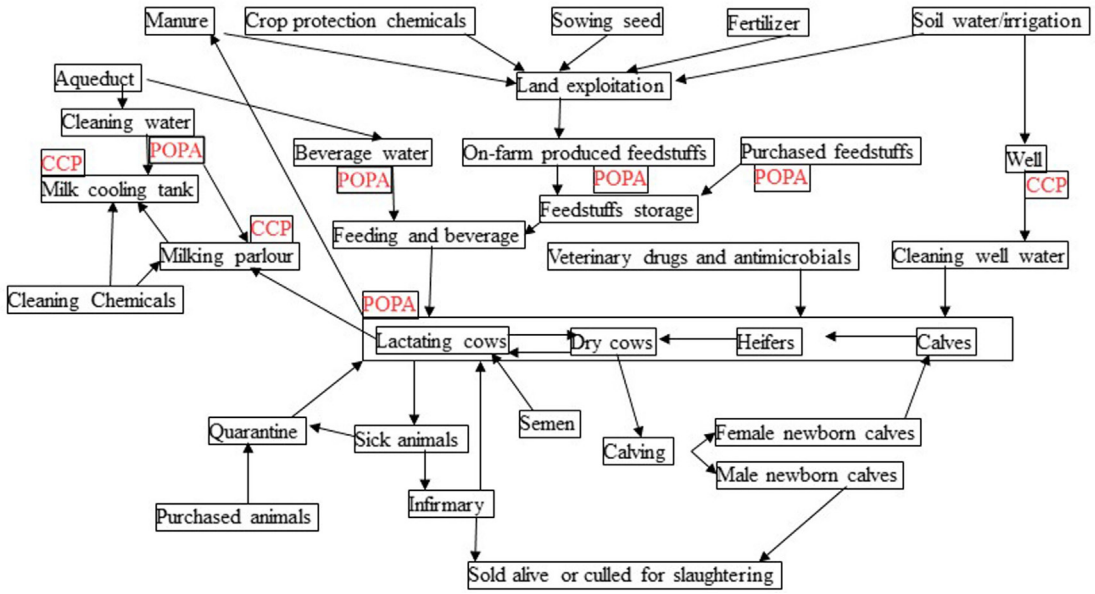

FIGURE 1 | General flow diagram of the production process in the dairy farm in central Italy.

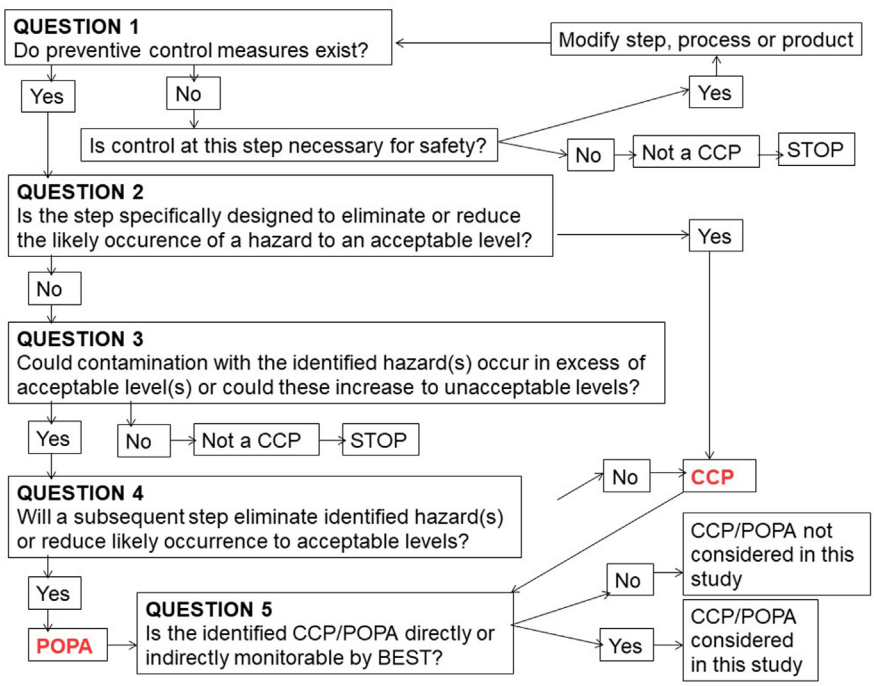

FIGURE 2 | Decision-tree approach in the dairy farm in central Italy. The approach is to determine whether a control point is critical (CCP) or not points of particular attention (POPA). Only POPAs and CCPs monitorable by BEST were considered and monitored.

According to the design of the BEST platform, POPAs are critical points where anomalous trends are measurable and, through anomalous variations in relevant control charting, can drive early risk management procedures in HACCP-like plans (9).

Based on the model presented in Noordhuizen et al. (3), the farm management *Decision tree* is drawn, under the One Health view (environment, animal health, food safety), with special attention to POPAs and CCPs that can be monitored with the BEST platform.

Farm Characterization: Laboratory Analysis at POPAs and CCPs

Well Water, Feed, and Silage

Metals, pesticides, and mycotoxins in feed and well water resulted below the respective legal thresholds or below the limits of quantification/detection, except for pirimiphos-methyl—an organophosphorus pesticide found in one feed sample (0.2 mg/kg). Water (both for drinking or cleaning) showed good microbiological standards (18, 19) (**Table 8**).

Coprological Analysis

Zoonotic agents were not detected from any fecal sample.

Bulk Milk

In accordance with EC Regulation 853/04, 37 bulk milk samples were processed for total bacterial count (CFU*1,000/mL), somatic cell count (cell*1,000/mL), fat (%), protein (%), lactose (%), aflatoxin M1 (μg/kg), and antimicrobial residues. Data show a good milk quality (20, 21) (**Table 9**).

DISCUSSION

The One Health concept applied to toxicant-related zoonoses requires the analysis of risks in the web of interactions at the environment–animal–human interfaces (8).

No environmental pollution sources were identified by the checklists. The farm is located in a not nitrate-prone area that is suited to agricultural activity, and near to protected natural areas (22). In the surroundings, there are no chemical industries or waste disposal sites, but only small-size dairy farms, characterized by good management and good milk quality standards. Cropping systems do not require a broad use of agrochemicals, making it unlikely a significant contamination of the vegetables used for feeds and of the water system. Groundwater contamination (**Table 8**) was highly variable and the results may not be representative of any temporal problems, thus highlighting the importance of a in continuum monitoring offered by the BEST.

Overall, the study farm presented a good standard of farming (23), agricultural, and sanitary practices. These observations were confirmed by the results of laboratory analyses. For instance, the absence of residual inhibiting substances and aflatoxin M1 indicate good animal husbandry, good management of feed as well as a conscious use of antimicrobial drugs (24). Aflatoxin alerts have become relatively common in Northern Italy due to climate changes, land usage and cropping errors, inadequate irrigation, parasites and insect attacks, and harvest preservation disorders (25). All these factors may lead to fungal colonization and toxins production. Prevalence may reach peaks higher than 10% of total processed samples. Based on the overall scenario, risk of aflatoxin B1 contamination can be considered mainly during and shortly after summer drought. As the legal thresholds are exceeded, milk have to be destroyed by local Authorities, thus causing important economic losses for farmers.

Breeding techniques ensure good standards of welfare and animal health. Paratuberculosis is widely diffused in Italy; the farm prevalence can be considered quite low, thus highlighting the possible eradication by mean of the new regional prophylaxis program.

Based on the HACCP-like approach and farm management decision tree, the analysis carried out in the sequential POPAs of the farm identified a limited set of farm-specific CCPs. In particular, we consider the following concepts.

(1) Well water should be periodically checked for pollution by synthetic fertilizers (ammonium nitrate and urea), as well as for bacterial contamination; indeed, the management of litter could lead to the risk of fecalization of the groundwater, as suggested by previous finding of "environmental" bacteria in fore-milk and water (*E. coli*). Well water is vulnerable to pollution by pesticides and their degradation products; even though the analyses did not reveal the presence of residues, monitoring is warranted.
(2) Bulk milk represents the end-stage product of dairy farms. Information gathered on bulk milk is obviously pivotal for food safety (e.g., residues, contaminants, somatic cells, and total bacterial count). Finally, milk may represent an indicator of the environmental quality, both of surrounding areas out of the farm (e.g., residues of heavy metals or pesticides) and inside the farm as determined by farming management systems (e.g., residues of veterinary drugs, disinfectants, aflatoxin M1). Overall, milk can be considered as a real "One Health" biomarker as it can provide a cluster of data relevant to food safety, animal health, farming management

TABLE 8 | Water quality parameters (mean values).

	Cleaning water	Water at watering[a]
Fecal coliforms	0 MPN/100 mL	0 MPN/100 mL
Total coliforms	0 MPN/100 mL	1 MPN/100 mL
Escherichia coli	0 MPN/100 mL	0 MPN/100 mL
Total bacterial count (22°C)	<1 CFU/mL	23 CFU/mL
Total bacterial count (37°C)	<1 CFU/mL	<1 CFU/mL
Fecal streptococci	0 MPN/100 mL	1 MPN/100 mL

[a]*Water collected from drinking troughs.*

TABLE 9 | Bulk milk quality.

	Fat (%)	Protein (%)	Lactose (%)	Somatic cell count (cell*1,000/mL)	Total bacterial count (CFU*1,000/mL)	Aflatoxin M_1 (ng/kg)	Antimicrobials residues
Mean	**3.81**	**3.33**	**4.77**	**220**	**38**	<30	**<MRLs**
SD	**0.13**	**0.11**	**0.04**	**44**	**20**	–	**<MRLs**
Min	3.51	3.14	4.68	133	12	<30	**<MRLs**
Max	4.07	3.50	4.84	328	101	<30	**<MRLs**

and environmental quality (26), thus protecting health and preventing food losses.

Databases of laboratory analysis provide interesting information for investigation and comparison in other farm systems.

The BEST system of early (bio)markers of anomalies can be applied as monitoring system at well water (POPA) and bulk milk (CCPs). The grid of markers (in environmental matrices) and biomarkers (in animal fluids) of the BEST platform (sensors and biosensors) is flexible, so as to host new probes depending on site-specific requirements (27–30). The grids of (bio)markers recommended in the selected POPAs and CCPs of the study farm are reported in bold in **Table 2**. Indeed, through new (automated) technologies like BEST account for the potential for "cocktail" effects from multiple residues and contaminants with different half-lives, metabolism, persistence, tissue accumulation, and targets. Multiarray signals covering oxidative stress, mitochondrial dysfunction, interactions with nutrients (vitamins, essential elements) leading to lipid/glucose dysmetabolism are promising sets of biomarkers early alerting on significant anomalies occurring in the farm, with important One Health implications.

The use of BEST at watering, milking parlor, and bulk milk is expected to facilitate daily monitoring of farm environment and management, milking efficacy and efficiency, process hygiene, and milk safety. Indeed, the user-friendly and self-instructed (by control charting) BEST system operating on-line and providing timely and continuous information can support the maintenance of production quality (31) as well provide early warnings that trigger appropriate decision trees (32).

Daily maintenance of a good farm management means time and cost-effective preparedness to unwanted and/or unexpected events of both microbiological and toxicological nature. Prevention strategies based on an HACCP-like self-monitoring systems empowering primary food producers (33) and providing measurable (bio)markers to monitor anomalies (including toxicological hazards) in critical points are crucial for translational science in real life. Scientific advances in risk analysis-driven biomonitoring of sentinel animals (26) are exempla of health-oriented innovation in primary production that exploit the "One Health" framework (10).

CONCLUSION

The application of risk assessment using POPAs and CCPs for farm management is a valuable initiative to overcome challenges of translational science in (veterinary) public health. The understanding of complex systems is a condition to accomplish true innovation through new technologies. In the case of One Health technology, biomonitoring of sentinel animals like food producing animals is crucial. The framework discussed in this work demonstrates how the development of an HACCP-like self-monitoring system based on measurable markers in critical points of the primary production chain and in live animals is feasible. Scientific advances in risk analysis can be applied to prevent toxicant-related zoonoses in daily primary production of food, with simultaneous benefit (One Health) for the protection of human, animal, and environmental health.

ETHICS STATEMENT

Farm owners and farmers have been formally enrolled in the Consortium of the project ALERT and thus they consented to the collection and use of data. According to EU Directive 2010/63 of the European Parliament and of The Council of 22 September 2010 on the protection of animal used for scientific purposes and the Italian law "Decreto Legislativo 26/2016," the authors can assert that all the animals involved in the study were exclusively submitted to practices respecting animal welfare and undertaken for the purposes of recognized animal husbandry, in accordance with good veterinary practice. Thus, the study does not require any further specification regarding ethics approval by authors.

AUTHOR CONTRIBUTIONS

All the authors substantially contributed to: (1) the conception of the work and the acquisition, analysis of interpretation of data; (2) drafting and revising critically the work for important intellectual content, (3) final approval of the version to be published; and (4) agreement on accountability in all aspects of the work, in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Understanding Seasonal Changes to Improve Good Practices in Livestock Management

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Background and Aim: Food quality control techniques based on process control methods are increasingly adopted in livestock production systems to fulfill increasing market's expectations toward competitiveness and issues linked to One Health pillars (environment, animal, and human health). Control Charts allow monitoring and systematic investigation of sources of variability in dairy production parameters. These parameters, however, may be affected by seasonal variations that render impractical, biased or ineffective the use statistical control charts. A possible approach to this problem is to adapt seasonal adjustment methods used for the analysis of economic and demographic seasonal time series. The aim of the present work is to evaluate a seasonal decomposition technique called X-11 on milk parameters routinely collected also in small farms (fat, protein, and lactose content, solids-not-fat, freezing point, somatic cell count, total bacterial count) and to test the efficacy of different seasonal removal methods to improve the effectiveness of statistical control charting.

Method: Data collection was carried out for 3 years on routinely monitored bulk tank milk parameters of a small farm. Seasonality presence was statistically assessed on milk parameters and, for those parameters showing seasonality, control charts for individuals were applied on raw data, on X-11 seasonally adjusted data, and on data smoothed with a symmetric moving average filter. Correlation of seasonally influenced parameters with daily mean temperature was investigated.

Results: Presence of seasonality in milk parameters was statistically assessed for fat, protein, and solids-non-fat components. The X-11 seasonally-adjusted control charts showed a reduced number of violations (false alarms) with respect to non-seasonally adjusted control chart (from 5 to 1 violation for fat, from 17 to 1 violation for protein, and from 9 to none violation for solids-non-fat.). This result was achieved despite stricter control chart limits: with respect to raw data charts, the interval of control chart allowed variation (UCL–LCL) was reduced by 43% for fat, by 33.1% for protein, and by 14.3% for solids-not-fat.

Conclusions: X-11 deseasonalization of routinely collected milk parameters was found to be an effective method to improve control chart application effectiveness in farms and milk collecting centers.

Keywords: dairy chain, cow milk, seasonality, risk management, risk assessment, food safety, livestock management, One Health

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INTRODUCTION

Food security, including safety, from livestock systems is of highest importance in human nutrition and one of the multifaceted aspects of sustainability (1, 2). The breeding sector is regulated by economic, political, as well as socio-demographic drivers that, in their turns, cannot ignore sustainability issues linked to One Health pillars (environment, animal, and human health) and their interconnections.

Given the peculiarities of food production chains (usually entailing highly perishable products, low or large batches volumes, great variability in raw materials characteristics, processing, and distribution) a significant effort has been devoted to increase and guarantee the general quality of finished products using industrial management practices covering the whole production chain (3, 4). In the last decades food industry has been pushed to implement a wide range of food quality management protocols to reply the increasing consumers' expectations, especially following major food crises (4), environmental alerts, globalization of markets of food products and food producing animals, globalization of dietary habits (1, 5), and upset of toxicant related zoonoses (6, 7).

In the precision dairy farming era, food quality control techniques based on process control methods and quality improvement programs are gaining increasing attention. In fact, environmental factors (from essential nutrients to toxic contaminants and agro-zootechnical residues) at the environment-animal-human interfaces impact severely on food security and food safety (7, 8), with impact on current and next generation (8).

Close monitoring of all factors implied in food chains management allows early management of anomalous events, thus leading to a general increase in food quality and safety, general enterprise's competitiveness (demonstrable deep quality control) and profitability (including decreased risk of undesired events and subsequent food waste and losses) along with gain in environmental sustainability (9–12).

Process monitoring techniques are based in the strict monitoring of sources of variability in any production phase. Systematic investigation on the root causes of any unusual source of variability, together with variability reduction techniques are the pillars of process control methods.

In the last decade, several attempts have been done to apply Statistical Process Control (SPC) in dairy production systems and in general livestock management (13), mostly based on traditional Shewart control chart (or Cusum control chart) (14) The relevance of some studies, at least from the point of view of practical benefits, is somehow unclear [for dairy herd, see (15)].

Regardless of the specific SPC implementation, most of the process monitoring techniques aim at the separation of the overall variation in a routine variability (also known as "chance causes") and an exceptional variation (or "assignable cause") originating from a change in the process that would be worth analyzing (16). If the chance causes variability magnitude is comparable to the variability due to assignable causes, the task of extracting a meaningful alert signal indicating the need of intervention on the process can be compared to that of extracting a meaningful signal from measurements extremely corrupted by noise. An example of chance causes in dairy production can be the normal biological variation in milk composition, while assignable causes can derive from animal illness, feeding, unplanned variations in herd management, or their consequences.

In addition, dairy production parameters routinely collected both in farms and Milk Collecting Centers may be affected by seasonal variations that render impractical or ineffective the use of some of SPC techniques, like statistical control charts, which are typically based on the underlying assumptions of independence and stationarity of observations (16, 17).

A possible approach to this problem is to use or adapt seasonal adjustment methods routinely used by national statistical offices and central banks, whose work is frequently based on analysis of economic and demographic seasonal time series.

Between those techniques, an entire category of non-parametric methods has been developed starting in the 60's (18, 19) to decompose time series into unobservable components using iterative procedure based on successive filtering, such as the X-11 family of methods (X-11, X-11-ARIMA, X-12-ARIMA). The X-11 method was introduced in 1965 by the United States Census Bureau as practical tool for seasonal decomposition of time series. X-11 uses an iterative approach to estimate the components of a time series. At each step different moving averages filters are used to decompose the time series into a trend/cycle component (a long term evolution/a slow movement around the trend), a seasonal component (Intra-year variations repeating regularly year after year), and an irregular component (Random fluctuations).

The Seasonal component should represent fluctuations in the data recurring with the same pattern, intensity and timing. In certain models, a modification in the seasonal component over the years timeline can be coped for to represent long term changes which gradually evolve as a response of a global, systemic change. In the former case, a stable seasonality is present in the time series, while in the latter a moving seasonality is said to be present.

The Trend or Cycle component takes in account a steady tendency (trend of growth, or decline) over a significantly long period of time; sometimes another component, generally alternating over a period of time greater than the year, may be superimposed over the trend and is generally called Cycle component.

The Irregular component is what remains of the time series after adjustment for seasonality and trend. It should represent mainly measurement errors, calendar changes, or exceptional events which cannot be forecast and have a significant influence on the time series.

Different models have been proposed over time to model the influence of each component in the total variation represented in the time series. Basically, additive models assumes that the magnitude of the components are independent from each other; multiplicative models assume that all three components are dependent on each other; finally, pseudo additive models assume the independence of S and I, but the dependence of S and I from C (20).

Seasonality adjustment is increasingly considered as a useful tool in livestock management (21–24), under the pressure for improving general efficiency and consumer acceptance, reducing waste, and increasing trading margins.

Another growing application for seasonality adjustment is the regulatory area: seasonality adjustment is one of the adjustment techniques adopted by the Irish national Department of Agriculture, Food and the Marine (25) in their calculations over bulk tank somatic cell counts requested by EU Regulation 853/2004 (26).

The main aim of this work is to conduct an evaluation of basic X-11 seasonal decomposition technique on data routinely collected in small farms (fat content, protein content, lactose content, solids-not-fat, freezing point, somatic cell count, total bacterial count) and to test the efficacy of seasonal removal methods to improve the impact of statistical control charting.

As a case study, we provide an application example on data coming from a 3 year long measurement campaign on a small dairy farm.

MATERIALS AND METHODS

Data Collection and Management

Data collection was carried on between January, 2011 and December, 2013 within the framework of the ALERT project[1] for the monitoring of wholesomeness and quality in the cow milk chain from primary (dairy farm) to secondary production (transformation industry). During this time span, data were collected from raw milk production of a small farm (in the following, EP).

The dairy farm was representative of a well-conducted, medium-sized dairy farm of Central Italy (27). All diagnostics were carried on at the Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana (IZSLT), a public body operating in the frame of National Health Service with duties related to animal health and welfare and food safety.

EP milk production was sampled three times for month (mean inter sample day span = 9.85 days, $SD = 3.01$). A total of 110 samples were acquired and analyzed during the study period. Milk samples were refrigerated at 4 (± 2)°C and carried to the testing facilities of IZSLT. Raw milk samples were tested for fat content % (Fat), protein content % (Protein), lactose content % (Lactose), solids-not-fat % (SNF) (all % by weight), freezing point (m°C), somatic cell count (SCC, x1000 cfu/mL), total bacterial count (TBC x1000 cfu/mL).

All lab analyses were carried on the samples within an average of 3.18 days ($SD = 2.28$) from sample collection. All parameters were analyzed following accredited IZSLT testing methodologies described in **Table 1**.

All data coming from the data collection procedures were imported into a purposely designed relational database at Istituto Superiore di Sanità (ISS) facilities. All subsequent analyses were carried on extrapolating raw data from this database. Additional info on the environmental temperature for the whole sampling period was added in the database. Climate data (daily mean temperature) was gathered from the official Istituto superiore per la protezione e la ricerca ambientale (ISPRA) database (28), at the closest monitoring station (∼8 Km from the EP farm).

TABLE 1 | Sample analysis methods.

Parameter	IZSLT method/internal reference
Total bacterial count	Fluoro-opto-electronic method (POS CIP 021 INT rev 3 2010)†
Somatic cell count	Fluoro-opto-electronic method (POS CIP 018 INT rev 5 2009)§
Fat, lactose, protein content; freezing point	IR Spectrophotometry (POS CIP 018 INT rev 5 2009)§
SNF	Gravimetric analysis (Rapporti ISTISAN 1996/34, pp. 7–10, Met B)

† *Updated to rev 4 on 2013-02-01.*
§ *Updated to rev 6 on 2012-03-01 and to rev 8 on 2013-02-01.*

The presence of seasonality in data parameters was initially assessed by visual inspection in raw data.

A more detailed seasonality test was carried on converting the raw data points into a 36 point monthly series (all samples from the same month were averaged, resulting in a 36 point data series) and by execution of Friedman test and Kruskal–Wallis test on monthly averaged data.

A p-value lower than 5% was the limit set to reject the null hypothesis of no seasonal effect.

Control Chart Analysis

Parameters coming from EP raw milk production showing a marked seasonal effect (Fat, Protein, SNF) were analyzed using control chart for individuals with three alternative approaches:

Method A: Control Chart for Individuals Using Raw Data

Control chart for individuals were plotted using raw data.

Method B: Control Chart for Individuals Using X-11 Seasonally Adjusted Data

Monthly time series were adjusted for seasonality using JDemetra+, X-11 additive method (18).

The algorithm used can be described as follows:

1. derive an initial estimate of the trend-cycle TC1 by applying A symmetric moving average moving average to the raw data;
2. subtract this estimate from the original time series in order to get an initial estimate of the seasonal-irregular (SI) component;
3. apply a moving average to the SI to obtain an initial estimate of the seasonal component S1;
4. subtract the initial S1 component from the raw data to obtain an initial estimate of the seasonally adjusted series SA1 (i.e., the trend-cycle/irregular);
5. apply a Henderson moving average to obtain a second estimate of the trend-cycle TC2;

[1] www.alert2015.it

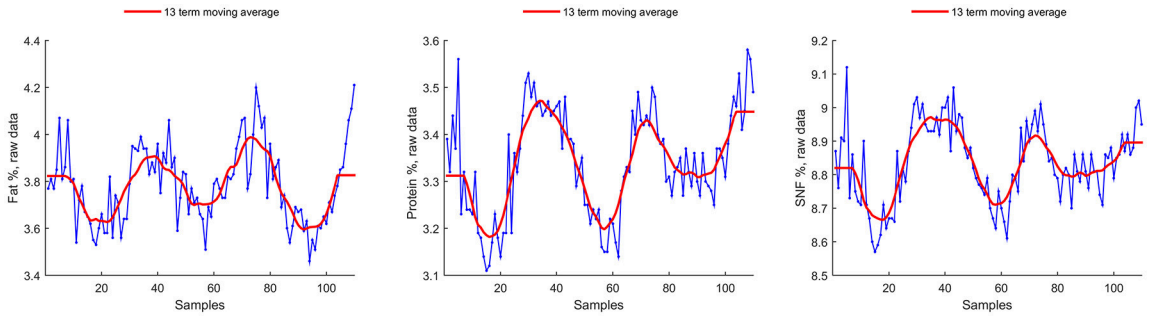

FIGURE 1 | Seasonal parameters raw data, with superimposed Witthaker–Henderson filtering (Method C).

6. subtract TC2 from the raw data to obtain a second estimate of the SI (SI2), and apply a moving average to obtain final estimates of the seasonal component (S);
7. subtract S from the raw data to obtain a final estimate of the seasonally adjusted series (SA2) and apply a Henderson moving average to obtain a final estimate of the trend-cycle TC;
8. subtract S from the SI2 to obtain an estimate of the irregular component (I).

Trading days and Easter effect were neglected. Control chart for individuals were constructed in Matlab using the seasonally adjusted time series.

Method C: Control Chart for Individuals Using Moving Average Seasonally Adjusted Data

Raw data was smoothed using moving average Whittaker–Henderson 13-term filter (29, 30) in order to get a gross approximation of seasonal component (GSC). A season-adjusted time series was derived subtracting the smoothed data from the raw data. Control chart for individuals were constructed in Matlab on the time series obtained subtracting the GSC from the raw data.

For all control charts Upper and Lower Control limits (UCL and LCL) were calculated using the following relationships

$$UCL = \mu_p + 3^*\sigma_p$$
$$LCL = \mu_p - 3^*\sigma_p$$

Where

μ_p: Estimated process mean;

σ_p: Estimated process standard deviation

Both estimated process parameters were calculated using the Matlab's control chart implementation.

Performance of the three algorithms was compared visually examining the identified trends (where available), and comparing the resulting estimated process means, standard deviations, range, Upper and Lower Control Limits, and number of process violations.

All correlational and statistical analyses were carried on in Matlab (The MathWorks, Inc., Natick, Massachusetts, USA), directly interfacing the relational database. For specific topics, data extracted from the database were analyzed using statistical package R (31), and JDemetra+[2] (rel. 2.2.0). Wherever applicable, all seasonality analysis were carried on following the European Statistical System (ESS) guidelines on seasonal adjustment (32).

RESULTS

Preliminary Analysis

As a general outlook, milk data showed both seasonally variable and seasonally stable parameters.

Visual inspection reveals a seasonal effect on fat %, protein %, and solid non-fat % (**Figure 1**). A red trend line (method C) was superimposed to facilitate the identification of the general trend.

Freezing point seasonality is unclear by visual inspection, as well for somatic cell count, and lactose (**Figure 2**). A clear outlier is present in total bacterial count.

Descriptive statistics (Mean, standard deviation *SD*, Range, Minimum and Maximum) for the original time series and for the monthly time series are reported in **Table 2**.

Statistical Analysis for Seasonality

Statistical analysis (JDemetra+) confirmed the presence of seasonality effect on fat, protein, and solid non-fat; there was no confirmed seasonality for freezing point, somatic cell count, total bacteria count, and lactose. For TBC, no evidence of seasonality was present even removing the clear outlier present. Results of this analysis are reported in **Table 3**.

Control Chart Analysis

Method A

Control chart for individuals were plotted on raw data and are shown in **Figure 3** for Fat %, Protein %, and SNF %. Main numerical results of Method A can be found, for each milk component, in **Table 4**.

Method B

Seasonal analysis on monthly time series (MTS) resulted in four time series, representing the seasonal component (S), the

[2]https://ec.europa.eu/eurostat/cros/content/software-jdemetra_en) (JDemetra is a software tool officially recommended for the seasonal and calendar adjustment of official statistics in the EU).

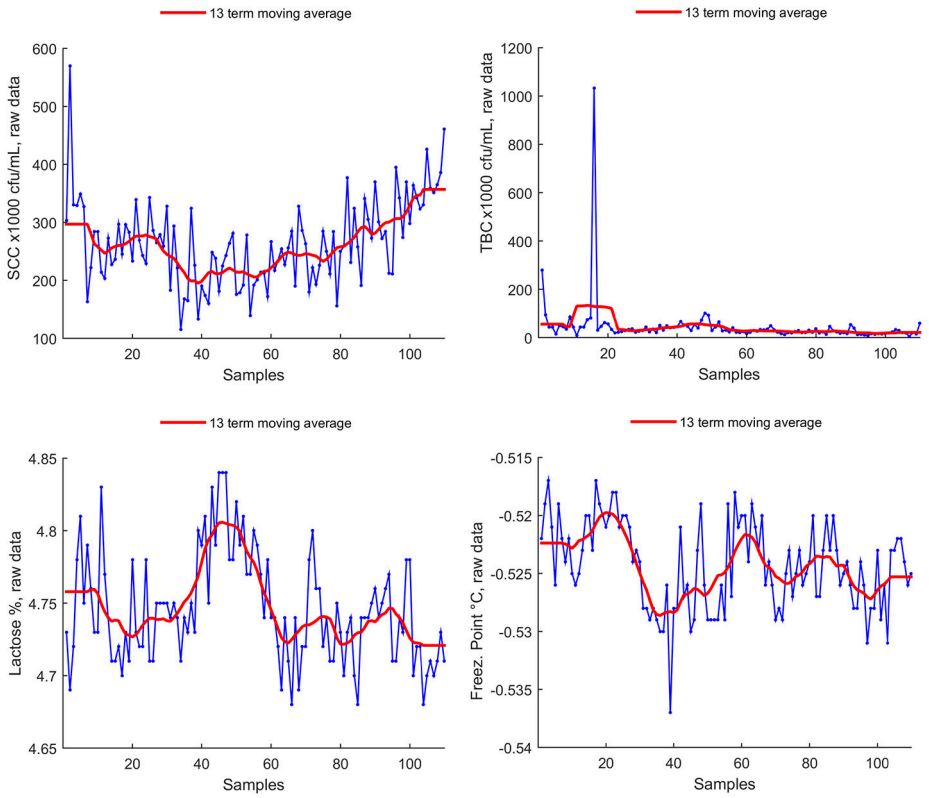

FIGURE 2 | Non-seasonal parameters raw data, with superimposed Witthaker–Henderson filtering (Method C).

irregular component (IRR), the trend (T) component, and a seasonal-adjusted component (SA). Under the additive modality of analysis, the following relationships stand true:

$$MTS = S + IRR + T \quad (1)$$

$$SA = IRR + T = MTS - S \quad (2)$$

Control chart for individuals for seasonally adjusted series (SA) are shown in **Figure 4** for Fat %, Protein %, and SNF %.

The JDemetra+ package outputs an overall measure of quality of decomposition called Q statistic, whose value is considered satisfactory if less than unity. Q statistics for Fat %, Protein %, and SNF % were, respectively, 0.55, 0.42, and 0.65.

Another indirect evaluation of the quality of decomposition is the negative correlation between Fat, Protein, and SNF seasonal components (S) and daily mean temperature, as shown in **Figure 5**.

Pearson correlation coefficients and their associated *p*-levels are reported in **Table 5**.

An estimation of the relative (%) contribution of the seasonal component (S) to the overall time series MTS (Equation 1) is given in **Figure 6** for fat, protein, and SNF.

As shown in the figure, the seasonal component for Fat % is in average about 2.81% of the MTS series. For Protein % and SNF % the seasonal component accounts respectively for 2.79 and 0.81% of the MTS.

TABLE 2 | Descriptive statistics of original data.

		N	Mean	*SD*	Range	Min	Max
Original time series	Fat %	110	3.7831	0.1659	0.7500	3.4600	4.2100
	Protein %	110	3.3406	0.1142	0.4700	3.1100	3.5800
	SNF %	110	8.8336	0.1176	0.5500	8.5700	9.1200
Monthly time series	Fat %	36	3.7868	0.1478	0.5892	3.5375	4.1267
	Protein %	36	3.3389	0.1073	0.4000	3.1433	3.5433
	SNF %	36	8.8312	0.1057	0.3787	8.6133	8.9920

Main numerical results of Method B seasonal adjustment can be found, for each milk component, in **Table 4**:

- For Fat component, Method B seasonal adjustment led to a reduction of control chart violations from 5 to 1 (**Figures 4**, **8**, rectangles). The data Range [max(Fat)–min(Fat)] was reduced from 0.75 to 0.33, and the interval of control chart allowed variation (UCL–LCL) was almost halved (from 0.59 to 0.34) with respect from raw data control chart (Method A), corresponding to a 42.3% reduction.
- For Protein component, Method B seasonal adjustment led to a reduction of control chart violations from 17 to 1. The data Range [max(Protein)–min(Protein)] was reduced from 0.47 to 0.22, and the interval of control chart allowed variation (UCL–LCL) was reduced from 0.32 to 0.22) with respect from

TABLE 3 | Statistical seasonality assessment in raw data.

	Friedman test		Kruskall–Wallis test	
	F	*p*	*H*	*p*
Protein	29.8718	**0.0017**	31.7087	**0.0008**
Fat	29.0000	**0.0023**	30.3273	**0.0014**
Lactose	16.9487	0.1094	18.0150	0.0812
SNF	29.4615	**0.0019**	30.5195	**0.0013**
Freez. Point	14.6923	0.1970	16.2192	0.1332
SCC	9.5128	0.5747	10.3333	0.5007
TBC	19.6667	0.0501	18.4294	0.0721

In bold, the significant values ($p < 0.05$).

raw data control chart (Method A), corresponding to a 31.3% reduction.

- For SNF component, Method B seasonal adjustment led to the absence of control chart violations (from 9 to 0). The data Range [max(SNF)–min(SNF)] was reduced from 0.45 to 0.25, and the interval of control chart allowed variation (UCL–LCL) was reduced from 0.39 to 0.34 with respect from raw data control chart (Method A), corresponding to a 12.8% reduction.

Method C

In this method, a smoothed time series is subtracted from raw data, in order to get an estimate of the variability of the time series not due to seasonal variation.

Smoothing is achieved using a 13 term Henderson filter, which is a symmetric moving average type filter designed to let annual trends to pass unchanged through the filter. The smoothed time series, resulting from the filter action is shown in red in **Figures 1**, **2**.

Control chart for individuals on the resulting time series data are shown in **Figure 7** for Fat %, Protein %, and SNF %. Main numerical results of Method C can be found, for each milk component, in **Table 4**.

Final Results and Comparison of Methods

For each of the three methods, estimated process mean and standard deviation, range and Upper and Lower Control Limits for the resulting time series are given in **Table 4**, together with the number of data points exceeding lower or upper control limits (violations).

Finally, a data plot showing Fat, Protein, and SNF raw data control chart highlighting UCL and LCL violations detected by the three methods is shown in **Figure 8**.

DISCUSSION

The current study focused on the application of control charts, a statistical process control technique, to seasonally influenced bovine milk parameter routinely collected from raw milk production of a small farm. While the application of control charts in herd management has been already advocated (13–15), literature on successful applications of control charts to monitor and manage trends in animal production systems is still relatively scarce, and results clearly demonstrating practical benefits are still lacking (13). As pointed out by other authors (14), autocorrelation of time series resulting from seasonality of the observed parameters complicates the application of control charts in biologically derived time series. Another relevant obstacle for the application of any statistical process control technique is the presence of missing data, either derived by technical glitches or by loose management techniques. In this study, which encompassed three complete years, careful planning of data collection procedures led to a complete dataset of 110 measurements without any missing data. Seasonality presence, usually investigated through linear ANOVA models (33), was assessed in method B using Friedman's test on monthly averaged data, which, being non parametric, does not need normality assumption, a condition than can be unmet in practice, and which is usually addressed through logarithmic transformations (23). This study's choice, while being relatively irrelevant for the implementation of control chart techniques—which are considered to be robust to deviation from normality (16)—may however represent in advantage in assessing the presence (or absence) of seasonality on collected milk parameters. In our study, seasonality was found in fat, protein, and SNF components of raw bovine milk, thus corroborating previous studies. Regarding fat and protein components, in fact, there is a general accordance on the presence of seasonality (34–38). For SNF, our study assessed seasonality not confirmed by other authors (33, 34), even though both cited papers reported statistically significant increase of SNF component in early autumn.

We could not assess statistically significant seasonality for lactose, somatic cell count, total bacterial count and freezing point parameters. While we did not found sufficient literature on freezing point seasonality, lactose content seasonality is still somehow debated [see (33, 35) for presence of seasonality, and (34, 36, 37) for unclear presence or absence of seasonal effects on lactose content], as well as total bacterial count seasonality [(23, 35, 38) for presence—(33), for absence]. We found no seasonal effect on somatic cell count, despite prevalent literature consistently reports on seasonality [(33, 34, 36–38), an exception being (35)]. Our data (**Figure 2**) show both a trend and several spikes, but—quite unexpected—no evidence of cyclic patterns. Investigation on this aspect is still ongoing.

The application of the X-11 algorithm (Method B) asked for monthly averaging of collected data, which can be a drawback because of the inherent loss of information deriving from the averaging process. This choice could be a limitation, since the amount of raw data collected for the study was considerably bigger than in previous studies (33, 34, 36). However, we could demonstrate a relevant reduction of the number of control chart limits violation on seasonally adjusted data, in comparison with the application of the same technique on raw data (Method A); this reduction was achieved, given the additive model used, by subtracting from the original data a seasonal component which accounts for (**Figure 6**) only a few percent of the raw time series.

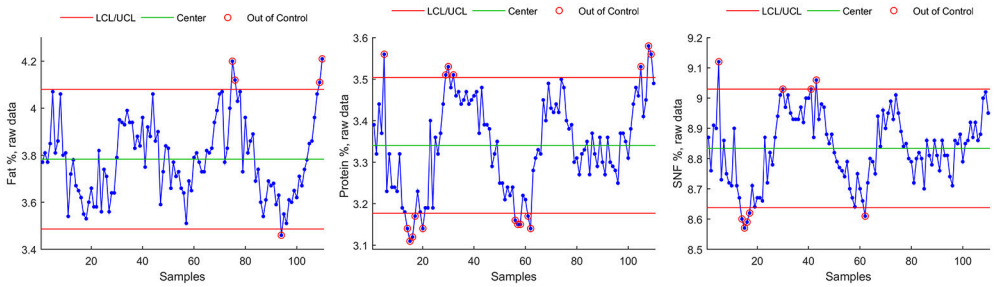

FIGURE 3 | Control Chart for individuals for raw data (Fat, Protein, SNF).

TABLE 4 | Control Chart estimated process mean μ_p and standard deviation σ_p, range, Upper and Lower Control Limits and number (#) of process violations for the three methods.

		μ_p	σ_p	Range [Min Max]	UCL	LCL	# Violations
Method A	Fat %	3.783	0.099	0.75 [3.46 4.21]	4.08	3.49	5
	Protein %	3.341	0.055	0.47 [3.11 3.58]	3.50	3.18	17
	SNF %	8.834	0.065	0.55 [8.57 9.12]	9.03	8.64	9
Method B	Fat %	3.787	0.056	0.33 [3.67 4.00]	3.96	3.62	1
	Protein %	3.339	0.036	0.22 [3.22 3.44]	3.45	3.23	1
	SNF %	8.831	0.056	0.25 [8.70 8.95]	9.00	8.66	0
Method C	Fat %	0.008	0.095	0.61 [−0.22 0.38]	0.29	−0.28	1
	Protein %	0.005	0.050	0.36 [−0.12 0.25]	0.15	−0.15	1
	SNF %	0.004	0.059	0.42 [−0.12 0.30]	0.18	−0.17	1

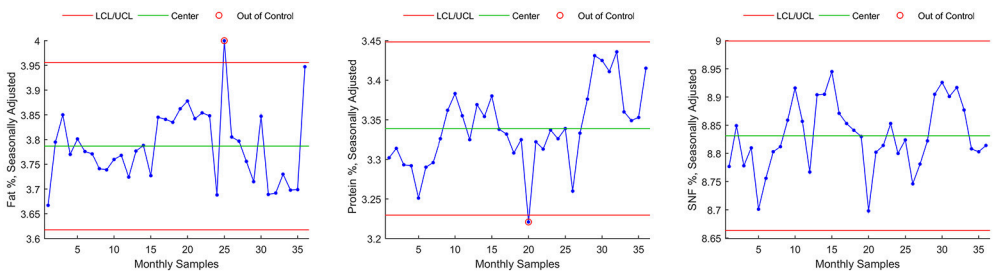

FIGURE 4 | Control chart for individuals for seasonally adjusted series.

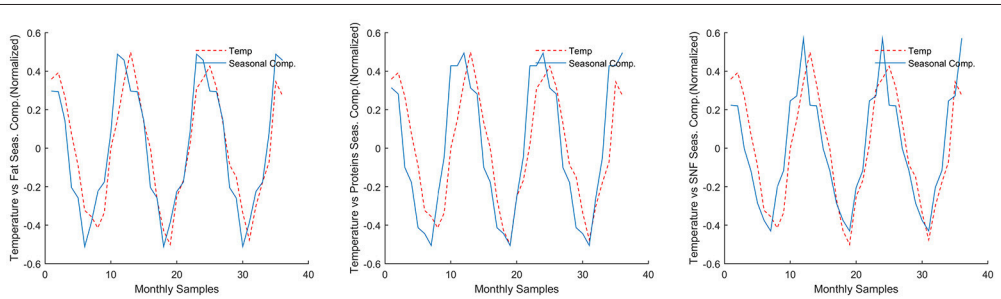

FIGURE 5 | Correlation of seasonal components with daily mean temperature. Both data mean-normalized, temperature y axis is inverted.

TABLE 5 | Pearson correlation coefficients between daily mean temperature and Fat, Protein, and SNF seasonal components.

Daily mean temperature vs:	*r*	[95% CI]
Fat %	−0.90**	[−0.82 −0.95]
Protein %	−0.81**	[−0.65 −0.89]
SNF %	−0.85**	[−0.73 −0.92]

$^{**}p < 0.001$.

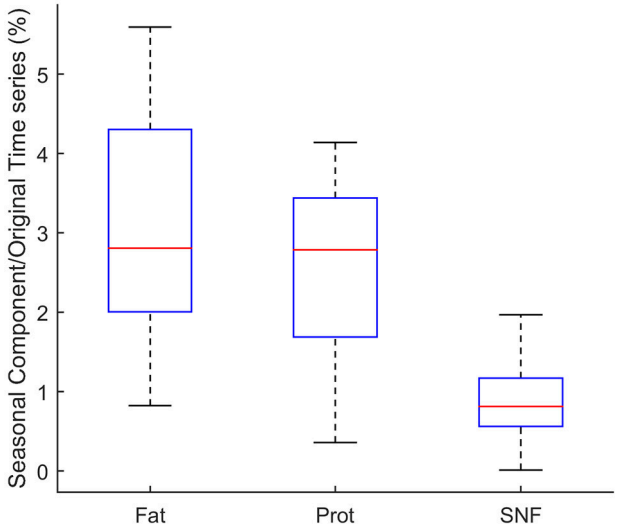

FIGURE 6 | Relative (%) contribution of the seasonal components (S) to the overall time series.

This observation summarizes that the total effect of both the averaging process and the deseasoning method on the amount of information present in the raw time series could be considered somehow limited.

It must be noted that the reduction in number of violations of control chart limits has been achieved despite a marked reduction (bigger for fat component, smaller for SNF component) of the interval of allowed variation (ULC–LCL). As a consequence, seasonally adjusted control charts could be more suitable than raw data control charts in revealing sudden deviations of in bulk milk components.

The proposed seasonal adjustment process (Method B) in statistical control charting could be of interest for additional reasons, besides the removal of parameter's seasonality. Following a general decomposition model, X-11 method decomposes the observed time series in three fundamental components, namely Seasonal (S), Trend or Cycle (T or C), and Irregular (I). The Seasonal component should isolate the periodic pattern, while the Trend component should contain linear or nonlinear long-term trends, and cycles with periodicity greater than the Seasonal period. The irregular component is usually defined as the cumulative component of all unpredictable effects and sampling errors. This decomposition could be of interest in dairy production systems. As an example, the isolated seasonal component (S) in both Fat, Protein, and SNF time series showed a strong correlation with daily mean temperature, thus corroborating previous works (33–37). Trend and Cycle components could be subject to further analysis, in order to investigate correlations with herd management techniques, or general animal's health status.

In this study, a reduction in control limit violations is obtained also through Method C. This method has a simple implementation but it showed to be ineffective in detecting parameter's shifts that are easily detected by both methods A and B (**Figure 8**, rectangular areas). Method C also showed sensitivity to outliers and time series extremes. This latter aspect is due to the symmetry of the applied Henderson filter whose performances degrades, by construction, at the beginning and at the end of the time series.

Interpretation and Relevance of Study Findings

In the social and economic contexts, seasonal adjustment is often used to remove the seasonal component from time series, mostly because it can be a confounding factor for movements in other components of greater economic significance (20). Similarly, the Irregular component is seen mostly as background noise, deriving from sampling errors or unpredictable events.

A major distinguishing factor in the application of seasonal adjustment in farming industry is that all seasonal, trend, cycle, and irregular components may be of interest.

In the food/farming industry, evidence suggests that a slightly different interpretation of the relevance of the three components should be adopted. While it is certainly true that the removal of the seasonal component may reveal hidden trends, it should be noted that this component is a manifestation of a biologically and physiologically relevant process. For this reason the seasonal component may itself contain valuable information on animal health, and any intervention leading to its modification could be of economical relevance.

The irregular component, which represents both the background noise of the process but also the effects of sudden changes in biological processes, may be of extreme interest in all those contexts where strict temporal monitoring of dynamically evolving parameters may be a driver of quick corrective intervention on animal's health and wellbeing, covering nutrition and herd management in general.

As an additional remark, some relevant topics in classical applications of seasonality adjustment may not be useful in milk production systems: for instance, trading days and holidays effects, which are usually taken into account in a socioeconomic analysis, may be of little relevance, since milk production process is primarily influenced by herd physiology and natural effects. Trading days, holidays/Easter effects could arise only indirectly from animal management (feeding, milking). However, in the farm involved in the study, all animal management activities

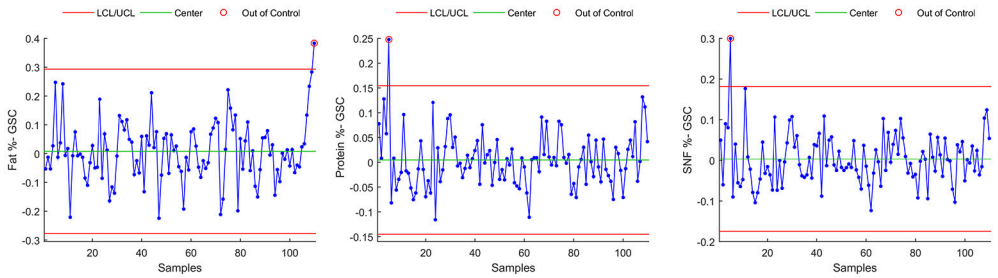

FIGURE 7 | Control chart for individuals on the H13 smoothed time series.

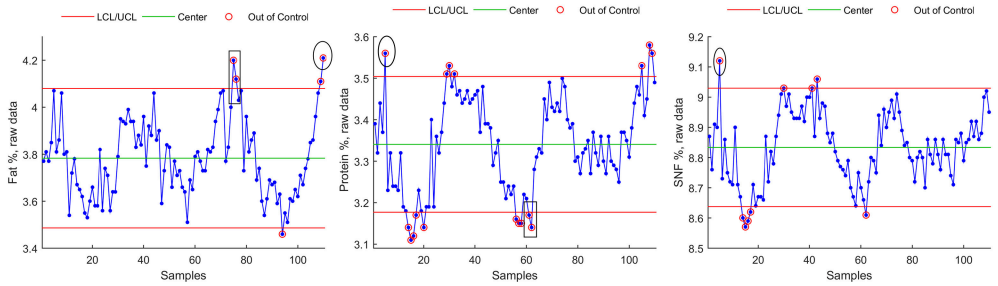

FIGURE 8 | Raw data control charts with Method A (small circles), Meth B (rectangles) and C (ellipses) violations.

are carried on in the same way every day, 365 days per year.

The study confirmed the correlation between Fat %, Protein %, SNF %, and environmental temperature. While this finding does not offer, in line of principle, new insight on seasonally sensitive parameters in respect to what can be found on available literature, the correlation strength may suggest that the seasonal component could be used as monitoring parameter in dairy herd management. Seasonally biologically sensitive processes, in fact, are influenced by herd management (e.g., feeding) that can have an impact on the seasonal components of the time series and, indirectly, on the nutritional composition of raw milk. Reshaping seasonality by feeding and other good practices (39), however, need further confirmation and deserves further applied research.

The present work, in terms of statistical control charts, is a phase I study, where historical data are used to construct control limits; these limits are being applied in an ongoing phase II study.

AUTHOR CONTRIBUTIONS

FM and CF conceived and planned the research, planned and performed data acquisition and analysis. FM, CG, and CF contributed to the interpretation of the results. FM and CG took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript drafting and revising critically the work and approved the final version to be published.

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frontiers in Public Health

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Portable Bio/Chemosensoristic Devices: Innovative Systems for Environmental Health and Food Safety Diagnostics

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This mini-review covers the newly developed biosensoristic and chemosensoristic devices described in recent literature for detection of contaminants in both environmental and food real matrices. Current needs in environmental and food surveillance of contaminants require new simplified, sensitive systems, which are portable and allow for rapid and on-site monitoring and diagnostics. Here, we focus on optical and electrochemical bio/chemosensoristic devices as promising tools with interesting analytical features that can be potentially exploited for innovative on-site and real-time applications for diagnostics and monitoring of environmental and food matrices (e.g., agricultural waters and milk). In near future, suitably developed and implemented bio/chemosensoristic devices will be a new and modern technological solution for the identification of new quality and safety marker indexes as well as for a more proper and complete characterization of abovementioned environmental and food matrices. Integrated bio/chemosensoristic devices can also allow an "holistic approach" that may prove to be more suitable for diagnostics of environmental and food real matrices, where the copresence of more bioactive substances is frequent. Therefore, this approach can be focused on the determination of net effect (mixture effect) of bioactive substances present in real matrices.

Keywords: agro-food supply chain, milk, on-site diagnostics, electroanalytical methods, biosensoristic devices, surface plasmon resonance, lab-on-a-chip

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ENVIRONMENTAL HEALTH AND FOOD SAFETY: SCENARIO AND NEEDS

Over the last few years, the abiotic contaminants levels in the environmental compartments and food increased to the point where they can cause potential human health effects due to exposure to chemical toxic substances. In particular, the interactions between environment and food supply chain that mainly occur at primary production level (including harvesting, milking and farmed animal production prior to slaughter, hunting and fishing, and harvesting of wild products) can cause serious both short- and long-term detrimental effects on human health.

Environmental and food safety remains a major global challenge, in particular in developing countries, where socioeconomic status predisposes a large share of the population to a direct environmental-origin contamination and/or consumption of contaminated food products.

To minimize the negative and dramatic impacts (especially in developing countries) of chemical toxic substances of anthropic origin on environmental and human health, several focused actions are needed. For instance, promoting a sustainable use of chemicals and agrochemicals (e.g., pesticides, veterinary antibiotics, and food additives); the development of toxicovigilance practices and systems (1) and more effective primary prevention strategies (raising users' awareness and promoting the use of good practice codes); moreover, implementation of legislative regulations; and the development of proper infrastructures and effective protocols for safely recycle and dispose of hazardous wastes are necessary.

Against this background, such critical issues have produced a great demand for simplified, sensitive, and rapid screening methods (2, 3), without (or with reduced) sample pretreatments, suitable for environmental monitoring and surveillance at critical control points throughout the entire agro-food supply chain. In fact, recent progress and challenges in the field of analytical chemistry are focused on improving analytical methods with reduced environmental impact and developing new analytical sensoristic devices for continuous monitoring and diagnostics oriented toward environmental health and food safety.

On-site, cost-effective sensoristic devices capable of routine, sensitive, and selective detection of a range of targeted contaminants present in the environment and foods can be employed, for instance, to overcome time limitations and to reduce costs of sample collection and transport to laboratories, thus providing benefits for a rapid diagnostics and early corrective actions.

EMERGING ROLE OF PORTABLE BIO/CHEMOSENSORISTIC DEVICES IN ENVIRONMENT AND AGRO-FOOD SUPPLY CHAIN MONITORING

For on-site diagnostics and environmental/food monitoring purposes, the application of standard and traditional analytical techniques (very sensitive and selective techniques, but costly, time consuming and requiring trained personnel with technical skills to perform the analysis) is in contrast with the current need of rapid, cheap, easy-to-use, and portable devices (4).

For these purposes, chemosensoristic and biosensoristic devices (herein collectively referred to as bio/chemosensoristic devices) are promising tools with interesting analytical features, which can be potentially exploited for on-site real-time applications, diagnostics, and screening for both environmental and food matrices. Such devices could be employed, e.g., to overcome existing limitations in measurements currently used in environmental and agro-food fields. While those measurements are mainly focused on the independent analyses of various parameters and analytes, complexity of environmental and food matrices requires a new holistic-like approach (4).

For instance, regarding nutritional and toxicology characterization of foods, a broader modern vision based on the concept of "whole food" is taking off (4, 5). Environmental and food matrices are complex mixtures of bioactive molecules, whose complex interactions between individual components could eventually produce different and hardly theoretically predictable "net effects." "Net effect" is necessarily different from single effects of each individual substance, and it could be additive (when it is equal to the sum of contributions of individual substances), synergistic (when it is greater than the sum of contributions of the individual substances), and antagonistic (when it is less than the sum of contributions of individual substances).

For a more proper and complete characterization of food matrices (and environmental ones) "as a whole," integrated analyses of physical, chemical, and biological parameters through sensoristic devices could be more suitable. The multichannel platform BEST (6) is a HACCP-like monitoring system that follows this approach for the generation of integrated analytical information. More specifically, BEST focuses on identification, control, simultaneous, and non-stop monitoring of anomalous variations throughout agro-zootechnical productions, developed to allow simultaneous collection and analysis of multiple signals. Such signals are produced from a battery of selected analogical and/or digital bio/chemosensoristic devices (or probes), integrated with each other and functioning simultaneously. The simultaneous acquisition of multiparameters and integrated information can be useful in determining correlations and relationships among different data (through multivariate data analysis), and it can constitute a flexible grid of indexes and multiple markers in series. Such integrated analytical approach helps to define a "fingerprint" and to identify new marker indexes of food matrices. A field validation of BEST prototype is taking place in a farm in the Lazio region (Italy) within project ALERT (7). This project, funded by the Italian Ministry of Economic Development under the Call Industria 2015 New technologies for Made in Italy (www.alert2015.it), aims at developing the BEST prototype for industrial-scale production. Another new interesting approach for innovative monitoring and diagnostics of the environment and the agro-food supply chain is provided by a recent patented physicochemical sensing device called SNOOP (8). SNOOP is a multiparameter and multisignal sensoristic device that uses advanced and appropriately designed sensitive materials. Such sensitive materials can be both biological materials (e.g., whole cells, enzymes, and aptamers) and chemical materials (newly synthesized and/or functionalized inorganic and organic materials), whose one of the main features is the specific interaction with the target analyte(s) present in real and complex matrices. Such interactions can produce specific or aspecific physicochemical (electric or optic) responses, and the simultaneous use of different sensitive materials and the combination/integration of outgoing signals can significantly increase the screening ability of SNOOP.

Electrochemical Bio/Chemosensoristic Devices

The field of electrochemical and optical bio/chemosensoristic devices has grown rapidly in the past few years. Thanks to advantages provided by intrinsic analytical features and the development of new advanced sensitive materials, the employment of these devices has proved to be very useful for chemical contaminants detection in environmental and food matrices (Tables S1 and S2 in Supplementary Material). In particular,

biosensors or biosensoristic devices (*an integrated receptor–transducer device, which is capable of providing selective quantitative or semiquantitative analytical information using a biological recognition element*) (9) hold promise to be relatively cheap and portable devices for *in situ* detection of environmental and food contaminants (10, 11).

A recent review on key research interests in the development of biosensors in South Africa has highlight a particular interest on the development of electrochemical (amperometric, impedimetric, potentiometric, and voltammetric) biosensor due to low fabrication and analytical equipment costs, in particular, for pesticides and heavy metals detection. Other research areas include nanotechnology, identification and validation of biomarkers, and development of biorecognition agents (antibodies and aptamers) and new biosensor design approaches (e.g., development of new materials) (12).

In recent literature, enzymes (13–15) and whole cells (16) seem to have been replaced with antibodies (17–23) and aptamers (24–29) as recognition elements in electrochemical and optical biosensoristic devices.

Regarding electrochemical devices, they possess unique features to address the challenges of field and on-site analytical chemistry: possibility of miniaturization and portability, sensitivity, selectivity, a wide linear range of detection, minimal power requirement, and cost-effective instrumentation. Voltammetry is one of the most widely used electroanalytical techniques for electrochemical detection in bio/chemosensoric devices (see Tables S1 and S2 in Supplementary Material). In fact, various voltammetric techniques possess intrinsic analytical advantages and features and included excellent sensitivity, rapid analysis times, and possibility of simultaneous determination of different analytes. In voltammetric pulse techniques, through different modulation of the applied potential, a higher speed of measurement and sensitivity (useful for determination of species at trace levels) can be achieved. In particular, differential pulse voltammetry and square-wave voltammetry have been extensively described in the recent literature for detection of various chemical contaminants in environmental samples (24, 25, 30–35). Other widely used electrochemical techniques includes cyclic voltammetry (for studies on redox behavior of analytes) (31, 36, 37) and stripping techniques (characterized by preconcentration step of the analyte onto or into the working electrode to achieve a greater sensitivity) (38–42). In addition, the latter are commonly applied for determination of metal speciation (chemical form can influence bioavailability of metals) useful for environmental risk assessment of metal pollution (43). Amperometry is another widely used electrochemical technique in bio/chemosensoristics (13–17, 44–51). Together with voltammetric techniques, electrochemical impedance spectroscopy is an extremely useful technique for a broad range of applications, including characterization of materials and detecting interaction between recognition elements (e.g., antibodies and aptamers) of sensoristic devices and analyte, through measures of changes in electrical surface properties of electrodes (26, 28). To improve analytical features and performances of electrochemical techniques, the last decades have witnessed a tremendous development of innovative sensitive materials for surface functionalization of electrodes. Several advances in the development of bio/chemosensors (in particular for electrochemical devices) have been achieved through the employment of (modified) electrodes (Tables S1 and S2 in Supplementary Material). Traditional mercury-based electrodes (39, 41) have gradually been replaced (because of low mechanical stability and toxicity of mercury) by other electrodes made of better suitable materials. As replacement of mercury, alternative materials (with similar or better analytical features) have been employed and/or developed: bismuth (a non-toxic element with high hydrogen overpotential and good mechanical stability) (52), boron-doped diamond (with a wider electrochemical potential window and reduced fouling compared to traditional materials) (34), nitrogen-doped graphene (doping converts an excellent conductor as graphene into a p- or n-type semiconductor) (49), and single and multiwalled carbon nanotubes and nanoparticles. Looking at recent literature, a considerable attention has been paid to the development and exploitation of nanostructured materials (nanoparticles, nanowires, or nanotubes) for sensoristic purposes: carbon-based (e.g., single-walled and multiwalled carbon nanotubes) (17, 31, 35, 42, 45, 50) and nanoparticles with different chemical composition (13, 24, 26–30, 32, 40, 50, 51). These nanomaterials (also functionalized) can modify surface architectures and functions of electrodes by, for instance, (i) enlarging active surface (e.g., increasing of docking sites for biological recognition elements) and (ii) enhancing electron transfer or electrical properties and amplify signals in general. Another interesting supramolecular-based approach to develop innovative materials for electrode modification is the synthesis of molecular and ion imprinted polymers. These are synthetic polymers able to mimicking biological recognition elements, like antibodies and aptamers, useful for the design of high-specificity sensoristic devices (30, 35, 38, 50, 53). Basically, these polymers are obtained from a copolymerization process of suitable monomers in the presence of a molecular or ionic template (the target analyte); the successive removal of the template leaves in the polymer structure binding sites that can re-host the analyte. Although they bring several advantages in terms of durability and cost-effective production (compared to aptamers and antibodies), it is still necessary to solve some problems related to heterogeneity of binding sites that can bring to non-specific bindings.

Optical Biosensoristic Devices and Lab-on-a-Chip (LOC)

The recent interest in optical biosensoristic devices for food analysis, with fluorescent, bioluminescent or chemiluminescent labels for detection, as well as the direct (label-free) detection (i.e., no reporter elements to generate a signal are needed) (54, 55), is increasing. The development of label-free technologies and in particular label-free surface plasmon resonance (SPR) has become the greatest example of employment of the technology as a routine analytical method in such fields (56).

Actually, biosensoristic devices based on SPR are ideal platforms for the label-free detection of molecular monolayers as they allow for qualitative and quantitative multiplexing measurements of biomolecular interactions in real-time without requiring a labeling procedure in the framework of food safety (57, 58).

Indeed, by using SPR-based immunosensors, one can obtain robust and quantitative results with narrow- or broad-spectrum specificity in relatively short time. In the case of milk, SPR circumvents the issues related to turbidity and protein fouling (both are generally limiting factors for optical-based biosensoristic devices application for milk testing) by measuring the refractive index modulation on the reverse side of the metal film where the biological selective element is immobilized (18, 59).

Since late 90s, SPR biosensoristic devices have become the main tool for the study of biomolecular interactions in life science, with successful applications in the field of food safety (58).

Although there are great advantages of the SPR technology, some disadvantages are evident: high cost of the readout instrumentation and a still high cost of the consumables (sensing chip and reagents) and large instrumentation footprint.

In recent years, nanoplasmonics (e.g., noble metal nanoparticles, nanometallic gratings, or a combination of metallic nanocavities organized in nanogratings) has shown a great potential in overcoming the technological/commercial limits of SPR (60) and for developing nanoplasmonic detection platforms. Even though important technological effort is still to be done to be competitive with point-of-care screening technologies, the integration on the same disposable and miniaturized platform of low-cost photonics devices with multiplexing nanoplasmonic and advanced microfluidics system can be considered as new and non-disruptive technology able to ensure competitiveness from both the economic and the detection point of views.

The development of advanced photonic biosensoristic devices has to be brought beyond the state of the art of the point-of-care-diagnostic systems by the synergetic integration of the different technological building blocks with consequent improvement of the single-component outputs. Moreover, the introduction of outperforming light-excitation/detection scheme allows for unraveling the potentiality of the sensor in terms for disposability, reliability, miniaturization, and multiplexing while providing laboratory quality analysis (61).

Within the current point-of-care diagnostic market, there is a limited number of systems that operate without the requirement for a dedicated desktop reader, and there are no quantitative, portable diagnostic platforms with multiple detection methods. The components from existing laboratory equipment are too bulky, fragile, and expensive and require too much mechanical integration to be consolidated into a point-of-care device (55).

Miniaturization (from microelectrodes/nanosensors to microfluidic platforms) is an increasing trend as a response to these needs to develop new miniature and portable analytical devices for environmental and food monitoring and diagnostics.

In this scenario, LOC devices have shown themselves to be highly effective for laboratory-based research, where their superior analytical performance has established them as efficient tools for complex tasks and a promising tools for a number of environmental monitoring applications, i.e., continuous surveillance of selected parameters and contaminant concentrations (62) and for agricultural and food safety (63). Referring to the state of the art in the recently developed LOC methods (64), it can be observed that they are based on nucleic acid amplification, biosensoristic devices, flow cytometry, spectrometry techniques, and multisensors systems.

However, to date, they have not been well suited to point-of-care or in-the-field applications: although the chips themselves are cheap and small, they must generally be used in conjunction with bulky optical detectors, which are needed to identify or quantify the analytes or reagents present. Furthermore, most existing detectors are limited to analysis of a single analyte at a predetermined location on the chip. The lack of an integrated, multiplexing, and fast detection scheme (one which is miniaturized, integrated, and able to monitor multiple locations on the chip) is a major obstacle to the deployment of diagnostic devices in the field. This issue has prevented the development of more complex tests where rapid, kinetic, or multipoint analysis is required.

CONCLUSION

Development of improved electrochemical and optical bio/chemosensoristic devices represents a technological challenge to broaden boundaries of field diagnostics and monitoring environmental and food samples. In particular, specific improved features of integration, portability (e.g., sensors equipped with built-in reading systems), cheapness, simplification of experimental protocols (less time- and labor-demanding protocols), and development of efficient high-throughput approaches are required. Concerning LOC devices, fast detection scheme and the ability to monitor at multiple locations on the chip could ensure a high selectivity and sensitivity for the analyte of interest. All these devices could be employed for the identification of new quality and safety marker indexes in real matrices as well as for the determination of mixture effects of bioactive substances.

AUTHOR CONTRIBUTIONS

All authors have made equal contributions in the writing and revising of this mini-review.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fpubh.2017.00080/full#supplementary-material.

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Engaging One Health for Non-Communicable Diseases in Africa: Perspective for Mycotoxins

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The role of mycotoxins—e.g., aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins, and ergot alkaloids—has been recognized in the etiology of a number of diseases. In many African countries, the public health impact of chronic (indoor) and/or repeated (dietary) mycotoxin exposure is largely ignored hitherto, with impact on human health, food security, and export of African agricultural food products. Notwithstanding, African scientific research reached milestones that, when linked to findings gained by the international scientific community, make the design and implementation of science-driven governance schemes feasible. Starting from Nigeria as leading African Country, this article (i) overviews available data on mycotoxins exposure in Africa; (ii) discusses new food safety issues, such as the environment–feed–food chain and toxic exposures of food producing animals in risk assessment and management; (iii) identifies milestones for mycotoxins risk management already reached in West Africa; and (iv) points out preliminary operationalization aspects for shielding communities from direct (on health) and indirect (on trade, economies, and livelihoods) effects of mycotoxins. An African science-driven engaging of scientific knowledge by development actors is expected therefore. In particular, One health/One prevention is suggested, as it proved to be a strategic and sustainable development framework.

Keywords: food safety, food security, immune system, risk assessment, risk management

MYCOTOXINS EXPOSURE AND ONE HEALTH (OH)

The burden of non-communicable diseases (NCDs) increasingly falls on the low- and middle-income countries and highlights the need for prevention of NCDs to be a part of development initiatives to reduce poverty and associated social and health inequalities. NCDs and novel (toxicant-related) zoonoses are linked with new issues in food safety, such as the environment–feed–food chain and toxic exposures of food producing animals (1). OH is the *joint effort of different discipline and sectors working at national, regional, and global level, to achieve the best possible health for communities, animals and the environment* (2). The OH concept acknowledges the web of links and interrelations that exist between human, animal, and environmental health. Broad institutional changes, implying transdisciplinary, multidimension, multisector, and multiactors

approaches, and including transboundary harmonization and involvement of health and non-health sectors, are required for OH to become a widespread approach to health policy (3), both at local and global levels (4).

The role of mycotoxins has been recognized in the etiology of a number of NCDs. Mycotoxins are toxic secondary metabolites of fungal origin (e.g., *Aspergillus*, *Penicillium*, and *Fusarium* genera) and contaminate agricultural products and feeds before or under postharvest. Despite differences in contamination levels, exposure to mycotoxins is apparent globally: calculations show that approximately 24–50% of all the commodities produced globally, especially basic foodstuffs, can be contaminated by mycotoxins (5–7). In economically developed countries where food safety regulations are in place and climate is temperate [e.g., European Union (EU)], mycotoxins are a problem deserving continuous monitoring, control, and efforts to improve management. In many African countries, the public health impact of mycotoxins exposure is largely ignored even in face of rising incidence of liver cancer (8), esophageal cancer (9, 10), neural tube disorders (11), stunted growth (12–14), and other outcomes associated with mycotoxins (15). Moreover, mycotoxins in feeds and derivatives reduce livestock and crop production and influence or even impede export for safety reasons (6). In zootechny, economic losses due to animal consumption of mycotoxin [e.g., aflatoxins (AFs)] contaminated feeds are associated with reduced feed intake, feed refusal, poor feed conversion, diminished body weight gain, increased disease incidence (due to immune suppression), and reduced reproductive capacities (7, 16). Examples of mycotoxins of greatest public health and agro-economic significance include AFs, ochratoxins (OTs), trichothecenes (TCTs), zearalenone (ZEN), fumonisins (Fs), tremorgenic toxins, and ergot alkaloids (17–19). Differences in regulations exist between countries. In the case of AF, for instance, the EU sets limits for AFB1 and for total AFs (B1, B2, G1, and G2) in nuts, dried fruits, cereals, and spices. Limits vary according to the commodity, but range from 2 to 12 ng/g for B1 and from 4 to 15 ng/g for total AFs. There is also a limit of 0.050 ng/g for AFM1 in milk and milk products. Limits of 0.10 ng/g for B1 and 0.025 ng/g for AFM1 have been set for infant foods (20). US food safety regulations include a limit of 20 ng/g for total AFs (B1, B2, G1, and G2) in all foods except milk and a limit of 0.5 ng/g for AFM1 in milk. Australia and Canada set limits of 15 ng/g for total AFs (B1, B2, G1, and G2) in nuts, the same as the international limit recommended for raw peanuts by the Codex Alimentarius Commission (CAC).

Mycotoxins are substances with low persistence in the sense that they do not bioaccumulate. Some mycotoxins (e.g. aflatoxin, ochratoxin) are found as parent compound or their metabolites in milk and eggs. However, the main contribution comes from vegetable foods.

AFB1 contamination of feeds is a risk for the health of several farm animals, including fishes; milk is the only food of animal origin where a significant feed–food carryover may occur. A statutory limit (0.020 mg/kg feed) is established in Europe (21, 22).

Mycotoxins can enter the feed and food chains through direct or indirect contamination pathways. Direct contamination occurs when the food or feed becomes infected by a toxigenic fungus, with the subsequent formation of mycotoxins (23). Indirect contamination occurs when an ingredient has been previously contaminated by a toxigenic fungus and, even though the fungus has been eliminated during processing, mycotoxins remain in the final product (6).

The changing climate may increase the burden of mycotoxins contamination of feeds and foods globally and affect livestock production in terms of both food safety and security (24). The most common mycotoxins reported in Africa are AFs (43.75%) followed by Fs (21.87%), OTs (12.5%), ZEN (9.38%), deoxynivalenol (DON) (6.25%), and beauvericin (BEA) (6.25%) (25). Rampant and *in utero* AF exposure in some African countries, including Nigeria, has been found with hematological evidence (biomarkers) in at least 98% of the population (26). Following the approach of the environment–feed–food chain, OH strategies should be adopted in Africa for the prevention of mycotoxins exposure.

Mycotoxins in African Staple Foods

Human ingestion of mycotoxins occurs mainly through contaminated plant food products or carryover in animal food products such as meat and eggs; noticeably, ingestion of mycotoxins' metabolites occurs through dairy products (6). The most risky food commodities are wheat, maize, rice, beans, oleaginous seeds, cocoa, coffee, grapevine, wine, fruits, nuts, spices, and dried food (27–29).

In general, diet in Africa pivots on starchy staple foods/food products based in maize (especially corn fufu), cassava (*Manihot esculenta*) (e.g., water fufu and garri), plantain, rice, yams/cocoyams, and potatoes (30).

Specifically in Nigeria, carbohydrate intake, such as cassava, yam, and rice constitutes the main diet. Produced by cassava, garri is a roasted granular hygroscopic carbohydrate, popularly consumed by several millions of people regardless of ethnicity and socioeconomic class, making it the most common food product consumed in Nigeria. Garri can be consumed directly in the dry form with peanut, coconut, smoked fish, soaked in water or milk or boiled in water as porridge, popularly called "eba" and eaten with various types of African soups (31). Various groups of molds have been reported to be associated with garri during storage and distribution (32). When present, they can affect the nutritional quality of garri and lead to mycotoxin contamination in case of toxigenic species. OTA has been detected in cocoa and cocoa products in Nigeria (33), and very few reports of its incidence in other crops in Nigeria are available. A high level of 150 ng/g of the OTA was detected in maize (34) and moldy rice (35) from northern Nigeria. Ayejuyo et al. (36) found very low levels of OTA (0.0–2.1 ng/g) in 25 brands of imported rice marketed in Lagos metropolis. Data concerning mycotoxins levels in rice from Nigeria are sparse. Makun et al. (35) report the presence of AFB1, ochratoxin A (OTA), and ZEN in moldy rice, and other studies have been based on AFs (37). Ayejuyo et al. (36) assessed and found OTA in imported rice marketed in Lagos metropolis. Makun et al. (38) provided for the first time the mycotoxin profile of home-grown Nigerian rice with respect to seven of the most important mycotoxins worldwide, namely, AFs, OTA, ZEN, DON, T-2 toxin, fumonisin B (FB), and patulin (PAT). The study reports AFs detected in all samples, total AF concentrations ranging from 28 to 372 ng/g. ZEN (53.4%), DON (23.8), FB1 (14.3%), and FB2 (4.8%) were also found in rice,

although at relatively low levels (38). The acceptable limits for ZEN, FBs, and DON are 30–200, <1,000, and 750–2,000 ng/g, respectively (39, 40). AF levels exceeding limits (10 ng/g) set by the 77 countries, including the EU, that regulate AFs were found in the homegrown Nigerian rice (38–40). OTA was found in 66.7% of the samples, with concentrations (134–341 ng/g) above the maximum levels (2–50 ng/g) in cereals for human consumption. Mycotoxins levels in some agricultural crops and foods in some African countries are shown in **Table 1**. The limit of quantification varies between 0.3 and 10 μg/kg depending on the mycotoxin.

Mycotoxins in African Street Food

Common local street-vended snacks in Nigeria include beans cake (*akara*), roasted, dried and milled maize and groundnuts (*donkwa*), groundnut cake (*kulikuli*), fibrous powdery form of cassava (*lafun*), cheese curds (*wara*), and yam flour. Reports on mycotoxin contamination of these snacks have mainly focused on *Aspergillus* and *Penicillium* mycotoxins, such as AFs and OTA with scanty record on other fungal metabolites including *Fusarium* mycotoxins (66, 67). Snack samples made separately from corn, groundnut, and wheat were contaminated by total AFs concentrations at levels exceeding the limits for total AFs in foods (15 ng/g) as recommended by the National Agency for Food and Drug Administration and Control (NAFDAC), that is the regulatory body in Nigeria (66, 67). Noticeably, peanut cake, popularly called "*Kulikuli*," is highly consumed due to its high protein and lipid content as well as its affordability by the many low- and middle-income people in sub-Sahara Africa (63). The AFB1 levels in *kulikuli* from different parts of Nigeria were about 200-folds more than the 10 μg/g NAFDAC limit and also higher than levels reported previously in peanut and peanut products (64, 82, 83). Rubert et al. (84) reported high levels of AFs (26 ng/g) in Nigerian baked coconut; α-zearalenol (α-ZOL) (54 ng/g) was found in coconut candy. Taken together street-vended snacks (cassava-, coconut- and groundnut-based types) in Nigeria seem contaminated by AFs. In Benin, Nigeria's closest neighbor, AFB1 was detected in 93.3% of peanut cake samples at concentrations above the EU limit (85). The consumption of peanut cakes with high levels of AFB1 portends a public health concern since the consuming population is school-aged children and young adults in their active economical and reproductive age.

Aspergillus flavus and *Alternaria tenuissima* have been isolated from local Nigerian foods (86, 87). The 75–94.1% prevalence of nephrotoxic OTA at level (5 ng/g) regarded as unsafe by the EU in maize, that is a major component of weaning foods and animal feeds in Nigeria, makes its contamination by OTA a serious issue (70, 88, 89). Aflatoxigenic strains of *A. flavus* and *Aspergillus parasiticus* have been reported in peanut and peanut products in Africa (82, 90). *A. flavus* SBG is morphologically similar to *A. flavus* S-type strains and not only produces small sclerotia but also can synthesize large amounts of both AFs B and G. The SBG strain type has a more limited distribution and may be an important source of AF contamination in West Africa (91, 92). Perrone et al. (93) investigated the incidence of *Aspergillus* sect. *Flavi* and the level of AF contamination in 91 maize samples from farms and markets in Nigeria and Ghana. There was higher contamination of the farm samples than the market samples, suggesting that AF exposure of rural farmers is higher than previously estimated. High levels of AFs B and G and lower income of *A. flavus* SBG strains suggest that long-term chronic exposure to this mycotoxin are much higher health risk in west Africa than is the acute toxicity due to very highly contaminated maize in east Africa (93).

Dietary Exposure to Mycotoxins' Mixtures

Daily exposure to mycotoxins' mixtures through consumption of single food sample is proven. Data on the co-occurrence of the principal mycotoxins in foods and beverages are increasing worldwide due to the availability and use of modern and sensitive LC–MS/MS methodologies suitable for simultaneous determination of mycotoxins and other fungal metabolites (94). The presence of mixtures of AFB1, OTA, and ZEN was reported in samples of breakfast cereals commercialized in Spain (94, 95). The study conducted by Solfrizzo et al. (94) on mycotoxins exposure in southern Italy confirmed the presence of DON and OTA in almost all urinary samples. In this study, 6% of urine samples contained AFM1, i.e., a metabolite of mycotoxin mainly found in maize (AF M1 is not present in Maize) and derivatives although these products are not staple foods in Italy where they are consumed as chips, polenta, popcorn, beer, cornflakes, snacks, muesli, and mixed cereals. From a risk assessment stand point, the co-occurrence of mycotoxins is very important though vaguely understood: indeed, recent *in vitro* data highlight potential additive or synergistic interactions (96–99). Notwithstanding this, also in Europe there are few published studies on the co-occurrence of mycotoxins [e.g., Ref. (100, 101)]. Co-contamination with AFs, OTA, and ZEN is very common in Nigeria, and up to five mycotoxins were detected in a single rice sample; AFs (B1, B2, G1, and G2) were found in all samples (38). The presence of AFs and OTA in this Nigerian staple food at levels exceeding the limits set by international regulatory bodies along with the co-occurrence of other toxicants with possible toxic synergistic effect made the studied rice sample unsuitable for human and animal consumption and raise national public health concerns (38). Kimanya et al. (102) confirmed co-occurrence of AFs with DON and Fs from maize based meals in northern Tanzania. In a survey of mycotoxins in traditional maize based opaque beers in Malawi, it was estimated that consumption of 1.0–6.0 L of this local beverage results in a daily FB1 and FB2 exposure of 29–174 μg/kg body weight (bw)/day [i.e., >provisional maximum daily intake of 2 μg/g bw/day set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA)] and AF exposure of 1.5–9.0 μg/kg bw/day for a 60 kg adult (103). This is of significant public health importance since this singular source alone can add to the body burden due to AFs and Fs dietary exposure among beer consumers (103). OTA, ZEN, DON, NIV, and other less reported mycotoxins such as citrinin, alternariol, cyclopiazonic acid, sterigmatocystin, moniliformin, BEA, and enniatins were detected in various food samples from Burkina Faso and Mozambique (41). The quantification of at least 28 toxic fungal metabolites in a single sample strongly suggests the huge variety of mycotoxin co-exposure in Africa (41).

Ngoko et al. (104) report 50–26,000 ng/g Fs, 100–1,300 ng/g DON, and 50–180 ng/g ZEN in maize samples from Cameroon.

TABLE 1 | Mycotoxins levels (μg/kg) in the crops and foods in some African countries.

Country	Mycotoxin	Food stuffs	Concentration (μg/kg)	Reference
Mozambique	Fumonisin B_1	Maize	159–7,615	Warth et al. (41)
	Fumonisin B_2	Maize	27.7–3,061	
	Fumonisin B_3	Maize	26.6–777	
	DON	Maize	116–124	
	DON-glucoside	Maize	12.6–32.5	
	NIV	Maize	20.2–45.9	
	ZEA	Maize	10.9–18.1	
	Citrinin	Maize	276–5,074	
Malawi	AF	Sorghum	1.7–3.0	Matumba et al. (42)
		Sorghum for thobwa drink	6.1–54.6	
		Sorghum for beer	4.3–1,138.8	
Botswana	AFs	Peanut	12–239	Mphande et al. (43)
Sudan	AFs	Sesame oil	0.2–0.8	Idris et al. (44)
		Groundnut oil	0.6	Elshafie et al. (45)
		Peanuts butter	21–170	
	AFB_1	Sesame unpeeled	0.4–14.5	Kollia et al. (46)
Tanzania	FUMs	Maize	11,048	Kimanya et al. (47)
	AFs	Maize	158	
Tanzania and DR Congo	AFs	Maize	0.04–120	Manjula et al. (48)
Zambia	FUMs	Maize	20,000	Mukanga et al. (49)
	AF	Maize	0.7–108.74	Kankolongo et al. (50)
Uganda	AFs	Groundnuts, cassava, millet, sorghum flour	0–55	Kitya et al. (51)
Kenya	AFs	Animal feed and milk	>5	Kang'ethe and Lang'a (52)
		Maize	>20	Daniel et al. (53)
		Maize	1–46,400	Lewis et al. (54); Mwihia et al. (55)
		Peanut	0–7,525	Mutegi et al. (56)
Ethiopia	AFs	Shiro and ground red pepper	100–525	Fufa and Urga (57)
	AFs	Sorghum, barley, teff, and wheat	0–26	Ayalew et al. (58)
	OTA	Sorghum, barley, and wheat	54.1–2,106	
	DON	Sorghum	40–2,340	
	FUM	Sorghum	2,117	
	ZEA	Sorghum	32	
Nigeria	AFs	Rice	28–372	Makun et al. (38)
		Edible tubers "tiger nuts"	454	Adebajo (59)
		Edible tubers "tiger nuts"	10–120	Bankole and Eseigbe (60)
		Sorghum	10–80	Salifu (61)
		Dried yam	27.1	Bankole and Mabekoje (62)
		Dry roasted groundnut	52.4	Bankole et al. (63)
		Groundnut cake	20–455	Akano and Atanda (64)
		Peanut cake (*kulikuli*)	13–2,824	Ezekiel et al. (65)
		Corn-based snacks	12.0–30.0	Ezekiel et al. (66, 67)
		Nut-based snacks	0.0–6.0	
		Wheat-based snacks	0.0–50.0	
		Fin fish	1.05–10.00	Olajuyigbe et al. (68)
		Shell fish	4.23–5.90	
	OTA	Rice	134–341	Oluwafemi and Ibeh (69)
	AFs	Weaning food	4.6–530	
	OTA	Maize	0–139.2	Makun et al. (70)
		Millet	10.20–46.57	
		Sorghum	0–29.50	
		Sesame	1.90–15.66	
		Fonio (acha)	1.38–23.90	
		Cassava (garri)	3.28–22.73	
Ghana	AFs	Maize	0.7–355	Kpodo (71)
	Fs	Maize	70–4,222	Kpodo et al. (72)
Benin	AFs	Maize	5	Hell et al. (73)
		Chips	2.2–220	Bassa et al. (74)
		Dried yams	2.2–220	Mestres et al. (75)
		Cowpea	nd	Houssou et al. (76)

(*Continued*)

TABLE 1 | Continued

Country	Mycotoxin	Food stuffs	Concentration (μg/kg)	Reference
Benin, Mali, and Togo	AFs	Dried vegetables		
		Baobab leaves, hot chili, and okra	3.2–6.0	Hell et al. (77)
Burkina Faso	AFs	Groundnuts	170	Yameogo and Kassamba (78)
	DON	Maize	31.4	Warth et al. (41)
	ZEN	Maize	11.0–15.8	
	Citrinin	Maize	531–5,074	
	Alternariol	Maize	5.1–16.0	
	Altertoxin I	Maize	3.4–10.8	
South Africa	FUMs	Maize	222–1,142	Burger et al. (79)
	Fs	Compound feeds	104–2,999	Njobeh et al. (80)
	DON	Compound feeds	124–2,352	
	ZEN	Compound feeds	30–610	
Lesotho	ZEN	Sorghum beer	50	Gilbert (81)

nd, not detectable; ZEN, zearalenone; DON, deoxynivalenol; AFs, aflatoxins; OTA, ochratoxin A; NIV, nivalenol.
Limit of quantification: DON = 10, NIV = 10, ZEN = 5, OTA = 0.3 μg/kg.

Detectable levels of AF ranged between 5.2 and 14.5 ng/g in other widely consumed foods in Cameroon, namely, cassava balls and cassava pellets (105). A simultaneous occurrence of mycotoxins (FB_1 41%, AF 51%, ZEN 57%, DON 65%, and OTA 3%) in human food commodities from Cameroon has also been reported (80). In another study from Cameroon, total AF levels exceeded the maximum limits of the European Commission (EC) regulations (30). Taken together, the widespread nature and high levels of multiple mycotoxins occurring in staple foods suggest high exposure levels that could have severe health implications in sub-Sahara Africa.

AFM1 in human breast milk is an important health risk for infants (16). The chronic intake of AF contaminated food could increase stillbirths and neonatal mortality, immune suppression with increased susceptibly to infectious diseases such as pneumonia, stunting of growth (33), and HIV/AIDS (106). In many countries, because animals are usually milked individually at the household doorstep mycotoxins consumption can be very high (107–109). Although the minute of mycotoxins through food of animal origin may be seemingly innocuous in the general population, vulnerable groups may not be spared, especially the genotoxic carcinogens such as AFs.

Additional Exposure Route: Mycotoxins in African Indoor

Inhalation of contaminated airborne aerosols can represent an additional route of mycotoxin exposure. Nowadays, people spend about 90% of their time in indoors environment due to working or resting (31). However, in many parts of the world, homes, schools, and workplaces are contaminated with airborne molds and other biological contaminants (110, 111).

Mycotoxins can be found in airborne particulates of environments where susceptible commodities are treated, such as warehouses, harbors, laboratories, and specific occupational settings where products/materials that are commonly contaminated (e.g., waste, feed, and animal production) are handled (112–115).

Poultries fungal burden is mainly affected by the kind of litter applied in pavilions (112), whereas in swine it is mainly affected by the feeding operations due to feed fungal contamination (113, 114). Waste management industries pose another challenge regarding workplaces fungal contamination, in waste water treatment plants and in solid waste management industries the main source are the waste water and the waste that need to be treated (115).

Moreover, the presence of mycotoxins in domestic households as a consequence of inappropriate hygiene conditions has been demonstrated, with immunosuppressive effects due to the inhibition of phagocytosis and of alveolar macrophage functions (27). Children, elderly, patients on immune suppressants, and with respiratory diseases are more susceptible to contamination by indoor fungi (110). *A. flavus* has been isolated from indoor environment like hospitals in Nigeria (116, 117). Although the presence of indoor fungi by mold contamination is related with dampness of the indoor environment and swampy locations, researches have indicated fungal presence as well in houses without these characteristics (111). The highest isolation rates (*Rhizopus* sp., for instance) were achieved from high residential density areas, probably an effect of overcrowding, poor sanitation and high arthropod infestation. Factors such as absence of basic facilities for drainage and waste disposal and dumps in proximity of residential homes do favor indoor mold contamination (118, 119).

RISK ASSESSMENT

Mycotoxins are metabolized in liver and kidneys and also by microorganisms in the digestive tract (7). Chemical structure and toxicity of mycotoxin metabolites excreted by animals or found in their tissues are different from the parent molecule. Toxicity depends of factors such as type of toxin, dose ingested, duration of exposure, age, and sex (29). The WHO (120) estimated that AFs were responsible for nearly 20,000 deaths each year, 3,000 of them on the African continent. The International Agency for Research on Cancer (IARC) classified AFB1 in group 1 "*carcinogenic to humans*." AFB1 is the most potent natural carcinogen and is usually the major AF produced by aflatoxigenic strains. The no observed-adverse effect level is not applied for genotoxic carcinogens, therefore no threshold is assigned to AFB1.

In particular, AFs are potent hepatotoxins. Chronic exposure to small doses of AF for prolonged periods (e.g., through the diet) has been associated with human hepatocellular carcinomas, which may be compounded by other carcinogens, such as hepatitis B virus. Hepatocellular carcinoma (HC) is the third most common cause of death from cancer in Africa (121). Approximately 250,000 deaths are caused by HC in sub-Saharan Africa annually and can be attributed to risk factors such as high daily intake (1.4 μg) of AF and high incidence of hepatitis B (17, 19). As well as causing liver cancer, AFs have been associated with other health problems in people such as stunting in children and immune suppression (16). Chronic exposure to AFs is associated with impaired immunity and malnutrition, therefore also with malaria and HIV/AIDS (21, 22, 122, 123). A study in Ghanaian adults reported that AFs could cause impairment of human cellular immunity that could decrease resistance to infections (19). Kwashiorkor, a disease usually considered a form of protein energy malnutrition, has long been linked to AF exposure, along with chronic gastritis and childhood cirrhosis (14, 124). Acute exposure to large doses (>6,000 μg) may precipitate severe acute liver injury with high morbidity and mortality (125). Symptoms of acute toxicity include reduced liver function, derangement of blood clotting mechanism, icterus (jaundice), and a decrease in essential serum proteins synthesized by the liver. Acute AF exposures have been associated with epidemics of acute toxic hepatitis in Africa with death rates ranging from 10 to 60% (6, 17). Other general signs of aflatoxicosis are edema of the lower extremities, abdominal pain, and vomiting. An outbreak of acute aflatoxicosis in Kenya in 2004 caused 125 deaths among 317 people that consumed AF contaminated maize (92).

Aflatoxin M1, OTA, and FB1, FB2 are classified in group 2B "*possibly carcinogenic to humans*." Chronic ingestion of Fs has been linked as possible risk factor for the occurrence of esophageal cancer in areas, such as the former Transkei region of South Africa, where Fs exposure from contaminated maize is high (126). There is a specific *p53* codon 249 mutation in the plasma of liver tumor patients from West Africa (Gambia) after exposure to AFs (127, 128). In a study of HIV and hepatocellular and esophageal carcinomas, related to consumption of mycotoxin-prone foods in sub-Sahara Africa, the relation between cancer and food suggested that Fs contamination rather than AF is the most likely factor in maize promoting HIV (129). OTA could also be associated with immunotoxic and neurotoxic effects (29).

Other mycotoxins, i.e., PAT, ZEN metabolites, some TCTs, in particular T-2 toxin, nivalenol (NIV), and DON, are considered by IARC as "*not classifiable as to its carcinogenicity to humans*" (group 3).

With special emphasis on infertility, that is an ongoing global reproductive health problem, also in Africa, *in vivo* and *in vitro* studies have shown that ZEN and metabolites [α-ZOL and β-zearalenol (β-ZOL)], DON, OTA, and AFB1 adversely affect fertility by arresting steroidogenesis. Exposure to these mycotoxins precipitate deleterious effects on the spermatozoa, Sertoli and Leydig cell function, oocyte maturation, and uterine and ovarian development and function in *in vivo*, *ex vivo*, and *in vitro* experimental models (130–134). Mycotoxins can induce oxidative stress and result in damage of sperm DNA (135), reduced fertilization rates and embryo quality (136). Mycotoxins have also been implicated as endocrine disruptors altering the steroid hormone homeostasis and interfering with receptor signaling (137–140). Concentrations of AFB1 significantly higher in the semen of infertile men than in controls (semen of fertile) have been reported by Ibeh et al. (141), thus suggesting that exposure to AFB1 could be a causative factor in male infertility in Nigeria. At least 50% of infertile men with high seminal concentrations of AFB1 had a greater percentage of abnormalities in sperm count, motility and morphology compared with the fertile men (10–15%) (141). These observations were comparable to male rats fed with AFB1 contaminated feeds (8.5 μg/g of feed) for 14 days (141). Similarly, semen and blood levels of AFB1 which ranged from 700 to 1,392 ng/mL and exceeded the WHO recommended level have reported in infertile men attending the infertility clinic in Nigeria (142). The high prevalence of male infertility in Africa (20–35%) (143–146) as well as the declining sperm count (147) motivate reproductive health experts in investigating the role of mycotoxins (148). Since endocrine disrupting chemicals are known to cause endometriosis, premature ovarian failure, and polycystic ovary syndrome, mycotoxins may also be involved in female reproductive disorders (149).

Markers and Biomarkers

Mycotoxins are measured in feeds, food, air, or other environmental samples for environmental monitoring purposes, whereas the presence of adducts and metabolites are assayed in human or animal tissues, fluids, and excreta for biological monitoring (150). A challenge in the field of internal exposure assessment is to develop accurate and reliable biomarkers. The biomarker approach is a promising tool for measuring toxin-mediated biological perturbations or the amounts of mycotoxins present in the matrix (28). In molecular epidemiology, it is possible to demonstrate the association between putative carcinogens and specific cancers (150). Biological markers of AFs, OTA, and Fs exposure have attracted the attention for mycotoxin biomonitoring studies. However, while AFs and OTA biomarkers have been successfully applied and validated over the last decade, large drawbacks remain to find a suitable Fs biomarker (28).

Biomonitoring of AFs can be done by quantifying AF metabolites in blood, milk, and urine. Indeed, the first studies in which biomarkers where used to determine human exposure to food pollutants involved AFB1. In these studies, correlation between AFB1 intake and urinary AFM1 excretion was statistically achieved and the exposure biomarker validated. The mean urinary AFM1 level in Cameroon (30) was similar to that observed in adults in Ghana (range: nd–0.115 μg/L) (151) and fully weaned that of Guinean children (152). A similar range was observed among pregnant women in Egypt (0.004–0.409 μg/g creatinine) (153). Ghana and Guinea are recognized as high-risk regions for AF exposure, whereas Egypt is regarded as moderate when compared with sub-Saharan Africa (152, 154–156). The estimates of tolerable daily intake of several mycotoxins are exceeded in Africa (30). In a pilot, cross-sectional and correlational study conducted in eight rural communities in northern Nigeria to investigate mycotoxin exposures in volunteers, urinary

biomarker levels were correlated with mycotoxin levels in foods consumed the day before urine collection in all age categories, suggestive of chronic (lifetime) exposures (10). In the urines with detectable AFM1, it was estimated that the mean intake of AFB1 was 0.67 ng/kg bw/day (max = 2.5 ng/kg bw/day). Higher AFM1 urinary levels have been detected in children from Sierra Leone children (157).

Albumin-bounded AFB1 and AFB1-DNA adducts in urine have also been explored for exposure assessment studies (28). Numerous studies have shown that carcinogenic potency is highly correlated with the extent of total DNA adducts formed *in vivo* (150). Excreted DNA adducts and blood protein adducts can also be monitored: the AFB1-N^7-guanine adduct represents the most reliable urinary biomarker for AF exposure but reflects only recent exposure (158). High AF-albumin adduct levels in maternal blood, cord blood, infant blood, and children's blood have been associated with poorer growth indicators and impaired markers of human immunity as shown by lower levels of secretory immunoglobulin A in saliva of Gambian children (159, 160). High levels of AFB1-albumin adducts were associated with low percentages of certain leukocyte immune phenotypes in Ghana (161). The chronic/dietary exposure to AF is evident from the presence of AFM1 in human breast milk (162) and umbilical cord blood samples (163), with serious implications for the next generation (109). Home-grown maize contamination led to arguably the largest fatal aflatoxicosis outbreaks in rural communities of Kenya, in which AF-albumin adducts were independently confirmed in the exposed (164). In another study from Kenya, wasting in children was related to consumption AF contaminated flour (165). In Ghana, low birth weight was shown to have an association with mothers' AF-albumin adduct levels (166). There is a dose-dependent decrease in height and weight for age in AF exposed children in a study carried out in Togo and Benin in West Africa (123, 167).

Mycotoxin-producing molds have lately been found to infect the intestinal tract to cause leaky gut, thus exerting important immunosuppressive activity, and produce neurotoxins (168). OTA, that has nephrotoxic, hepatotoxic, immunotoxic, and genotoxic effect and induces carcinogenicity, teratogenicity, and mutagenicity, has also been seen to cause dysregulation of several gene expression including the upregulation of SOX9 (169), i.e., a gene involved in the development of the male phenotype and has been detected in autistic cases (170). It has recently been posited that single nucleotide polymorphisms in NLGN4X 3′UTR and illegitimate microRNA-inducing OTA could be a possible biological mechanism reflecting the gene–environment interaction in patients without causative mutations (171, 172) and suffering from dysbiosis and leaky gut (173). Although there seem to be no published data on population-based estimates of prevalence of pervasive developmental disorders from African region, the prevalence of autism spectrum disorder (ASD) among children with developmental disorders in Egypt and Tunisia has been documented as 33.6 and 11.5%, respectively (174, 175). The ASD is an increasing neurodevelopmental disorder with a broad phenotype, appearing by 3 years of age: it often shows comorbid situations, such as mental retardation, epilepsy, and recurrent gastrointestinal abnormalities. In Nigeria about 0.9% of the children under the age of 3 years manifested neurodevelopmental delays in a recent survey (176). It is even feared that this value may be higher considering late diagnosis (176). Like most aspects of ASD, the mycotoxin impact on this prevalence remains unknown.

Human health risk assessments of Fs hinge on maize consumption. Maize consumption can be <10 g/person/day in various European countries, but up to 400–500 g/day in rural Africa (177), with a 90 percentile value of over 700 g/person/day (178). The implication of this socio-geographical dietary variation with respect to attaining the provisional maximum tolerable daily intake (PMTDI) of 2 ng/g bw/day of Fs is enormous. Whereas a European consumer at an assumed bw of 60 kg would need to consume 10 g maize at an Fs contamination level of 12,000 ng/g, an African who consumes 500 g/day would exceed the PMTDI if the contamination level was above 240 ng/g (179). The detrimental effects of Fs on the developing fetus and young infants are now known from both experimental and epidemiological researches. Transkei region in South Africa and Tanzania where Fs exposure is high is known to have elevated incidences of neural tube defects and growth retardation (180, 181). Fs interfere, *via* depletion of sphingolipids, with the folate receptor, thus inhibiting the uptake of folate and eventually leading to cellular folate deficiency and neural tube defects (182), that can be prevented in experimental animals by folate supplementation (183).

PERSPECTIVES FOR RISK MANAGEMENT IN WEST AFRICA

Many African countries have some mycotoxin regulations but only for AFs (or a few other mycotoxins) in specific foods, or no regulations at all. Even when standards are in place, severe mycotoxin-poisoning outbreak occurs in Africa (92). Indeed, good practices and recommendations for the field management of risk of mycotoxins occurrence would be strategic for investment of public, non-governmental organization, and private funds at the scale of the subsistence farmer, the smallholder, and through to a more advanced value chain (184).

The multiplicity of origins of fungal infections implies that strategies for prevention of mycotoxins contamination must be applied at an integrative level along all the food production chain. There are three steps of intervention that must be of concern: prevention (i) before any fungal infestation, (ii) during the period of fungal invasion of plant material and mycotoxins production, and (iii) when agricultural products have been identified as heavily contaminated (7, 185).

Risk mitigation practices cover pre- and postharvest:

(i) *Predictive models*. Weather conditions (e.g., hot and humid tropical climate that favors fungal proliferation) are the most influential parameter on mycotoxins contamination and fungal infection and growth (186, 187). Predictive models for mycotoxins occurrence based on regional weather data would be a valuable tool to estimate the risk of contamination (188). In a study that examined AF exposure

in pregnant Gambian women having staple food in refined white rice, millet, or maize with groundnut sauce, AF exposure throughout pregnancy was found, with higher levels in the dry season. Women in later stages of pregnancy showed higher levels of AF-albumin adducts than those in earlier stages of pregnancy in the dry season (189).

(ii) *Preharvest interventions*. Good agricultural practices such as sanitation, early sowing date, balanced nitrogen fertilization, moderate plant density, breeding for resistance to drought, insect pest damage or fungal infections, biological control, early harvesting, and moisture levels and proper handling during harvesting. An integrated program involving plant maturation, nutrition, and insect control is crucial (9, 185), along with proper and timely crop rotation, tillage, and fungicide administration (7, 150). Biogeographical agricultural models of cultivated plants could also be useful.

(iii) *Postharvest strategies* (transportation, marketing, and processing). Control of factors such as temperature, humidity, pH, packaging, cross contamination by practices like sorting and complete drying decrease contamination during storage (46). In case of toxin manifestation, measures are required that act specifically against certain types and groups of toxins (7, 150).

(iv) *Detoxification strategies* for contaminated feeds are studied to reduce or eliminate the adverse effect of mycotoxin. The addition to the animal's diet or the treatment of contaminated feeds with mycotoxin-binding agents may be useful to protect animal health and avoid milk contamination by the carcinogenic AFM1 metabolite. However, mycotoxin binders may impact animal health, e.g., by interfering with the absorption of nutrients or medications (7, 190). Traditional techniques that could reduce/detoxify mycotoxins during food processing are studied (191).

(v) *In house protective practices*, such as proper food storage, dietary diversity—where possible—, and vaccination against HBV to prevent the synergism of AF exposure and chronic HBV infection in liver cancer risk (7, 95, 150, 192–194). Significant building blocks for mycotoxins risk management do exist in West Africa, such as the following:

- *Surveillance and monitoring of environmental/food matrices experiences*. Biomonitoring of mycotoxins in biological fluids such as blood or urine is useful to generate reliable information on internal exposure at individual level compared with dietary assessments (10). Validated *biomarkers of exposure* are available, such as urinary metabolites, DNA, and protein (albumin) adducts (15, 192). The OTA levels found in Nigerian-grown rice and maize are within the lower limits of concentrations (200–1,000 ng/g) that have been linked to porcine nephropathy in Bulgaria (195). There has been a speculation about the contribution of OTA to raise the incidence of chronic renal diseases in Nigeria in conjunction with malaria, hypertension, and diabetes conditions. Poor record of renal *registry* in Nigeria has hampered the tracking of chronic renal disease; however, available hospital data revealed that chronic renal failure accounts for about 10% of medical admissions in Nigeria, and extrapolating this, puts the frequency figure between 200 and 300 patients per million of population (196).
- *Application of biomarkers*. In a pilot study using multi-urinary biomarkers among rural residents in northern Nigeria, Ezekiel et al. (10) detected mycotoxin in all age categories. Their observations suggest chronic/lifetime exposures, and some exposures were higher than the tolerable daily intake. The study developed in Cameroon by Abia et al. (30) used for the first time in Africa a novel multi-mycotoxin assay utilizing LC–MS/MS to determine the frequency of occurrences and levels of several mycotoxins, or their metabolites in urine.
- *Experiences of total diet studies (TDSs)*. Dietary intake estimate should include data on consumption of raw and processed foods (100) to assess average dietary exposure and identify excessive consumer subgroups. TDSs are often used as a risk assessment tool to evaluate exposure and—when performed periodically- exposure trends in the general population and (more vulnerable consumers such as children or diseased subjects, or higher consumers) high-risk subgroups. TDSs differ from traditional food monitoring in two major aspects: (i) chemicals are analyzed in food in the form in which it is consumed and (ii) cost-effectiveness, because composite samples (more ingredients grouped) after kitchen processing are analyzed. As made by European participants in the SCOOP [Scientific Cooperation on Questions relating to Foods (197)] exercises, African countries could group by region and collect, and harmonize knowledge on the status of mycotoxins contamination of raw material and food products (197). Preliminary experiences of TDSs do exist in West Africa, along with its methodology and methods (198).
- *Seminal governance framework based on OH*. OH integrates efforts for building a governance national strategy based on the linked and mutually supported protection of environment, farm animals and human well-being (199).
- *Seminal toxico-vigilance (TV) system*. The TV system aims at updating (and harmonizing) registers on information on incidence of poisoning in communities (200).
- *Risk assessment and advices for food regulations*. Mycotoxins regulations have been established in about 100 countries, out of which 15 are African, to protect the consumer. As in the case of Europe (the European Food Safety Authority), an African independent body could be established with the task of independent science-based risk assessment on food and feed. So far, the JECFA, that is an international committee administered jointly by FAO and WHO, serves as an independent scientific committee which performs risk assessment and provides advice to FAO, WHO, and the member countries of both organizations. The requests for scientific advice are for the main part channeled through the CAC in its work to develop international food standards and guidelines under the Joint FAO/WHO Food Standards Programme.

African Turning Point on Mycotoxins

Mycotoxins are now recognized as major cause of food intoxications in SSA. Many economically developing countries have realized that reducing mycotoxins level in foods will not only reduce financial burden on health care but also confer international trade advantages such as exports to more attractive and remunerative markets. Moreover, reducing mycotoxins level means facing lowered animal production, lowered yields in agriculture, and lower market value (5, 7, 17).

The study from Somorin et al. (101) concerning the co-occurrence of AFs, OTA, and citrinin in egusi melon seed from Nigeria is one of the examples to explain the basis for increasing border rejection of melon seed consignments from Nigeria to EU as highlighted in the European Rapid Alert System for Food and Feed (RASFF) (201). This led to the enactment of legislation which mandates that 50% of consignments of egusi and their derived products from Nigeria be checked before being allowed entry into the EU (202, 203).

Pivoting on what has already started in Africa, mentioned "building blocks" deserve strengthening and improvement. Based on the OH approach, mycotoxin reduction and control are dependent on the concerted efforts of all actors and stakeholders along the food production chain. We highlight here:

- *Political will* to address mycotoxins exposure and support capacity for testing commodities, which determines whether requirements can be enforced (162). As in the General Food Law issued by the EC, that clearly describes the food safety framework in the EU, including the role and responsibilities of the different parties involved from farm to fork, a envisaged African general food law could have a hierarchic and network character (21).
- *Strengthened laboratory capacities*, including efficient, cost-effective sampling, and analytical methods. Indeed, scientific research is moving toward reliable but cost-effective and sustainable user-friendly techniques for the acquisition of analytical data under field conditions and environmental stress (204).
- *Nationwide surveillance and regular monitoring* capacities by increased food and feed inspections (200).
- Established *early warning systems* as well as *risk management systems* allowing timely corrective actions and avoiding both food losses and waste (205).
- *Training and empowerment of farmers* and food producers on the good agricultural and good management practices. Indeed, communities are the foundation of Public health (205).
- *Improvement of facilities*. Many African countries do not have the infrastructures to prevent and control food contamination (e.g., **Figure 1**): science could give low cost solution to long lasting problems of infrastructures.
- *Consumer awareness and education*. According to Ezekiel et al. (65), at least 85% of the consumers of *kulikuli* in Nigeria are not aware of the risk of AF contamination of vended peanut cake. Consumers should prefer food producers adopting good practices.
- *Dissemination* of information *via* national media (radios, television, newspapers and magazines, and town hall meetings) and the web (206).

FIGURE 1 | Granaries in Burkina Faso, 2012 (courtesy of Ilaria Proietti, NOODLES Alliance).

FIGURE 2 | Photograph of mycotoxin contaminated food: reproduced from Environmental Health Perspectives; September 2013, Volume 121, Issue 9, doi:10.1289/ehp.121-A270.

- *Food processors or industry* should contribute to an improved economic sustainability and enhanced international trade [see, e.g., reflections in Ref. (207)].
- *The "luxury" of choice* **Figure 2**. In countries where populations are facing starvation or where regulations are either not enforced or non-existent, chronic intake of AF may occur liver cancer incidence rates are 2–10 times higher in economically developing countries than in economically developed ones. Unfortunately, strict limitation of AF contaminated food is not always an option. A joint FAO/WHO/United Nations Environment Programme Conference report stated that in some economically developing countries, where food supplies are already limited, drastic legal measure may lead to food security problems, e.g., lack of food and excessive prices. It must be remembered that people living in these countries cannot exercise the option of starving to death today to live a better life tomorrow (150).

The following are some more aspects that deserve deep attention:

- *Risk analysis* is increasingly recognized as an essential component in modern science-based food safety systems and plays a growing and important role in guiding food safety authorities. Informed by the risk assessment process, risk management in its broadest sense involves the consideration and implementation of food policy options, while taking due cognizance of tolerable levels of risk. Risk communication involves the interchange of information concerning risk and its perception among all stakeholders in food safety, including policy makers, industry, and consumers (208, 209).
- *Risk to benefit assessment*. Interestingly, some of the food items that are prone to mycotoxins contamination are component of healthy diet. Based on RASFF reports, the most predominant category of mycotoxins is AF in pistachios, peanuts, almonds, hazelnuts, and Brazil nuts. OTA occurs mainly in beverages (raw coffee and derivatives, cocoa powder), fruit and processed fruits (mainly raisins/sultanas and figs), spices and condiments (mainly pepper), vegetables, cereals, and other crops (202). Models for risk to benefit assessment are increasingly available (210).

CONCLUSION

Operationalization of OH for mycotoxins can shield population from direct (on health) and indirect (on trade, economies, and livelihoods) effects of mycotoxins. Mycotoxins effects on public health and economy in Africa are not directly measurable, though its existence is indicated by environmental, toxicological, and clinical data. The contamination of food and feed by mycotoxins represents a serious health problem as well a considerable economic obstacle in African countries, where the trade balance is based on the exportation of commodities. Nowadays, the poorest regions of the world have neither the infrastructures to prevent and control food contamination, nor the luxury to allow the rejection of contaminated food. Operationalizing mycotoxins in the OH frame is useful to build a risk management frame that is sound and understandable in terms of empirical observations by local institutional stakeholders expected to issue risk management programs in Africa. Indeed, governance schemes for early prevention of toxic exposures deserve inclusion in development initiatives.

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CL, CF, and OO contributed equally from the literature search, write-up, and revision of this article.

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REVIEW
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The Hotspot for (Global) One Health in Primary Food Production: Aflatoxin M1 in Dairy Products

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One Health involves the multifaceted environment-animal-human web: nevertheless, the role of toxicological issues has yet to be fully explored in this context. Aflatoxin B1 (AFB1) contamination of feeds is a risk for the health of several farm animals, including fishes; milk is the only food of animal origin where a significant feed-food carry over may occur. The main AFB1-related compound present in milk is the hydroxy-metabolite aflatoxin M1 (AFM1). Besides contamination of raw milk, AFM1 is of concern for the whole dairy chain; AFM1 may also contaminate the milk of several other ruminants used for milk/dairy production. In a One Health perspective, milk represents a sentinel matrix for AFB1 vulnerability of the agro-food system, that is crucial in a phase when food/nutritional security becomes a global issue and climatic changes may affect agricultural productions. In the global setting, food chain exposure to long-term toxicants, such as AFM1, is a growing concern for economically developing countries, whereas global trade and climatic change makes AFM1 an emerging hot issue in economically developed countries as well. We critically review the state of the art on AFM1 risk assessment and risk management using two scenarios as case studies: a European Union country where the health system aims at ensuring a high-level protection of food chain (Italy) and the world's largest (and economically developing) producer of dairy products by volume (India). The case studies are used to provide building blocks for a global One Health framework.

Keywords: toxicology, risk assessment, risk management, climatic change, food security, food safety, India, Italy

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INTRODUCTION

Aflatoxin B1 (AFB1) is a major toxic contaminant of foods and feeds; it is secondary metabolite of several *Aspergillus* spp. fungi affecting many food ingredients and feed materials, especially nuts (e.g., peanuts) and grains (mainly corn). *Aspergillus* molds can also concurrently produce other, less toxic AF metabolites (B2, G1, and G2). AF-producing *Aspergillus* spp. behave differently: *Aspergillus parasiticus* is more adapted to a soil environment, whereas *Aspergillus flavus* is more adapted to the aerial parts of plants. Contamination from *Aspergillus* may arise both in the field, as stressed plants may become infected, and/or during storage and transport (1). In the past, AFB1 contamination was thought to be mainly a problem of economically developing countries; in the last decade, climate

changes have brought suddenly the attention to an enhanced risk in industrialized countries too, including Europe (2).

AFB1 is a potent hepatotoxicant and liver carcinogen; since it acts through a genotoxic mechanism, a tolerable daily intake cannot be set and human exposure should be reduced to levels as low as achievable. Tolerable levels (in the range from micrograms to nanograms per kilogram) have been set in Europe in various susceptible plant-derived food commodities as well as feed materials and complete feeds, based on the calculations of margins of exposure (1, 3). With regard to food-producing animals, AFB1 contamination of feeds is a risk for the health of several farm animals, including fishes; however, milk is the only food of animal origin where a significant feed-food carry over may occur (1). Thus, in a One Health perspective, milk may also represent a sentinel matrix for AFB1 vulnerability of the agro-food system, which may be crucial in a phase when food/nutritional security becomes a global issue and climatic changes may affect agricultural productions.

The main AFB1-related compound present in milk is the hydroxy-metabolite aflatoxin M1 (AFM1). Albeit less potent than AFB1, AFM1 presents similar toxicological hazards: in Europe, maximum levels for AFM1 have been set for consumable milk (0.05 μg/kg) and infant formulae (0.025 μg/kg) as parameters to reduce human exposure to the minimum, reasonably achievable level. Besides contamination of raw milk, AFM1 is of concern for the whole dairy chain, as it may be carried out to dairy products (4). Upon intake of contaminated feedingstuffs. AFM1 is also present in the milk of other ruminants used for milk/dairy production, such as water buffalo, camel, sheep, and goat (5). Since most of the available evidence deals with cow's milk, AFM1 should be considered as a concern for all dairy productions.

GLOBAL ASPECTS

Global Trade and Food Security

In 1996, the Food and Agriculture Organization (FAO) stated that food security is set in "when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life." Therefore, food safety is an essential part of food security. With global trade and climatic changes, food safety has emerged as a hot issue whose problems and solutions are transnational.

The global market has made AF contamination of feeds and milk in emerging countries a relevant problem in the industrialized world too. Already in 1989–1990, a UK survey on feed materials revealed high AFB1 levels in a number of samples imported from India, other parts of Asia and South America (6): ingredients at higher risk included those derived from sunflower, corn, and other oily seeds and cereals (7, 8). Whereas feed materials based on seeds, nuts, and grains draw most of the attention, the international trade of dairy products is a vulnerable segment as well.

India is the world's largest producer of dairy products by volume, accounting for more than 16% of world's total milk production, and it also has the world's largest dairy animal population (9). Cattle and water buffalo milk are both major comparts of the Indian dairy sector, different from other dairy producing countries. The Indian dairy system is a low input–low output one; the average individual producer owns less than five cattle or water buffaloes and uses locally available feeds. This results in milk yields far below international averages and also in the world's lowest production costs. In the 1990s, imports (0.4%) and exports (0.3%) were almost equal, but from 2001, India became a net exporter of dairy products (10). In 2010, the government and the National Dairy Development Board have drawn up a National Dairy Plan that intends to nearly double India's milk production by 2020.[1] In India, about 70% of the population lives in rural areas and about 38% of them are poor. For these people, as well as for the large vegetarian segment of the Indian population, dairy products provide a critical source of calories and animal proteins; per capita mean consumption of milk has been estimated at about 250 g per day, corresponding to more than 90 kg per year.[2] Milk is consumed as whole milk by the majority of the Indian consumers, including infants and children, and liquid milk is a major component of the diet of Indian children.

In Italy, milk production in 2012 has been 10,876,191 t in the bovine sector, 192,000 t in the buffalo, 406,000 t in the sheep and 28,000 in the goat sectors (*CLAL, Dairy Economic Consulting firm*).[3] The production trend is still largely seasonal, with a peak level in March–May. The area with the highest milk production is the Po valley in northern Italy, featuring among the main intensive agricultural areas in Europe, and in particular the region of Lombardy. The production system based on milk quota has characterized the milk sector in Italy since 1984, when the European Union adopted the quota system, up to 2015. The quota system has induced in Italy a steady production in the last 20 years and has prevented the milk price level to increase, thus forcing the farmers to keep under control the production costs and the supplies of raw materials for feed production. Milk production in Italy is undergoing a serious crisis due in large part to lower costs in other EU countries, so the national dairy industry increasingly relies on imports. To cope with the crisis, high-quality products, such as many *made in Italy* cheeses, are strategic because, despite higher costs, they meet high demand from international markets. Mean individual consumption of dairy products in Italy is calculated in 55 L of milk, 22.6 kg of cheese, 9.3 kg of yogurt and fermented milk, and 2.3 kg of butter per year in 2012 (*CLAL, Dairy Economic Consulting firm*, see text footnote 3). Further to "quality" products, "traditional" Italian products (i.e., products whose methods of processing, storage, and ripening have been consolidated over time, at least for 25 years) may run into the international trade, whereas "typical" Italian products are allowed for marketing in the production site only (Reg. 1151/2012, November 21, 2012).

In general, safety and security of milk and dairy products directly impact on public health and socio-economic development. It should also be considered that several opinions of the European Food Safety Authority (EFSA) on feed additives (11)

[1] http://www.nddb.org/information/stats/milkprodindia.
[2] http://www.nddb.coop/ndpi.
[3] http://www.clal.it.

and contaminants (12) pointed out that infants and children have higher intakes of dairy foods compared to adults, hence, are more exposed to substances present in milk. Among dairy products, prevention and management of AFM1 contamination of milk is a priority issue due to potential concerns for consumer's health.

Food Safety: State of the Art on AFM1 Risk Assessment

Risk assessment in food safety is defined for all populations groups, with a special attention for those identified as potentially more vulnerable. The One Health international use of terminology for risk assessment is driven by three standard-setting organizations, the Codex Alimentarius Commission (CAC) in relation to food safety, the World Organization for Animal Health (OIE) for animal health and the International Plant Protection Convention (IPPC) for plant health, under the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) of the World Trade Organization of which the European Union is a member. Regulation (EC) 178/2002, which establishes EFSA, contains definitions of a number of risk-related general terms which are similar to those provided by CAC. Although the European legislator does not dictate which of the three methodologies (and associated terminology) has to be used, should the major purpose of risk assessment be the regulation of international trade, the EFSA Scientific Committee concluded that particular care must be taken that the principles of CAC, OIE, or IPPC are followed strictly. EFSA Scientific Panels should identify which specific approach is most useful in dealing with their individual mandates, recognizing that different risk analysis standards have an impact on the terminology used by different EFSA Scientific Panels (13). Of course, in their turn, EFSA activities may (and should) contribute significantly to the development and updating of the scientific basis underlying OIE, IPPC, and especially CAC standards.

The characterization of a toxicological hazard in the food chain starts from the identification of health effects and of groups that may have an enhanced biological susceptibility, as well as the relationship between the extent and severity of effects and the intake level. In parallel, exposure assessment should consider the extent of exposure, as well as the most vulnerable food commodities and the most exposed population group(s), which may not be the same as the biologically susceptible group(s). Accurate, comprehensive, and comparable data on food consumption are crucial to assess risks.

AFM1 in Milk: Considerations on Toxicology and Carry Over

In ruminants, a considerable part of the ingested AFB1 is degraded in the rumen and does not reach systemic circulation. The absorbed fraction of AFB1 is transformed in the liver into a number of metabolites, including the hydroxy-metabolites AFM1, AFM2 (the analogous metabolite of AFB2) and AFM4. All AFM are excreted with milk, but AFM2 and AFM4 occur in milk at much lower concentrations than AFM1, thus are not considered as priority issues *per se*. AFM1 is a major AFB1 metabolite: it enters the systemic circulation or is conjugated in liver to glucuronic acid and excreted via bile: in its turn, circulating AFM1 can be excreted via the kidneys or be carried into milk.

Overall, AFM1 toxicological hazards, in particular hepatotoxicity and hepatocarcinogenicity (including genotoxicity), are comparable to those of the parent compound, even though AFM1 has a lower carcinogenic potency compared to AFB1, i.e., one or two orders of magnitude in experimental studies (14): considering that AFB1 ranks among the most potent carcinogens, AFM1 still retains a carcinogenic potential that is definitely worth of concern.

AF toxicosis in dairy animals does not represent a reliable alert for AF exposure and carry over into milk. Indeed, ruminants are generally less sensitive compared to non-ruminants because AFs are partly degraded by the forestomach flora. Most clinical signs recall liver dysfunction, such as anorexia, icterus, hemorrhages, and ascitis; at necropsy, the liver centrilobular necrosis and bile duct proliferation together with kidney lesions are fairly characteristic. In cattle, clinical signs occur after exposure to concentrations of 1.5–2.2 mg/kg feed, and in small ruminants even after exposure to >50 mg/kg feed. Early alerts might be represented by reduced milk production, photosensitization and, most important, reduced immune response including reduced response to vaccination. For such subtle effects, it is difficult to set a no-effect level: however, there is a margin of safety of at least 75 between toxic exposure levels (≥1.5 mg/kg feed) and the statutory limit (0.020 mg/kg feed) in Europe, which likely affords adequate protection (15, 16).

The excreted amount of AFM1 in the milk of dairy cows may represent at least 1–2% of the ingested AFB1; however, it is modulated by several factors (17). High-yielding dairy cows may show a higher carry over rate of AFM1 into milk, even above 6% of the ingested AFB1 (18).

Model calculations in Europe show that vulnerable high-yield cows exposed to feed with the current European maximum levels for AFB1 might produce in some cases milk with AFM1 levels above the European limit (19): the consumers of milk or dairy products from intensive, high-yield farming might be more exposed to AFM1, thus corroborating the magnitude of the AF problem both in low-scale and intensive farming. An important feature of AFM1 is the binding with the protein fraction of milk, and in particular the preferential binding to casein during milk coagulation (20). Therefore, AFM1 is liable to concentrate in cheese and other dairy products with a high protein content. Finally, there is widespread evidence of AFM1 carry over into the milk of other ruminant species (5, 20), but a thorough framework to assess the species-specific kinetics is lacking.

Is Aflatoxicol an Issue?

Aflatoxicol in a main metabolite of AFB1 in many species, from humans (21) to salmonids (22). Aflatoxicol has been somewhat overlooked, as it is even not mentioned in the EFSA opinion on aflatoxins in feeds (1); however, this metabolite is suspected to be an endogenous reservoir of AFB1 in the organism. Indeed, in poultry, aflatoxicol is the main component of total AF residues, with highest content in liver (23).

In ruminants, the situation may be different: in calf liver preparations *in vitro*, M1 and Q1 were the major chloroform

soluble AF metabolites, with small amounts of aflatoxicol (22). In two cows given a single oral high dose (0.5 mg/kg_{bw}) of AFB1, aflatoxicol was just a minor component of AF residues in cow's milk: the ratio of the concentrations for aflatoxicol, AFB1 and AFM1 was approximately 1:10:100, respectively (24). Also in the milk of goats experimentally treated with AFB1, aflatoxicol was present in trace amounts only whereas AFM1 predominated (25).

This data are not in accordance with an extensive study carried out on pasteurized cow's milk marketed in Mexico. Aflatoxicol was detectable (≥0.05 μg/L) in 13% of samples, 8% showing levels ≥0.5 μg/L: the upper value was 12.4 μg/L. AFB1 was present mainly in traces, the highest value being 0.4 μg/L. Autumn samples were significantly more contaminated with aflatoxicol, while no relationship was found with milk fat content (26). Interestingly, the same Mexican survey found that aflatoxicol concentrations were overall of the same magnitude order as those of AFM1 (40% of samples ≥0.05 μg/L, 10% of the samples ≥0.5 μg/L, upper value 8.35 μg/L) (27).

In real-life situations, exposure to contaminated feed may be a prolonged, low-level one or may follow a repeated pulse-like pattern: it might be possible that these scenarios would result in different metabolism of AFB1 compared to findings of the limited experimental studies, using high-dose short-term exposures. On a practical ground, and pending more robust data, one cannot rule out altogether that aflatoxicol might be monitored in milk and dairy products concurrently with AFM1 in order to achieve a sound estimation of consumer's exposure.

Interestingly, an isolated paper reported that aflatoxicol may bind to bovine uterine estrogen receptors *in vitro,* although its potency is much lower than the strong estrogen-agonist mycotoxin, zearalenone (28): to our best knowledge, the role of aflatoxicol as endocrine disrupter in the disorders of reproduction or lactation of cattle has not been further explored, nor any possible significance for consumers safety.

Traslational Research: State of the Art on AFM1 Risk Management

The FAO states that *the primary goal of the management of risks associated with food is to protect public health by controlling such risks as effectively as possible through the selection and implementation of appropriate measures* (29). The overall objective is to undertake legitimate measures to protect the health of consumers (in relation to food safety matters) at a level they consider necessary (sometimes defined "protection goals") in a consistent and transparent way while prohibiting unjustified restrictions of trade; thus, risk management should encompass proportionate, targeted, and effective measures.

The established prevention strategy of AFM1 contamination of milk is mainly good practice along the feed production chain, including the primary production of feed ingredients. In fact, aerobic in nature, mycotoxic fungi need air, moisture, nutrients, and suitable temperature for their growth and metabolism.

Climatic conditions in India are most conducive for mold invasion, proliferation, and production of mycotoxins. The high-risk areas in India are Kerala, Western India, Gangetic plains, north eastern and coastal areas of Andhra Pradesh, Karnataka, and Tamil Nadu. Unseasonal rains and related flash floods are very common in India, and this enhances the moisture content of the grains and therefore its vulnerability to fungal attack (30).

The high-risk area in Italy is the Po valley that is at the same time also the most milk productive area and the area whose climatic gradient is at highest risk. The average humidity rate here is about 80%; Piacenza, a town located in the center of the valley, shows an annual average of 80.1%. Apart from climate, climate changes (i.e., aspects like changes in temperature, relative humidity, insect attack, drought, and stress condition of the plants) influence the ability of molds to produce mycotoxins (2).

Due to the worldwide recognized problems expected for food and feed safety in relation to climatic changes, AFs in cereal crops can be listed among emerging risks. The EFSA Scientific Committee in 2007 stated that "*an emerging risk to human, animal and or plant health is understood as a risk resulting from a newly identified hazard to which significant exposure may occur or from unexpected new or increased significant exposure and/or susceptibility to a known hazard*" (31). Thus, AFM1 in milk is a well-known risk which, due to changing scenarios, shows an increasing and still poorly predictable exposure pattern. The emerging risks identification requires a high level of expertise due to the data gaps and uncertainties in the evaluation process. Since 2010, EFSA has provided scientific criteria and recommendations to address consistent and up-to-date activities on emerging risks in Europe and European Member States; since 2012 a Standing Working on Emerging Risks is on place (32). In Italy, the National Reference Centre on Emerging Risks has been implemented in Milano (Lombardia Region) as a structure of the Istituto Zooprofilattico of Lombardia and Emilia (located in Brescia): currently, main activities concern procedures and methodologies to assess and collect data sources and reinforcement of a knowledge exchange network inside and outside Italy, involving other institutions and stakeholders in conformity with the Regulation CE 178/2002 (33). The Italian system is definitely in place for biological hazards and animal diseases; other aspects, including emerging toxicological hazards, deserve implementation and strengthening of the expertise network.

Prevention in the Dairy Chain: Manageable Aspects

Control of AFM1 is routinely practiced in many industrialized and emerging countries, but the cost to track contamination continuously is hardly sustainable. No doubt, a consistent net of controls performed according to validated methods provides a highly valuable support both to reducing consumer's exposure and mainly to monitoring the space and time trends; however, stand-alone controls would present a remarkable shortcoming. Rejection of milk as unfit for consumption, hence food wastage, would be the only possible solution, especially when a significant sample fraction exceeds a given regulatory limit. Therefore, controls should be intended as the downstream component of a prevention strategy aimed at reducing consumers' exposure, primarily through the prevention of AFM1 contamination. AF contamination of crops in the field is the most critical step in Europe. Apart from weather conditions, the following points impacting on the quality of raw feed ingredients represent the

main known manageable factors contributing to the occurrence of AF in milk.

Feed Chain Facilities

Since the 1990s, an increase attention toward AFB1 contamination in Mediterranean Europe revealed that corn silage is a vulnerable item: during ensiling, under unfavorable circumstances, high temperature can facilitate the growth of toxigenic *Aspergillus* spp. Here is a list of critical factors (34, 35):

- Soil contamination by *Aspergillus* spores may be increased by modern cultivation systems excluding crop rotation, frequent irrigations with fixed modern equipment, and leaving a presence of infested and damaged pods in the field.
- Cultivar selection that disregard vulnerability to *Aspergillus* spp. as a selection criterion.
- Moisture content of grain or relative humidity surrounding the substrate.
- Delay in harvesting corn.
- Breaking of grains due to threshing machines or insect/rodent attack, implying an increased presence of impurities and grain fragments.
- Poor storage conditions, in particular when grains are stored without artificial drying phase in the wet seasons.
- Transport conditions, when grains are loaded and/or transported in wet and closely packed conditions (lack of aeration).

Further to ethic and scientific responsibility, the legal aspects linked to the EC Regulation no. 178/2002 (33) require that feed business operator implement a traceability system for the identification of corn stocks.

The corn suspected of contamination should be clearly identified, stored in separate compartments of the premises that should be easily distinguished from those containing the safe product. The level of AFs contamination should be considered during the pre-marketing phase to make choices based on the results of self-monitoring: the different batches will be sold as human food ingredient, animal feed material, or other (e.g., industrial) purposes. Corn having AF levels greater than the maximum legally tolerated levels must be destroyed: also industrial usages are not allowed (33). The current EU legislation does not allow dilution of corn or other feed material batches with AF levels above the legal limits at feed factory level. The European approach considers that not allowing dilution is a powerful mean to stimulate all operators throughout the chain to apply the necessary prevention measures to avoid contamination as much as possible. Last but not least, the same approach applied to feedingstuffs for dairy cows must be applied to feedingstuffs for small dairy ruminants.

Farm

In both cases of feed manufacturing in-house and feed purchasing, the farmer should pay special attention to the preliminary check of corn stocks in order to verify safety through standardized sampling procedures.

The experienced check of quality and origin of feed materials at farm is all important, especially in economically developing countries, where most farmers do not have a consistent technological support. Clean livestock feed holds the key to clean milk. The majority of farmers in most milk-producing states in India feed cereals or agricultural/oilseed by-products to their dairy animals. Such AF-vulnerable feed materials as cereals (maize, sorghum, etc.) and oilseeds (peanuts, soybean, etc.) constitute more than 70% of cattle feed (30). Moreover, the food that is declared unfit for human consumption often finds its way as feed for animals. Indeed, a number of reports indicate the presence of high concentrations of AFs in cattle feed in India; the situation may be worsened by the adoption of new techniques for feed preserving without due considerations for safety, e.g., silages are more vulnerable to *Aspergillus* if anaerobic conditions are not strictly controlled (36).

Strategies to Minimize Feed Contamination by AFB1

Clean livestock feed holds the key to clean milk. Intervention practices point at reducing AF contamination in the field and preventing AF formation during storage. New techniques for preserving green fodder such as silages are unsafe if anaerobic conditions are not strictly controlled (e.g., artificial drying in the whet seasons).

Selection of Resistant Cultivars. Strengths and weaknesses of biological control (e.g., breeding for introduction of a atoxigenic strain to the crop environment to compete with toxigenic strain) and enhanced plant resistance (e.g., resistance to the fungus, inhibition of AF biosynthesis, resistance to insects) have been reviewed, as well as relevant challenges in economically developing areas (34, 35).

Silage Additives. Worldwide, a high proportion of the ruminant diet consists of silages made of forage crops. In practice, silages are often contaminated with mycotoxins, including AFB1: when silage conditions are inadequate, a significant production of toxins may occur also during ensiling. In the large mass of ensiled feed, mycotoxin may be not distributed homogenously, rather, it may occur in some hot spots. Several feed additives, either chemicals or bacterial strains, are proposed to improve the ensiling process in Europe. Thus, it is relevant to know their effect, if any, on AF production and persistence. The use of formic acid appeared to somewhat favor the production of AFB1 and is discouraged in Europe (37); conversely, interventions with microbial additives that can enhance aflatoxin degradation can be a promising strategy (38).

Feed Additives. Mycotoxin binders/adsorbing agents to *reduce AF bioavailability* are permitted only in complete feeds with levels of AF or other mycotoxins not higher than the maximum tolerated limit. Indeed, the EFSA has a quite strict approach toward feed additives intended as mycotoxin binders. Several compounds successfully reduce the bioaccessibility of AFs from contaminated feeds *in vitro.* The treatment of contaminated feeds with mycotoxin binding agents may be useful to protect animal health and avoid milk contamination by the carcinogenic AFM1 metabolite. However, mycotoxin binders may impact animal health, e.g., by interfering with the absorption of nutrients or medications (39). A potential alternative strategy is to act on the *Aspergillus* metabolism within feedingstuffs, by

inhibiting AF biosynthesis or promoting degradation into non-toxic metabolites by biotransforming agents such as bacteria/fungi or enzymes (39). The EFSA approach toward feed additives intended to reduce AF contamination is consistent with the general European policy identifying a high level of food safety (40) and prevents unsafe material to be recovered for use in the food chain. On the other hand, one might argue that making no attempt to recover contaminated feeds would eventually lead to wastage of resources and to a weakening of dairy chain sustainability, especially in economically developing countries scenarios other than Europe. Local practices in developing countries may be investigated for their effectiveness: interestingly, lactic acid fermentation of grain-based materials may result in AF degradation (41). In all cases, considering the serious risks for consumer's health related to AFM1 in milk, approaches to recover contaminated feedingstuffs should be strictly regulated and surveyed.

Strategies to Minimize AFM1 in Living Animals and Their Products

Animal Detoxification Systems. Once ruminants are exposed to AF, attempts may be made to support the animal's capacity for detoxification either in rumen or liver.

Processed Dairy Products. In India, processing of milk is limited to pasteurization or fermentation, and both these methods are not capable of reducing AF or its metabolite. In fermented dairy products, AFB1 is transformed into the non-toxic AFB2 and the less toxic aflatoxicol (42). Although no information is provided on AFM1, this finding may indicate that transformation of milk into fermented products could be a strategy for risk reduction in areas with high AFM1 contamination.

Operational Aspects

European Scenario

Similar to the approach adopted in different contexts for other high-concern contaminants, like dioxins, Europe considers two official thresholds for AFM1 in milk, a *alert threshold level* calling for action (0.04 μg/kg) and a *maximum tolerated level* (0.05 μg/kg) (43). When the *alert threshold level* is exceeded, the business food operator must inform the competent authority (CA) within 12 h and propose the corrective measures to apply; in general, these refer to good farming practices, e.g., modification of animal diet by reducing or cutting the feed material/source having the highest risk of contamination. Thus, whereas dilution of contaminated feed is not accepted as a standard risk management practice at feed factory level, it can be accepted as a temporary measure in the farms where the threshold level in milk is exceeded.

When the *maximum tolerated level* is exceeded, the business food operator must inform within 12 h the CA and all other food chain operators that have been supplied with the contaminated milk. Provisions are then dictated by the EU regulation and include suspension of milk delivery and/or sale, starting procedures for withdrawal from the market, and elimination of contaminated milk (44). A key tool to ensure the cross-border follow of information is RASFF, the Rapid Alert System for Food and Feed. RASFF ensures that urgent notifications are sent, received, and responded to collectively and efficiently. Currently, in Europe, the self-monitoring plan must assure the compliance with the maximum tolerated level of AFM1 (43). To make the monitoring effective, at least one sample of milk should be taken twice a week; most important, the plan should take into consideration risk categorization parameters, namely, the territory (e.g., climatic conditions), the production volumes, the results of previous controls as well as additional risk factors like the modification of the daily feeding rate or the opening of a new corn silage. A reliable tracking system for feed materials, and also for purchased animals, is a necessary complement to the self-monitoring plan at dairy farm level. At the level of dairy factory, a monitoring plan should be established taking into the risk categorization parameters mentioned above in order to identify farms, farm clusters or farming areas calling for an enhanced level of attention. At dairy factory level, where milk is often collected from multiple and different sources, it is especially critical to have a robust tracking system in place.

Finally, since the global market requires co-ordination of control activities and an overall strategy for risk management, since 2007 the EFSA is building a framework for collection of national dietary survey data from European Member States.

The Indian Scenario

Constraints in controlling AFM1 contamination are currently a complex problem in the emerging Indian scenario. Millions of small dairy owners who produce more than 60% of India's milk are resource-poor farmers with scant space and money for storing feeds and feed ingredients. The dairy industry that relies on milk supplies from such livestock owners needs to test samples for AF before pooling the milk for industrial processing; this may not be practical as testing and quantifying for each vendor is neither economical nor feasible. India has limited feed resources to meet the needs of a huge population of cattle and buffalo, while production of grains for direct human consumption has priority. This scarcity of feed resources forces the farmers and dairy owners to compromise on the safety and quality of feeds in order to fulfill the nutrient requirement of their livestock. Furthermore, these farmers, even though individually small and marginal, contribute altogether a major portion of milk to the dairy processing industries through milk unions/cooperatives; hence, traceability from such a multitude of rural enterprises remains a problem.

Several papers report data in AFM1 contamination of milk and dairy products in India (41); however, whereas many reports are issued, the reliability of findings and conclusions drawn is questionable. Several reasons do suggest caution. Sampling procedures may not be appropriate for ensuring true representation of contamination in the cattle population. Also, on many occasions, analytical methods used are either not appropriate or not properly validated so as to achieve desired accuracy. Further, these analyses may be done in non-accredited laboratories. There is a widespread recognition that a problem does exist, but the awareness on how to investigate it should be improved. However, recently the Food Safety and Standards Authority of India (FSSAI)

laid down regulations/guidelines (45) on sampling and analytical procedures to be followed for different chemical contaminants in various commodities/feed ingredients/mixtures for surveillance purposes. A legal limit for AFM1 is established at 0.5 μg/kg; however, while industrialized countries have set maximum permissible limits for AF levels in livestock feed, no legal limits exist for livestock feeds and fodder in India. Indeed, feed, rather than downstream control of milk, is the key point for AFM1 risk management.

In general, economically developing countries may adopt the maximum tolerated levels of AFB1 in feeds or AFM1 in milk as Europe or other industrialized countries; however, risk management may be different. In particular, in situations where food security is less consolidated than in Europe, consideration may be given to minimize wastage of food with high nutritional value. Besides the use of mycotoxin binders in feeds to reduce uptake by animals, dilution of contaminated feedingstuffs seem to be the preventive action of choice. In the case of contaminated milk, to date no reliable procedure to decontaminate milk for human consumption, other than dilution, is available.

Regulatory Aspects: The Food Safety Assessment and Management Structure in the Frame of the European Hygiene Package and the Role of Self-monitoring

The current regulations about food safety in Europe (Hygiene Package, collecting Reg. CE 852, 853, 854, 882, 2004), following the principles of the European Strategy for Food Safety (2000), clearly distinguish responsibilities and roles: the food business operator is the primary responsible assigned to guarantee the safety of feed and food that is put on the market. The tool in charge of the food operator is primarily the self-monitoring plan that is approved by the CA, systematically updated along with any foods process modifications, and then confirmed by the same CA. The programing of official monitoring activities is aimed to check the application of self-monitoring by the food-producing enterprise. Consequently, it is important that the public services responsible for food safety make available consistent, updated and evidence-based tools in order to support and facilitate risk prioritization and management by enterprises.

The toxicological characteristics and potential exposure of the general population, including children, make AFM1 a priority issue for the dairy chains; accordingly, a specific program should be in place for monitoring of AFM1 on raw milk delivered at processing plants. Such program should indicate the frequency of sampling, which should be based on both the production capacity and on-risk categorization indicators; the method of analysis, which must have been accredited; the tracking system of every single supplier; the corrective measures to be taken in the event of alert or maximum tolerated levels being exceeded; last but not least, operational guidelines should also include management actions in case of higher risk situations, such as when environmental and climatic conditions can increase the levels of contamination in corn or other major feed materials (34).

The high rate of increased levels of AFB1 in corn and AFM1 in milk in Northern Italy in 2003, in relation with highly unfavorable climate conditions (high temperatures, drought, and strong insect damage), was efficiently managed through a food chain approach that significantly reduced the chance for consumer exposure. The event of 2003 pointed out critical phases of self-monitoring. In Italy, there have been several recent alarms on corn contamination with AF related to changing climate conditions and the consequent presence of AFM1 in milk: this situation has prompted the Ministry of Health to issue a contingency plan (i.e., extraordinary operative procedure for the prevention and risk management of aflatoxins contamination in the dairy chain and in the production of corn for human and animal consumption in extreme climatic condition) to deal with emergency situations that may jeopardize both consumer's safety and the availability of nutrients from dairy products (46). The Italian Health system is highly characterized by One Health. It has two main characteristics. First, its remit includes all veterinary topics, including feeds, which is indeed rather unusual among EU member states. Besides reflecting the spearheading role of the Italian school in the development of veterinary public health, this approach has been adopted by European bodies (DG SANCO and EFSA) and it is consistent with the conceptual framework "from farm to fork." Second, the structure of the Italian Health system (in particular the food safety system, including official control and risk assessment in food safety) is shaped like a broad-based pyramid; the Ministry of Health provides the general policy to the regions, which have a strong autonomy in allocating resources. More in detail, the pyramid is structured at three levels: the Ministry of Health (first level) is the central CA for risk management; for risk assessment, the Ministry is assisted by the National Health Institute (ISS) and by the National Food Safety Committee, an independent expert body hosted in the Ministry premises. Since the system is a federal one, policies relevant to the management framework in the territory have to be negotiated within the State-Regions Council, that deals with all matters when the central authority overlaps with the (21) regional autonomies (second level). The federal approach to health matters is in place since 10 years and is now under debate because of several negative instances, including inconsistent approaches and lengthy political negotiations hindering decision. Within regions, the system is broken up in (146) local health units (LHUs), that are in charge of managing the risk on the territory (third level). Each LHU has a Prevention Department that includes a Veterinary service, divided in three areas (Animal Health and Welfare, Food Safety and Hygiene of establishments and premises). The LHUs lists the farms according to risk categorization criteria and assess both the resources available and the needs for intervention. The Food Safety area of the veterinary service is the territorial body in charge of both carrying out the official control in food safety and adopting suitable measures and actions for risk management, which include quantification of costs and reimbursements, if due.

The effectiveness of the official control system is continuously monitored through a randomized or targeted comparison with the self-monitoring system, which, to date, is based on farm's management documents and analytical data produced by the 10 Istituti Zooprofilattici (Institutes for the animals health and

safety of food products). Such comparison is usually mainly based on sample monitoring by the LHU system and its annual distribution of resources that must, of course, take into account also other items (compliance with international plans, audits). Last but not least, the European Commission developed, since the early 1990s, a hierarchical network of Community Reference Laboratories (CRLs) and National Reference Laboratory (NRL) in the Member States (47). This CRL-NRLs system aims at controlling and coordinating the work carried out by routine field laboratories commonly entrusted with analysis of residues and contaminants in Europe. The Institute for Reference Materials and Measurements of the European Commission Joint Research Centre is the CRL for AF, including AFM1, whereas the NRL is located at the Italian National Health Institute.

What Can Be Done More

India: What Can Be Done in the Frame of the Food Safety and Standards Act

In India, food safety has been recognized as an important component in protecting the health of people. However, in view of widespread poverty and malnutrition in economically developing countries like India, programs directed toward food security (to satisfy caloric needs and minimize hunger and malnutrition) have precedence over programs designed to ensure wholesomeness, quality, and safety of food.

In order to meet the global standards, the Government of India enacted an integrated food law called the *Food Safety and Standards Act* in August 2006, which came into effect from August 2011. The new FSSAI, established under this Act, has consolidated various policies setting the requirements for food safety, including machinery, premises, quality control, certification, packing, marking, and labeling standards for all food products; the Act aims at regulating food safety in India through one overarching regulation. Maximum tolerated levels for both domestically produced and imported milk and dairy products have been set by the authority for most of the contaminants and toxicants. The permissible limit for AFM1 in milk and dairy products is 0.5 μg/kg prescribed by the mandatory regulations of the country (FSSAI: Food Safety and Standards Rules 2011), in accordance with the CAC. As dairy product prices and income of dairy production continue to increase, the average dairy herd size is also increasing. In addition, interests from corporate investors have also facilitated construction of larger dairies partnering with dairy processors. Thus, Indian scenario is changing, and food safety standard and tools should cope with such change.

Integrating Biomarkers into the Control System

The European strategy for food safety (40) empowers the risk assessment approach and the "from farm to fork" principle. In the new EC perspective, the Official control must be increasingly integrated by renewed systems for self-monitoring by food business operators.

The ethical, scientific, and legal responsibility of food operator in the safety and quality of food products they put on the market requires the definition of good practices, self-monitoring plans (including Hazard-Analysis and Critical Control Points, or HACCP, of course) and traceability systems. On the other side, self-control plans like the mentioned two analyses per week have the weakness of being carried out basing only on statistical and economical criteria. Innovation in the food chain requires the optimization of results obtained from the resources devoted to self-control activities. In this view, the drivers for decision-making in self-control plans should be increasingly based on scientific inputs rather than statistics only.

On its side, scientific research is called to develop cost- and time-effective field methods/tools that can be transferred for self-control purposes. Innovative methods are also expected to complement the consolidated European system for official control: this is based on sophisticated and expensive laboratory instruments and techniques that require extensive sample pretreatment and personnel training, e.g., multi-analytic method based on liquid chromatography-electrospray ionization tandem mass spectrometry (48). Moreover, costly analytical methods imply that the sample is transferred from the field to the laboratory. This approach needs integration by validated biomarkers that can be increasingly emerging as measurable biochemical or molecular (parent toxin itself) indicators of contamination. They should be monitored directly on the farm or dairy factory to screen daily production and eventually allow timely corrective action. These biomarkers should be transferable, i.e., validated by the establishment of a dose–response relationship, and reliably measured under conditions of use and by food business operators. Biomarkers should be sampled in living animals; thus, matrices are blood/serum, milk, urine, feces. AFM1 in milk is a direct and relevant biomarker of exposure of AF in ruminants; further research is needed to identify biomarkers of effective dose, i.e., indicating that concentrations of AFM1 are reaching levels that may have relevant biological activities.

The biomarkers approach should be developed to complement the consolidated European system for official control (based on sophisticate laboratory instruments), thus implementing an integrated top-down and bottom-up approach (49, 50). This is particularly important for primary productions in economically developing countries, where environmental conditions and poor resources stress both chances of contaminations and challenges for prevention (51).

Promising technologies are being developed to prevent (e.g., heat, humidity, and antioxidant power of the environment) and early detect fungal contamination and remove materials containing fungi: tools include tests for chemical or physical changes occurring with fungal growth like electronic noses and tongues. Among possible field tools, biosensors for AFB1 are based on indirect assays, i.e., the presence of the AF is established by its interaction with a biological medium immobilized on the surface of the probe, either an antibody that selectively binds the antigenic AF (immunosensor) or an engineered micro-organism (bioluminescent whole-cell biosensor). Recently, proposed sensors are based upon the inhibition of enzymes. The biochemical (binding or inhibition) event triggers a signal that can be

detected by its optical, acoustic, or electrochemical properties: the advantages of electrochemical assays may include the low cost of production of the electrodes, amenability to miniaturization, and multiplexing (52). Critical points during development of field methods are matrix effects and use conditions (farm is not a university laboratory) as well as the need for a time-effective sample preparation, as well as measurement. Mammalian cell-based biosensors may detect active concentrations of toxic substances and are promising for field application due to their high speed, low cost, and considerable sensitivity (53). Some early metabolic effects might be useful to develop biomarkers of effective dose. AFM1 impairs the mitotic process, without effects on cell viability (54). AFB1 in rats has been associated with hypocalcemia, a decrease in absorption of calcium, and the impairment of availability of bile salts; the mechanism was the decrease of Vitamin D3 production and lipid absorption, which might be early effects at intestinal and/or feed conversion level. Additionally, AF affect also the bioavailability of other essential minerals including iron, phosphorus, and copper (55). Effects on these essential minerals would likely be related to reduced antioxidant response and also reduced immune response (e.g., impaired immunoglobulin production), which have both been related to AF exposure in farm animals. It would certainly be worthwhile to assess whether these early metabolic changes can be used as early biomarkers in milk, in order to support early intervention under self-monitoring practices (50).

Of course, no single metabolic parameter would have the appropriate specificity to signal a possible presence of an active concentration of AFM1; however, a panel of different parameters may be investigated as an AF "fingerprint." Such approach requires the investigation of the dose–response relationship linking the intake of AFB1, the presence of AFM1 in milk, and the possible metabolic biomarkers. Analogously, co-occurrence of mycotoxins different from AFM1 in milk should be investigated (56).

Endorse Scientific Research

With regard to AF, the following research needs are highlighted:

- Selection of cultivars of maize and other relevant crops that have reduced susceptibility toward the fungal infestation. The maize, third worldwide crop, needs protection at the production level.
- Integrated prevention strategies at pre-harvest or postharvest times, including (when required and feasible and upon a risk-benefit analysis) the search for methods of mycotoxin decontamination.
- Field study to assess prevention strategies in the field (including cultivar selection) as well as in feedingstuffs. Applicability (field studies) of prevention methods should be verified in the presence of climatic and pedoclimatic conditions as well as different farming methods.
- Sensitive and cost-effective methods for detection and screening of AF (including aflatoxicol) in feed and milk exploiting immunochemistry and sensor/biosensor technology. (Bio) sensor arrays have the potential to become widely accepted as a system for early alert and self-monitoring applications, provided that robust results on fully automated platforms are successfully generated and grids of (bio)markers are validated. This will result in higher protection of animal and human health and enormous cost saving to food business operators through the prevention and reduction of product recalls and reduced treatment costs. Fabrication techniques of the microelectronics industry, microchemical sensors and biosensors, novel artificial receptors for recognition of specific mycotoxins in conjunction with, for example, microchemical sensors, offers novelty in both recognition and transduction process. Such tools offer a realistic route to the development of analytical measurement systems for the rapid, on-site (out-of-laboratory) assessment of food raw materials and processed food.
- Update of estimate model for AFM1 carry over in consideration of developments in production systems and animal nutrition and, most important, in all relevant milk-producing species. These considerations and the toxicological risks related to AFM1 call for prevention, rather than management upon a crisis onset, considering that there is clear evidence that also feed ingredients from advanced economies may expose to high levels of AFB1.
- Strategies for farmers' information and risk perception to support the empowerment and proactive role of food primary producers in the protection of public health.
- Development of models for the prediction of biogeographical agricultural scenarios of cultivated plants as well as the related molds/mycotoxins.

CONCLUSION

The detection of AFM1 in milk is the direct and most appropriate biomarker of internal dose to assess and measure whether a dairy animal is exposed to the toxicity of AFB1, as well as to assess and verify the efficacy of any corrective action. At the same time, the detection of AFM1 is also a biomarker of human dietary exposure to a toxic contaminant such as AFM1. Under this view, the possibility of daily management of AFM1 level through biomarkers is a challenge for both human and animal health, i.e., for the One Health framework. The project ALERT[4] focuses on self-monitoring in the dairy chain. Indeed, milk is both highly consumed by infants, highly vulnerable to toxic contaminants, suited sentinel matrix for environmental monitoring purposes, and business core of a particularly precious and suffering group of food business operator like farmers. ALERT has the purpose of identifying and characterizing innovative metabolomic-based biomarkers for early warnings based on production and product anomalies and self-monitoring purposes, designing modern HACCP plans including tools to manage the toxicological risks, and establishing a long-term dialog between producers and research bodies for strengthening innovation (49). Regulatory (i.e., top-down) measures may have little impact in remote rural

[4]http://www.alert2015.it.

areas and in family farming communities in economically developing countries: here, bottom-up and communication activities are particularly crucial (49).

AUTHOR CONTRIBUTIONS

The authors contributed equally to the paper.

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REVIEW
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A Novel Strategy to Predict Carcinogenicity of Antiparasitics Based on a Combination of DNA Lesions and Bacterial Mutagenicity Tests

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Genotoxicity and carcinogenicity testing of pharmaceuticals prior to commercialization is requested by regulatory agencies. The bacterial mutagenicity test was considered having the highest accuracy of carcinogenic prediction. However, some evidences suggest that it always results in false-positive responses when the bacterial mutagenicity test is used to predict carcinogenicity. Along with major changes made to the International Committee on Harmonization guidance on genotoxicity testing [S2 (R1)], the old data (especially the cytotgenetic data) may not meet current guidelines. This review provides a compendium of retrievable results of genotoxicity and animal carcinogenicity of 136 antiparasitics. Neither genotoxicity nor carcinogenicity data is available for 84 (61.8%), while 52 (38.2%) have been evaluated in at least one genotoxicity or carcinogenicity study, and only 20 (14.7%) in both genotoxicity and carcinogenicity studies. Among 33 antiparasitics with at least one old result in *in vitro* genotoxicity, 15 (45.5%) are in agreement with the current ICH S2 (R1) guidance for data acceptance. Compared with other genotoxicity assays, the DNA lesions can significantly increase the accuracy of prediction of carcinogenicity. Together, a combination of DNA lesion and bacterial tests is a more accurate way to predict carcinogenicity.

Keywords: genotoxicity, carcinogenicity, antiparasitics, risk evaluation, DNA lesions

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INTRODUCTION

Antiparasitics are used widely throughout the world in humans and animals to kill or eliminate parasites *in vivo* and *in vitro*, and in public health to control diseases and prevent the spread of parasitism from livestock to humans. According to the pharmacological effects and the target parasite species, antiparasitics can be divided into three main groups: anthelmintics, antiprotozoal agents, and insecticides. Chemically based treatment remains the most frequently chosen tool to control parasitism. Unfortunately, the use of antiparasitics does not always result in the expected therapeutic success. The toxic effects were found to be responsible for the therapeutic failure of drug treatment (1). In the 1970s of the last century, it was reported that the chemicals had the capacity to cause cancer in both

animals and humans (2, 3). Genetic and carcinogenic damage was found to have important health implications for the induction of diseases, such as lung cancer (4), pancreatic cancer (5), bladder cancer (6), leukemia (7–9), and non-Hodgkin's lymphoma (10). Therefore, the regulatory agencies of Europe, the USA and Japan suggested that genotoxicity and carcinogenicity studies should be conducted to learn the benefit/risk ratio before commercial approval of pharmaceuticals.

It was recommended by the regulatory agencies that genotoxicity testing, which was considered to be a fundamental part of the carcinogenic risk assessment, should be performed prior to commercialization. It was forbidden to use compounds with proven genotoxic properties on humans except in rare cases with adequate justifications (11). According to the present guidelines for genotoxicity testing of pharmaceuticals (12–15), a standard test battery contains: (a) a test for gene mutations in bacteria, (b) an *in vitro* test with cytogenetic evaluation of chromosomal damage using mammalian cells or an *in vitro* mouse lymphoma thymidine kinase$^{\pm}$ gene mutation assay, and (c) an *in vivo* test for chromosomal damage using mammalian hematopoietic cells. These assays were considered the best approach for genotoxic hazard identification and potential carcinogenic risk prediction. However, some limitations of this standard test battery in detecting genotoxicity were found. The current revised guidelines of the Veterinary International Conference on Harmonization and ICH S2 (R1) suggested that it can detect the genetic toxicity of most substances. However, for some special chemicals such as antimicrobial, it was required to supply the bacterial assay with a validated *in vitro* test for gene mutation in mammalian cells to detect the genetic toxicity (12, 15).

How can we identify and analyze positive genotoxicity results, especially *in vitro* cytogenetics? Two main factors including cytotoxicity and the highest testing concentration of the tested chemicals have very important effects on the result of genotoxicity. The Organization for Economic Cooperation and Development (OECD) had changed over the years to find the most suitable toxicity required at the highest concentration. In the 1999 revision, it was recommended that at least 50% toxicity should be induced. The ICH S2B suggested that *in vitro* genotoxicity tests should be conducted up to a top concentration of 10 mM in 1997 (16). In fact, when the dose level exceeds 100 μM, the physiological biological reactions will be disorder and then result in positive findings in *in vitro* genotoxicity tests. Moreover, a study sponsored by the European Center for the Validation of Alternative Methods indicated that the high testing dose should be reduced because the false-positive results in *in vitro* genotoxicity occurred at concentration levels from 1 to 10 mM. Recently, the ICH updated the genotoxicity guidelines (**Table 1**) (11, 17). It reduced the highest dose to 1 mM and supported the *in vivo* genotoxicity assays.

Antiparasitics were used in the market for many years, and for a large proportion of them, genotoxicity and carcinotoxicity assays were performed prior to 1980, when the bioassays were not concordant with the present guidelines. Thus, it is necessary to re-evaluate the old data (especially the cytogenetic data) under the current guidelines of ICH S2 (R1) (17).

TABLE 1 | Summary of the ICH (S2B) and ICH S2 (R1) proposed revision to S2.

ICH (S2B)	ICH S2 (R1)	
Bacterial mutation (Ames) (positive)	**Bacterial mutation (Ames) (negative)**	
	Option 1	**Option 2**
In vitro mammalian cell test (10 mM)	*In vitro* mammalian cell test [1 mM]	No requirement
Chromosome aberrations or TK gene mutation test	Chromosome aberrations or TK gene mutation test or micronucleus test	
In vivo cytogenetic assay	*In vivo* cytogenetic assay	*In vivo* cytogenetic assay
	Suggest to be integrated into acute toxicity assays of 28 days	

ICH, International Committee on Harmonization of Requirements for Registration Pharmaceuticals for Human Use. It is a summary of the difference between the current ICH (S2B) guideline for testing of pharmaceuticals and the revised guideline of ICH S2 (R1) (15, 18).

For pharmaceuticals, whose clinical use is continuous for at least 6 months or intermittent in chronic recurrent conditions, the long-term carcinogenicity studies in rats and mice using lifetime treatment are required (19). This has remained the most frequently chosen testing strategy since proposed by regulatory authorities in 1970s. The objective of carcinogenicity studies is to discover whether a drug has the ability to cause carcinogenicity in animals and whether this tumorigenic potential poses a relevant risk to humans (19, 20). To make an evaluation of carcinogenic risks to humans, the International Agency for Research on Cancer (IARC) in the 1–101 volumes of IARC monographs was published in the years from 1972 to 2011 (21). It examined 940 drugs in various groups: the carcinogenicity studies were sufficient for 107 drugs (11.4%), limited for 59 drugs (6.3%), and inadequate for 266 drugs (28.3%); and the remaining 508 drugs (54.0%) were not classifiable in terms of their carcinogenicity to humans. However, it included only 10 antiparasitics: 2 antiparasitics (Metronidazole and Dichlorvos) were classified as possibly carcinogenic to humans (Group 2B), and 8 antiparasitics (Chloroquine, Chlordimeform, Danex, Deltamethrin, Fenvalerate, Malathion, Permethrin, and Pyrimethamine) were considered non-classifiable in terms of their carcinogenicity to humans (Group 3).

Based on the above mentioned, it is meaningful to verify the extent of antiparasitics having the available results of genotoxicity and carcinogenicity studies. It is also necessary to re-evaluate *in vitro* genotoxicity results according to the present revised guidance. Due to the bacterial mutagenicity test alone produced misleading positive in predicting the carcinogens, we compared the combinations of bacterial mutagenicity test and other genotoxicity assays (such as cytogeneticity *in vivo* and *in vitro*, DNA lesions and mouse bone marrow micronucleus), aiming to work out a novel strategy to predict carcinogenicity.

The 136 antiparasitics that are listed in both the human and veterinary pharmacopeia were authorized by China. Forty-three and 107 antiparasitics were obtained from the human pharmacopeia and veterinary pharmacopeia, respectively. Since some parasites, including helminths, schistosome, and tapeworm,

can infect both humans and animals, simultaneously, 14 antiparasitics (Albendazole, Amoscanate, Artesunate, Bithionol, Diethylcarbamazine, Ivermectin, Levamisole, Piperazine, Pyramine, Praziquantel, Mebendazole, Metronidazole, Niclosamide, and Semduramicin Soditium) can be used on both humans and animals.

The methodology of the major carcinogenicity and genotoxicity tests were summarized in **Table 2**. The collected information of genotoxicity and/or carcinogenicity of antiparasitics was obtained primarily from peer-reviewed journals (e.g., *Medline*, *Toxline*, and the *Registry of Toxic Effects of Chemical Substances*) (22), the US National Toxicology Program, the edition of *Physician's Desk Reference* (23–25), the Center for Drug Evaluation and Research of the Food and Drug Administration and some relevant websites, such as http://www.updata.usa.com, http://www.osha.gov, http://www.toxnet.nlm.nih.gov, http://www.ntp.server.niehs.nih.gov, http://www.potency.berkeley.edu, http://www.fda.gov/cder, http://www.scirus.com, and http://www.inchem.org. For some antiparasitics, the genotoxicity and carcinogenicity data are incomplete in terms of the absence of the dose, the indication of an exogenous metabolic system in the genotoxicity assays, and the sex in carcinogenicity assays. In such cases, we presented our data in tables as obtained in these experimental conditions except for special markings. Moreover, regarding the present guidelines, the equivocal results that we found in extensive research were marked as positive in this review.

RESULTS

Genotoxicity and Carcinogenicity of Antiparatics

For the present analyses, an antiparasitic was regarded as genotoxic when it produced positive or equivocal results in at least one of the standard battery tests, and as a rodent carcinogen when it increased tumor incidence. **Table 3** covers the information available on genotoxicity and carcinogenicity findings for each tested antiparasitic. The following genotoxicity assays were used: Ames (bacterial mutagenesis), sex-linked recessive lethal, *in vitro* cytogenetics (chromosome aberrations), *in vivo* cytogenetics [chromosome aberrations, micronucleus and sister chromatid exchange (SCE)], unscheduled DNA synthesis *in vitro* (UDS), MLA (mouselymphoma L5178Y TK$^{\pm}$ assay), and other types of genotoxicity studies, including DNA fragmentation, mammalian mutagenesis HGPRT, SCE *in vitro*, DNA strand break analysis *in vitro*, and the micronucleus assay *in vitro*. The long-term carcinogenicity test was carried out in mice, rats, and other species.

Table 4 summarizes the total number of antiparasitics and the following are included: the number of antiparasitics with at least one genotoxicity or carcinogenicity test result and with data required by the present guidelines; the number of antiparasitics only tested for genotoxicity or carcinogenicity. It also presents the antiparasitics with results in *in vitro* data required by present guidelines; the number of antiparasitics that have at least one result in long-term carcinogenesis assays in rats or mice; and

TABLE 2 | The methodology of the major carcinogenicity and genotoxicity tests.

Test system	Materials	Principle of reference
Bacterial mutagenicity	The following fi-M Salmonella strains were used for the bacterial reverse mutation assay: TA97a, TA98, TA100, TA102, and TA1535. All strains were checked for maintenance of genetic markers prior to study	This test was performed by a plate incorporation procedure as outlined by OECD No.471, 46 Redbook 2000 IV.C.1.a (26), Redbook 2000: IV.C.1.a (27), and Chinese standard guidelines (28)
Mouse lymphoma assay	The mouse lymphoma assay using the thymidine kinase (Tk) gene of L5178Y Tk$^{\pm}$ −3.7.2C mouse lymphoma cell lines was found to be the closest to the *in vivo* environment among the different *in vitro* mammalian and bacterial gene-mutation testings	The MLA was performed according to FDA toxicological principles for the safety assessment of food ingredients and OECD guidelines for the testing of chemicals. IV.C.1.c Mouse Lymphoma Thymidine Kinase Gene Mutation Assay (29) and Test Guideline 490: *In Vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene (30)
Chromosomal aberration assay	The potential of tested compound to induce structural and numerical chromosome aberrations was evaluated in Chinese hamster lung fibroblast cells (V79)	Chromosomal aberration assay *in vitro* according to OECD No.473 (31), Redbook 2000 IV.C.1.b *In Vitro* Mammalian Chromosomal Aberration Test (32)
Bone marrow erythrocyte micronucleus assay	For each treated animal, at least 1,000 polychromatic erythrocytes (PCE) were counted to determine the micronucleus frequencies and record the micronucleus occurrence rate per one thousand PCE, and the proportion of PCE to normochromatic erythrocytes (NCE) was evaluated by counting a total of 1,000 erythrocytes	This assay was conducted in accordance with OECD Guideline No.474 (33) and Redbook 2000 IV.C.1.d. Mammalian Erythrocyte Micronucleus Test (27)
HGPRT mutation test	Mutations were expressed during a period of 6–7 days, including two subculturing steps. Subsequently, mutant frequencies (mutants/106 cells) and cloning efficiencies were scored	This assay was carried out following standard test procedures (34)
Unscheduled DNA synthesis assay	Prior to drug treatments, peripheral blood lymphocytes were isolated from healthy individuals. The radioactivity was determined by Beckman Ls3801 liquid scintillation spectrometry	This assay was performed according to the OECD guideline number 482 (26, 34)
Long-term carcinogenesis assay in rodent	The animal were randomly assigned to four groups based on their body weights, and each group of animal were fed the basal diet mixed with tested compound for a total period of 78 weeks (mice) and 104 weeks (rat)	Long-term carcinogenesis assay was conducted according to the guidelines of Ref. (35, 36)

TABLE 3 | Genotoxic and carcinogenicity effects of antiparasitics.

Test system	Dose or concentration (LED or HID)	Result	Reference
1. Acriflavine (8048-52-0)			
Salmonella typhimurium (none), TA1537, TA1538, TA98	50 µg/plate	–	(37)
Salmonella typhimurium (rat, liver, S-9, aroclor1254), TA1537, TA1538, TA98	50 µg/plate	+	(37)
Gene mutation, *Aspergillus nidulans*		+	(38)
Chinese hamster ovary (CHO), CHO-K1-BH4 (HGPRT)	0.5–4 µg/l	+	(39)
Chromosome aberrations *in vivo*, Mammalian or early embryo		–	(40)
Forward and reverse gene mutation, host-mediated assay, *Salmonella typhimurium#*		–	(41)
Sex-linked recessive lethals and sex-chromosome loss		+	(42)
Micronucleus test *in vivo and in vitro*, chromosome aberrations, mammalian polychromatic erythrocytes, mammalian cell culture, non-human		+	(43)
Mitotic recombination or gene conversion, *Saccharomyces cerevisiae*		NC	(44)
Sperm morphology, mouse		+	(45, 46)
2. Albendazole (54965-21-8)			
Bacterial mutation (Ames)		–	(47)
SCE and micronucleus (MN) on human lymphocytes *in vivo*	15 mg/kg p.o. in diet for 28 days	+	(48)
Micronuclei in cultured peripheral blood lymphocytes *in vitro* and in cultured human lymphocytes	10–100 µg/ml	+	(49)
Cytogenetics *in vitro* and *in vivo*		–	(47)
Micronucleus assay with CHO-K1 cells *in vitro*		+	(50)
Long-term carcinogenesis assay, mice	400 mg/kg/day	–	(24)
Long-term carcinogenesis assay, rats	20 mg/kg/day	–	(24)
3. Amitraz (33089-61-1)			
Salmonella typhimurium, TA98, TA100, TA97, TA102	0–200 µg/plate	–	(51)
Genotoxic in the vibrio test	10^{-3} to 10^{-5} µg/plate	–	(52)
DNA damage on hamster cells *in vitro,* comet assay	3.75 µg/l	+	(53)
Long-term carcinogenesis assay. rat (oral)	0, 15, 50, 200 mg/l in feed for 104 weeks	–	(54)
Long-term carcinogenesis assay. mouse (oral)		–	(54)
4. Amodiaquine (86-42-0)			
Salmonella typhimurium, TA100, reverse mutation	0.1–5,000 µg/plate	–	(55)
Salmonella typhimurium, TA97A, TA102, TA104	0.1–1,000 µg/plate	–	(56)
Salmonella typhimurium, TA100 (rat, liver S-9, Phenobarbital), reverse mutation	0.1–5,000 µg/plate	–	(56)
Salmonella typhimurium, TA97A, TA102, TA104 (rat, liver S-9, Phenobarbital), reverse mutation	0.1–1,000 µg/plate	–	(56)
5. Amoscanate (26328-53-0)			
Salmonella typhimurium, TA1537, TA1535, TA100, TA1538, TA98, reverse mutation	0.1–1,000 µg/plate	–	
Salmonella typhimurium, TA1537, TA1535, TA100, TA1538, TA98 (rat, liver S-9, aroclor 1254 or Phenobarbital), reverse mutation	0.1–1,000 µg/plate	–	
Salmonella typhimurium, TA100 (rat, liver S-9, aroclor 1254 or Phenobarbital), reverse mutation	20–160 nmol/plate	–	(57)
6. Amphotericin B (1397-89-3)			
Bacterial mutation (Ames)		–	(47)
Chromosome aberrations, peripheral blood lymphocytes		–	(58)
Cytogenetics *in vitro* and *in vivo*		–	(47)
MLA		–	(47)
7. Atovaquone (95233-18-4)			
Bacterial mutation (Ames)		–	(47)
Cytogenetics *in vitro* and *in vivo*		–	(47)
MLA		–	(47)
Long-term carcinogenesis assay, mice (liver tumors)	human AUC × 5	+	(24)
Long-term carcinogenesis assay, rats	NR	–	(24)
8. Bithionol (97-18-7)			
Salmonella typhimurium (none), TA98, TA100, TA97, TA102, TA100, TA1535, TA1537, TA97	0.1–1,000 µg/plate	–	(59)
	0.1–6.6 µg/plate	–	(60)
Salmonella typhimurium (rat, liver, S-9, kanechlor 400) TA98, TA100, TA97, TA102	0.1–1,000 µg/plate	–	(59)
Salmonella typhimurium (Hamster, liver, S-9, Aroclor 1254) TA100, TA1535, TA97, TA98	1–200 µg/plate	–	(60)
Micronucleus test *in vivo*, chromosome aberrations, mammalian polychromatic erythrocytes		–	(61)
9. Bromofenofos (21466-07-9)			
Salmonella typhimurium (rat, liver, S-9, kanechlor 400), TA100, TA98, TA1535, TA1537, TA1538; *Salmonella typhimurium* (none), TA100, TA98, TA1535, TA1537, TA1538	0.005–0.5 mg/plate	–	(61)
Micronucleus test *in vivo*, chromosome aberrations, mammalian polychromatic erythrocytes (mouse)		–	(61)

(*Continued*)

TABLE 3 | Continued

Test system	Dose or concentration (LED or HID)	Result	Reference
10. Chlordimeform (6164-98-3)			
Salmonella typhimurium, TA1535, TA1537, TA98, TA100	1–7,500 µg/plate	–	(62)
Salmonella typhimurium, TA98, TA100, TA1535, TA1537, TA1538	1–2,000 µg/plate	–	(63)
			(64)
Recombination assay, *Bacillus subtilis* (H17 vs. M45)		–	(65)
			(63)
E. coli polA (WP_2 uvra), recombination assay, DNA effects (bacterial DNA repair)	10^{-5} g/ml	–	(65)
E. coli	1–7,500 µg/plate	–	(62)
UDS *in vitro*, DNA effects (Human diploid fibroblasts FL cell)	10^{-6} to 10^{-3} g/ml	–	(66)
		+	(66)
Chromosomal aberrations *in vitro* and *in vivo* human peripheral lymphocytes	MTD	–	(67)
Chromosomal aberrations *in vivo,* Chinese hamster cells (CHO), Voles living donor bone marrow cells	MTD	–	(68)
SCE, bone marrow cells in mice, Voles living donor bone marrow cells, Voles fibroblasts	10 mg/kg	+	(67)
	80 mg/kg	+	(63)
Micronucleus test, mice bone marrow cells *in vivo*, peripheral lymphocytes	77 mg/kg	–	(69)
Mitotic recombination or gene conversion, *Saccharomyces cerevisiae*		–	(44)
Neoplasms		+	(70)
Carcinogenicity studies in mouse and rat		+	(71)
Chromosomal aberrations, mouse bone marrow cells *in vivo*	100 mg/kg	+	(55)
11. Chloroquine (54-05-7)			
Salmonella typhimurium, TA97, TA1537, reverse mutation	250 µg/plate	+	(72, 73)
	200 µg/l		
Salmonella typhimurium, TA1977, TA1535, TA1537, TA1538, reverse mutation	600 µg/l	–	(74, 75)
	10 000 µg/plate		
Salmonella typhimurium, TA98, TA100, reverse mutation	0–10,000 µg/plate	+	(56, 73)
Salmonella typhimurium, TA98, TA100, TA1537, TA1538, reverse mutation	5,000 µg/plate	NC	(73, 76)
Salmonella typhimurium, TA97A, TA1537, reverse mutation	5,000 µg/plate	–	(73, 77)
Salmonella typhimurium, TA98, TA100, TA97A, TA100, reverse mutation	50 µg/plate	–	(73, 78)
	10,000 µg/plate		
Salmonella typhimurium, TA102, TA104, reverse mutation	5,000 µg/plate	–	(56, 73)
E. coli WP2 uvra, reverse mutation	5,000 µg/plate	NT	(72, 73)
E. coli, reverse mutation	300 µg/plate	+	(78)
Salmonella typhimurium, TA97A, TA100, reverse mutation	20–50 µg/plate	+	(78)
Salmonella typhimurium, TA97A, TA100 (rat, liver S-9, phenobarbital); *Salmonella typhimurium*, TA102, TA104; *Salmonella typhimurium*, TA102, TA104 (rat, liver S-9, phenobarbital), reverse mutation	0.1–10,000 µg/plate	–	(79)
E. coli polA (W3119 vs. P3478) Rec-assay, DNA effects (bacterial DNA repair)	0.1–10,000 µg/plate	+	(55)
Chromosome aberrations, mammalian cell culture, non-human, micronucleus test *in vitro*		+	(43)
SCE, mouse bone marrow cells *in vivo*	12.5 mg/kg	+	(78)
Chromosomal aberrations, mouse bone marrow cells *in vivo*	100 mg/kg	+	(55)
12. Closantel (57808-65-8)			
Chromosomal aberrations *in vivo*, bone marrow cells	0, 5, 10, 15, 20 mg/kg	+	(80)
13. Coumaphos (56-72-4)			
Salmonella typhimurium (none), TA98, TA1535, TA1537, TA1538, TA100, TA100, TA98	3.3–3333.3, 3.3–10,000, 0.3–333.3 µg/plate	–	(81)
Salmonella typhimurium (rat, liver, S-9, aroclor 1254), TA98, TA1535, TA1537, TA1538, TA100, TA100, TA98	3.3–3333.3, 3.3–10,000, 0.3–333.3 µg/plate	–	(81)
Salmonella typhimurium (none), TA98, TA100, TA1535, TA1537, TA1538	667, 1.000, 3.333, 6.667, 10,000 µg/plate	–	(82)
E. coli WP2 uvra, (none); *E. coli* WP2 uvra (rat, liver, S-9, aroclor 1254)	3.3–10,000, 0.3–333.3 µg/plate	–	(81)
E. coli, mouse, liver, S-9; *E. coli,* hamster liver, S-9, aroclor 1254	3.3–10,000, 0.3–333.3 µg/plate	–	(81)
Chromosomal aberrations *in vitro*, CHO cells (rat, liver, S-9, aroclor 1254)	100, 300, 1,000 µg/l	–	(83)
Chromosomal aberrations *in vitro*, CHO cells (none)	99.5, 299, 995 µg/l	–	(83)
Micronucleus *in vivo*, polychromatic erythrocytes	480 mg/kg of coumaphos at 98.0% purity	+	(82)
Carcinogenicity studies, rats	0 (1% peanut oil), 1, 5, 25 mg/l in diet for 24 months	–	(82)
Carcinogenicity studies, mouse	0, 10, 20 mg/l in diet	–	(84, 85)
Carcinogenicity studies, rats	0, 10, 20 mg/l in diet	–	(84, 85)

(*Continued*)

TABLE 3 | Continued

Test system	Dose or concentration (LED or HID)	Result	Reference
14. Cyfluthrin (68359-37-5)			
Salmonella typhimurium, TA98, TA100 (none); TA98, TA100 (rat liver S9), reverse mutation	1,000–5,000 µg/plate	–	(86)
Gene mutation, Ames/micronucleus test in cultured human peripheral blood lymphocytes		–	(86)
Chromosomal aberrations in cultured human peripheral blood lymphocytes; chromosomal aberrations *in vivo*	1,000, 2,000 mg/ml 250, 500, 1,000 mg/kg b.w.	+	(86)
SCE, in cultured human peripheral blood lymphocytes	500, 1,000, 2,000 mg/ml	–	(86)
SCE in blood lymphocytes	500, 1,000, 2,000 µg/l		
Micronucleus (MN) formation in cultured human peripheral blood lymphocytes	500, 1,000, 2,000 mg/ml	+	(86)
DNA damage on the epithelial cells of human nasal mucosa	0.05, 0.1, 0.5, 0.75, 1.0 mg/ml	+	(87)
DNA damage and comet assay in fish species	5.6 mg/l beta-cyfluthrin for 48 h	+	(88)
Chromosomal aberrations *in vitro*	500, 1,000, 2,000 µg/l	–	(86)
Mouse bone marrow cells *in vitro*	1,000 µg/l	+	(86)
15. Cypermethrin (52315-07-8)			
Salmonella typhimurium, TA98, TA100, TA1535		–	(89)
Micronuclei formation in bone marrow cells in rats; DNA damage in blood cells in rats	25 mg/kg b.w. p.o. for 28 days	+	(90)
Micronucleus test in mice *in vivo*		NC	(91)
Chromosomal aberrations (CAs) on human peripheral lymphocytes; SCE on human peripheral lymphocytes	12.5 + 2.5, 15 + 5, 17.5 + 7.5, 20 + 10 mg/ml	+	(92)
Micronucleus (MN) tests on human peripheral lymphocytes	12.5 + 2.5, 15 + 5, 17.5 + 7.5 mg/ml	+	(92)
Excision-repairable DNA damage in ICR mouse hepatocytes		–	(93)
DNA strand breakage and DNA hypomethylation in ICR mouse hepatocytes		+	(93)
Chromosomal aberrations on human peripheral lymphocytes	5, 10, 15, 20 mg/ml	+	(94)
SCE on human peripheral lymphocytes			
Micronucleus (MN) tests on human peripheral lymphocytes	5, 10 mg/ml	+	(94)
Chromosomal aberration (CA) in highly mitotic kidney cells; micronucleus (MN) tests in erythrocytes of a freshwater fish	0.4, 0.8, 1.2 µg/l for 48 and 72 h	+	(95)
DNA damage in vital organs in mouse	12.5, 25, 50, 100, 200 mg/kg b.w.	+	(96)
DNA damage using alkaline comet assay	25, 50, 75 mg/kg b.w. for 6–15 days	+	(97)
Transplacentally genotoxic			
Peripheral blood for MN test	20, 30, 40, 50 mg/l	+	(98)
Excision repairable DNA lesions		–	(99)
Long-term carcinogenesis assay, rat	75, 1,500 mg/kg b.w.	–	(100)
Long-term carcinogenesis assay, mouse	240, 1,600 mg/kg b.w.	–	(100)
16. Danex (52-68-6)			
E. coli, WP2 (rat, liver S-9, aroclor 1254)	500–10,000 µg/plate	+	(101)
E. coli, WP2 UVRA (rat, liver S-9, aroclor 1254)			
UDS Human fibroblasis			(66)
Salmonella typhimurium, TA100, reverse mutation	1–5,000 µg/plate	+	(101)
Salmonella typhimurium, TA1535, TA1535 (rat, liver S-9, aroclor 1254), reverse mutation	1.25–5,000 µg/ml	–	(102)
Salmonella typhimurium, TA104, TA100 (rat, liver S-9, aroclor 1254), reverse mutation	5–25 mg/plate	+	
Salmonella typhimurium, TA104, TA100, TA1535, TA97, reverse mutation	1–25 mg/plate	+	(102)
Salmonella typhimurium, TA1535, TA97 (rat, liver S-9, aroclor 1254), *Salmonella typhimurium*, TA100, TA98, TA104		–	(102)
Salmonella typhimurium, TA100, TA98, TA97; *Salmonella typhimurium*, TA100, TA98, TA104 (rat, liver S-9, aroclor 1254)	0.1–25 mg/plate	–	(102)
Salmonella typhimurium, TA100, TA98, TA97, TA1535, TA1537 (rat, liver S-9, aroclor 1254), reverse mutation	500–5,000 µg/plate	–	(102)
Salmonella typhimurium, TA1535, TA1537, reverse mutation	100–10,000 µg/plate	–	(102)
Salmonella typhimurium, TA98, TA100, reverse mutation	33–10,000 µg/plate	–	(103)
Chromosomal aberrations, V79	0.4–4,000 mmol	–	(104)
	0.04–0.8 mmol	+	
Micronucleus *in vivo*, mouse	100 or 200 mg/kg	+	(105)
	3.13, 6.25, 12.5, 25 mg/kg		(106)
UDS human cells		–	(103)
17. Deltamethrin (52918-63-5)			
Salmonella typhimurium, TA98, TA100, TA1535, TA1537, and TA1538		–	(70)
Salmonella typhimurium, TA98, TA100	20–600 µg/plate	–	(107)

(*Continued*)

TABLE 3 | Continued

Test system	Dose or concentration (LED or HID)	Result	Reference
Salmonella typhimurium, TA98, TA100, TA1535, TA1537, TA1538	0–5,000 μg/plate	–	(108)
Chromosomal aberrations, CHO cells *in vitro*	0, 19, 38, 75, 150 μg/l	+	(108)
Micronucleus test, mice bone marrow cells *in vivo*	8.0–90.0 mg/kg	+	(109)
V79/6-thioguanine, Chinese hamater V79	4–40 μg/l	–	(107)
Carcinogenesis assay. mouse (dermal)	0, 1, 2,4 mg/kg b.w. for 32 weeks	–	(110)
Long-term carcinogenesis assay. Rat (intragastric)	0, 3, 6 mg/kg for 120 weeks	–	(111)
Long-term carcinogenesis assay. Rat (oral)	0, 25, 125, 500, 800 mg/l in feed for 2 years	–	(108)
Long-term carcinogenesis assay. Mouse (oral)	0, 10, 100, 1,000, 2,000 mg/l in feed for 97 weeks	–	(108)
Long-term carcinogenesis assay. Mouse (intragastric)	0, 1, 4, 8 mg/kg in diet for 120 weeks	–	(111)
18. Diaveridine (5355-16-8)			
Bacterial umu test, *S. typhimurium,* TA1535	0.1, 0.3, 1.0, 3.0 μg/l	–	(112)
Salmonella typhimurium, TA100, TA98, TA97, TA102	0.5, 1.0, 2.5, 5.0, 10, 25 μg/plate	–	(112)
E. coli, WP2 uvra/pkm101	0.5, 1.0, 2.5, 5.0, 10, 25 μg/plate	–	(112)
Chromosome aberration in cultured Chinese hamster CHL cells	12.5, 25, 50, 100 μg/l	+	(112)
Micronucleus test in rodent bone marrow, mice and rats	500, 1,000, 1,500, 2,000 mg/kg b.w.	–	(112)
Comet assay in five mouse organs *in vivo*	1,000, 1,500, 2,000 mg/kg b.w.	+	(112)
Salmonella typhimurium, TA98 (rat, liver, S9)		–	(113)
Salmonella typhimurium, TA98 (Hamster, liver, S9), TA100 (rat, liver, S9) reverse mutation		–	(113)
Salmonella typhimurium, TA100 (Hamster, liver, S9)		+	(113)
Salmonella typhimurium, TA97, TA98, TA100, TA102 (rat, liver, S9) reverse mutation	0.1–3.0 μg/l	–	(112)
Salmonella typhimurium, TA1535, TA1535 (rat, liver, S9)	10 μg/l	–	(112)
Chromosomal aberrations	100 μg/l,48 h	+	(112)
Mouse bone marrow cells *in vivo*, rat		–	(112)
Comet assay (liver, kidney, lung, spleen)		+	(112)
Comet assay (bone marrow)		–	(112)
19. Diazinon (333-41-5)			
Salmonella typhimurium, TA1535, TA1536, TA1537, TA1538 carcinogenicity studies *in vivo*			(114)
Salmonella typhimurium (TA98, TA100, TA1535, TA1537, and TA1538), reverse mutation		–	(115, 116)
Salmonella typhimurium, TA98, TA97, TA102, TA1535, TA1537, TA100 reverse mutation	20–80 mg/l, 100–10,000 μg/plate	–	(117, 60)
E. coli WP2 uvra, tryptophan reverse gene mutation		–	(101)
E. coli (rat, liver S-9, aroclor 1254), mouse, Hamster	0.3–333.3, 1–100, 10–10,000 μg/plate	–	(81)
MNs (micronuclei) in rat lymphocytes	150 mg/kg b.w.	+	(118)
SCE, non-human CHO cells *in vitro*		+	(119)
SCE, human Laz-007 B lymphoid cells *in vitro*		+	(120)
DNA effects (bacterial DNA repair), *Bacillus subtilis* (H17 vs. M45), recombination assay,		NC	(79)
DNA damage in human blood lymphocytes *in vitro*	750 μg/l	+	(121)
UDS *in vitro*, DNA effects human diploid fibroblasts		–	(66)
Mitotic recombination or gene conversion, *Saccharomyces cerevisiae*		–	(44)
Long-term carcinogenesis assay. mice	0, 100, 200 mg/l in diet	–	(122, 123)
Long-term carcinogenesis assay. rats	0, 400, 800 mg/l in diet		(84, 85)
20. Dichlorvos(DDVP) (62-73-7)			
Salmonella typhimurium, TA100	500–1,000 μg/plate	+	(124)
	100–6,666 μg/plate	+	(125)
	0.5–500 μg/plate	++	(126)
	100–5,000 μg/plate	+	(60)
	100–1,000 μg/plate	+	(127)
Salmonella typhimurium, TA98	100–6,666 μg/plate	+	(125)
	100–5,000 μg/plate	+	(60)
Salmonella typhimurium (TA98, TA100, TA1535, TA1537, TA1538), histidine reverse gene mutation		+	(115, 116)
Salmonella typhimurium, forward and reverse gene mutation, mitotic recombination and gene conversion, DNA effects, host-mediated assay		–	(128)

(*Continued*)

TABLE 3 | Continued

Test system	Dose or concentration (LED or HID)	Result	Reference
SCE *in vitro*, human lymphocytes		–	(129)
SCE *in vitro*, non-human	With dose response	+	(130)
SCE *in vitro*, human, human lymphocytes		NC	
E. coli (rat, liver S-9, aroclor 1254)	22.6 µg/l	+	(101, 131)
E. coli polA (W3119 vs. P3478), Recombination assay, DNA effects (bacterial DNA repair)		+	(79)
E. coli WP2 uvra, tryptophan reverse gene mutation		+	(101)
E. coli	5 mg/ml	+	(132)
		+	(101)
		+	(133)
Chromosome aberrations, mammalian polychromatic erythrocytes		NC	(134)
Chromosomal aberrations *in vitro*, CHO cells	16, 50, 100, 160 µg/l	+	(135)
	50, 160, 500, 1,600 µg/l	+	
	500, 750, 1,000 µg/l	+	
Chromosome aberrations, *Allium cepa*	With dose response	+	(136)
Chromosome aberrations, non-human bone marrow *in vivo*		–	(40)
Chromosome aberration, mammalian germ cells *in vivo*		–	(137)
Chinese hamster V79	1.25–5 µg/l	–	(104)
CHO, CHO-k1-bh4 (HGPRT)/6-thioguanine	50–150 µg/l	+	(138)
Mitotic recombination or gene conversion, *Saccharomyces cerevisiae*		+	(44)
Mouse lymphoma, L5178Y (TK+/TK–)	0–0.33 µg/l, 0–0.12 µg/l, 0–0.24 µg/ml	+	(130)
Micronucleus *in vivo*, erythrocytes		–	(130)
Mouse lymphoma, L5178Y (TK+/TK–)	6.25–200 µg/l	+	(125)
UDS human cells	6.5–650 mg/ml	+	(104)
UDS rat hepatocytes	0.005–1.25 mg/ml	–	(131)
UDS mouse forestomach epithelium	1–100 mg/kg	–	(139)
Sex-linked recessive lethal gene mutation, *Drosophila melanogaster*		–	(140)
Sperm morphology, mouse		NC	(45, 46)
Dominant lethal test, rodents	With dose response	NC	(137)
Recombination assay, spot test, DNA effects, *Bacillus subtilis* (H17 vs. M45)		+	(79)
Carcinogenicity studies *in vivo,* non-human		NC	(141)
Carcinogenicity studies, mouse	0, 317, 635 mg/l in diet	–	(142)
Carcinogenicity studies, rat	0, 150, 318, 326, 635 mg/l in diet	–	(142)
Long-term carcinogenesis assay. Rat	0, 4, 8 mg/kg in corn oil for 105 weeks	+	(125)
Long-term carcinogenesis assay. Mouse	0, 10, 20 mg/kg in corn oil for 105 weeks	+	(125)
Long-term carcinogenesis assay. Rat	0, 0.1 mg in 0.2 ml water for 111 weeks	–	(143)
Long-term carcinogenesis assay. Mouse	0, 10, 20 mg/kg in corn oil for 104 weeks	+	(144)
Long-term carcinogenesis assay. Rat	0, 4, 8 mg/kg in corn oil for 104 weeks	+	(125)
21. Dimetridazole (551-92-8)			
Salmonella typhimurium, TA98, TA100, TA1535, TA1537, TA1538		+	(145)
Salmonella typhimurium, TA100		+	(146)
Salmonella typhimurium, TA97, TA98, TA100, TA102	50–200 µg/plate	+	(147)
Comet assay in human lymphocytes	354.3 mg/ml	+	(148)
22. Fenbendazole (43210-67-9)			
Salmonella typhimurium (none), TA100, TA97, TA98, TA102 (rat, liver, S-9, aroclor 1254), TA100, TA97, TA98, TA102.	5–1,000 µg/plate	–	(149)
Chromosomal damage in Chinese hamster lung (CHL) cells	0.78 mg/ml	+	(150)
Cytotoxicity to 10T1/2 cells	0.04–1.60 mg/ml	+	(150)
Morphological transformation in mouse embryo fibroblasts	0.08–0.4 mg/ml	+	(150)
23. Fenchlorphos (299-84-3)			
SCE, human somatic cells *in vitro*		+	(120)
24. Fenthion (55-38-9)			
Ames reverse gene mutation	0.1–20 µg/plate	–	(151)
Bacillus subtilis (H17 vs. M45)	20 µg/plate	–	(132)
E. coli polA (W3119 vs. P3478), recombination assay, DNA effects(bacterial DNA repair)		NC	(79)

(*Continued*)

TABLE 3 | Continued

Test system	Dose or concentration (LED or HID)	Result	Reference
E. coli WP2 uvra, tryptophan reverse gene mutation		–	(101)
SCE, non-human V79 cells *in vitro*		+	(152)
SCE, human somatic cells *in vitro*		NC	(120)
Mitotic recombination or gene conversion, *Saccharomyces cerevisiae*		–	(44)
Bacillus subtilis (H17 vs. M45), recombination assay, spot test, DNA effects (bacterial DNA repair)		NC	(79)
Drosophila melanogaster, sex-linked recessive lethal mutation		NC	(140)
UDS, human diploid fibroblasts *in vitro*		–	(66)
UDS, thymidine incorporation, rat hepatocytes	0, 5.0, 7.5, 10.0, 15.0, 30.0 µg/l	+	(153)
Chromosomal aberrations, CHO cells *in vitro*	0, 0.02, 0.04, 0.08, 0.15 µg/l	–	(153)
SCE *in vivo* and UDS *in vitro*		+	(154)
Chromosomal aberrations, human peripheral lymphocytes *in vitro*	0.5, 1.5, 2.5, 5.0 µg/ml	+	(151)
Long-term carcinogenesis assay. Mice	0, 0.1, 1, 5, 25 mg/l in diet for 2 years		(153)
Long-term carcinogenesis assay. Rats	0, 5, 20, 100 mg/l in diet for 2 years	–	(153)
Long-term carcinogenesis assay. B6C3F1 male mice	10 mg/l in diet for 103 weeks	+	(155)
Long-term carcinogenesis assay. B6C3F1 female mice	10 mg/l in diet for 103 weeks	–	(155)
Long-term carcinogenesis assay. F341 rat	200 mg/l in diet for 103 weeks	–	(155)
25. Fenvalerate (51630-58-1)			
Salmonella typhimurium, TA104	100–3,500 µg/plate	–	(156)
TA100	500–4,000 µg/plate	–	
TA97	100–4,000 µg/plate	–	
TA100	500–4,000 µg/plate	–	
TA98	100–3,000 µg/plate	–	
Micronuclei in bone marrow in mice *in vivo*	10, 20 mg/kg by i.p.	+	(157)
Peripheral blood for MN test	25, 50, 75, 100 mg/l	+	(98)
Chinese hamster V79 gene mutation	4–40 µg/l	–	(107)
Excision repairable DNA lesions		–	(99)
Chromosomal aberrations, Chinese hamster ovary (CHO-K1) *in vitro*	10, 25, 50, 100,150 µg/l	+	(158)
Long-term carcinogenesis assay. Rat (oral)	0, 1, 5, 25, 250 mg/l in diet for 2 years	–	(159)
Long-term carcinogenesis assay. Rat (oral)	1, 5, 25, 250, 1,000 mg/l in diet for 2 years	–	(160)
Long-term carcinogenesis assay. Mouse (oral)	0, 10, 50, 250, 1,250 mg/l in the diet for 2 years	–	(161)
Long-term carcinogenesis assay. Mouse (intragastric)	0, 40, 80 mg/kg in arachis oil for 120 weeks		(111)
26. Fipronil (120068-37-3)			
Salmonella typhimurium, TA98, TA100, TA1535, TA1537	0–0.5 mg/plate of 90.6% fipronil	+	(162)
Chromosomal aberrations, human lymphocytes *in vitro*	0, 4.69, 9.38, 18.75, 37.5, 75, 150, 300 µg/l	+	(162)
Sister chromatid exchanges (SCEs); DNA damage, comet assay *in vitro;* micronuclei (MN) in human peripheral blood lymphocytes	0.7,0.3 µg/l	+	(163)
Comet assay with gillsin, the fish Rhamdia Quelen; nuclear morphological alterations	0.05, 0.10, 0.23 µg/l	–	(164)
Micronucleus test in the Piscine	0.10, 0.23 µg/l	+	(164)
Chinese hamster V79 cells, HGPRT mutations	0, 0.8, 4, 20, 100, 500 µg/l	+	(162)
Bone marrow polychromatic erythrocytes, mouse micronucleus *in vivo*	0, 1, 5, 25 mg/kg b.w.	+	(162)
Long-term carcinogenesis assay. Rat (oral)	0, 0.5, 1.5, 30, 300 mg/l of 95.4% fipronil in diet for 104 weeks	+	(162)
Long-term carcinogenesis assay. Mouse (oral)	0, 0.1, 0.5, 10, 30 mg/l of 95.4% fipronil in diet for 78 weeks	+	(162)
27. Flubendazole (31430-15-6)			
Salmonella typhimurium (none), TA100, TA98; *Salmonella typhimurium* (rat, liver, S-9, aroclor 1254), TA98, TA100	0.01–10 µg/plate	–	(165)

(*Continued*)

TABLE 3 | Continued

Test system	Dose or concentration (LED or HID)	Result	Reference
28. Furapromide (1951-56-0)			
Chromosomal aberrations, V79 cells		+	(166)
Salmonella typhimurium, TA98, reverse mutation *Neurospora crassa*, forward gene mutation		+	(167) (168)
Chromosomal aberrations, V79, HPRT	10–120 µmol	+	(166)
Saccharomyces cerevisiae, mitotic recombination or gene conversion	7–567 µmol	+	(44)
Salmonella typhimurium (TA98, TA100, TA1535, TA1537, and TA1538)		+	(115, 116, 169)
29. Furapyrimidone (75888-03-8)			
Salmonella typhimurium, TA98, TA100; *Salmonella typhimurium*, TA98, TA100 (S-9), reverse mutation	0.01–10 µg/plate	+	(170)
30. Imidacloprid (138261-41-3)			
Salmonella typhimurium, TA100 (rat, Liver, S-9)	25–10 µg/plate	–	(171)
Salmonella typhimurium, TA98 (rat, Liver, with or without S-9)	25–100 µg/plate	+	(171)
Salmonella typhimurium, TA97, TA98, TA100, TA102 (S9)	40, 200, 1,000, 5,000 µg/plate	–	(172)
Micronuclei test in mouse bone marrow	23, 45, 90 mg/kg b.w.	–	(172)
Chromosome aberration in primary spermatocytes testicle	38, 75, 150 mg/kg b.w.	–	(172)
Micronucleus (MN) test *in vivo,* amphibian	165 mg/kg b.w.	+	(173)
Comet assay *in vivo,* amphibian	0.05, 0.1, 0.2, 0.5 mg/kg b.w.		
Bone marrow polychromatic erythrocytes in rats	100, 200, 300 mg/kg b.w.	+	(174)
Micronucleus *in vitro*, Human peripheral blood lymphocytes (rat, liver, S9)	0.2, 2, 20 µg/l	+	(175)
Micronuclei test in human peripheral lymphocytes SCE test in human peripheral lymphocytes	0.1, 0.5 mg/l	+	(176)
Comet assay, DNA damage, SCGE	0.05, 0.1, 0.2, 0.5 mg/l	+	(176)
Micronucleus (MN) formation in human lymphocytes *in vitro*	50 µg/l	+	(174)
SCE induction in human lymphocytes	Combination with metalaxyl at 100, 200 µg/l	+	(174)
SCE induction in human lymphocytes	0.1, 1, 5, 10, 50, 100 µg/l	–	(174)
Micronucleus in the rat bone marrow	200, 300, 400 mg/kg b.w.	+	(174)
DNA damage, Comet assay, SCGE		+	(177)
Micronucleus (MN) tests on *Hypsiboas pulchellus* tadpoles	25 mg/l for 96 h	+	(178)
DNA single-strand breaks on *Hypsiboas pulchellus* tadpoles	37.5 mg/l for 96 h	+	(178)
Nuclear abnormalities	12.5–37.5 mg/l	–	(178)
Chromosome abnormality on sperm deformity of the earthworm	0.2 mg/kg dry soil	+	(179)
DNA damage in human peripheral blood lymphocytes exposed *in vitro*		+	(180)
Long-term carcinogenesis assay. Rat (male)	0, 100, 300, 900, 1,800 mg/l	+	(181)
Long-term carcinogenesis assay. Mice	0, 100, 330, 1,000, 2,000 mg/l	–	(181)
31. Ivermectin (70288-86-7)			
Carcinogenicity studies, rats	0, 2 mg/l in diet for 1 year	–	(182)
32. Lindane (58-89-9)			
Salmonella typhimurium, *Serratia marcescens*, forward and reverse gene mutation, host-mediated assay		NC	(128)
MN-forming activity in MCF-7 and PC-3 cells	10^{-12}, 2×10^{-12}, 10^{-11}, 2×10^{-11}, 5×10^{-11} g/ml	+	(183)
Chromosomal aberrations in human peripheral lymphocytes *in vitro*		+	(184)
Micronucleus (MN) formation in bone marrow *in vivo*		+	(185)
Sex-linked recessive lethal gene mutation, *Drosophila melanogaster*		NC	(140)
Chromosome aberrations, *Allium cepa*		+	(136)
Chromosome aberrations, *Hordeum vulgare* (barley)		+	(186)
Chromosome aberrations, *Vicia faba*		+	(187)
Chromosome aberrations, Tradescantia species		+	(187)
Mitotic recombination or gene conversion, *Saccharomyces cerevisiae*		+	(44)
DNA damage and the risk for cancer on human tonsillar	0.5, 0.75, 1.0 mg/ml	+	(188)
Carcinogenicity studies in mouse	12.5, 25 and 50 ppm for 80 weeks	–	(189)
Carcinogenicity studies *in vivo*, non-human		+	(141)
Long-term carcinogenesis assay. AVy/AVy, AVy/a, A/a mouse	160 mg/kg/day	+	(190)
Long-term carcinogenesis assay. Rats	0, 0.05, 0.45, 4.5, 18.7 mg/kg/day (male) 0, 0.06, 0.57, 5.6, 23.1 mg/kg/day (female)	–	(54)

(*Continued*)

TABLE 3 | Continued

Test system	Dose or concentration (LED or HID)	Result	Reference
33. Malathion (121-75-5)			
Salmonella typhimurium, TA98, TA100, TA97A, TA102, TA1535, TA1537, reverse mutation	33–1,650, 80–400 mg/l	–	(191, 117)
E. coli WP2 uvra, tryptophan reverse gene mutation		–	(101)
Recombination assay, spot test, DNA effect (bacterial DNA repair)	NR		(79)
SCE, non-human, V79 cells *in vitro*	NR	+	(119)
SCE, human somatic cells *in vitro*	NR	+	(120)
Chromosome aberrations	NR	+	(186)
Micronuclei in bone marrow *in vivo* (mice)	2.5, 5, 10 mg/kg i.p. or p.o.	+	(157)
Chromosomal aberrations, mouse (injection)	400 mg/kg b.w.	+	(192)
Chromosomal aberrations, mouse (oral) bone marrow cells *in vivo*	240 mg/l for 4 or 8 weeks, 120 mg/l for 8 weeks		
Chromosomal aberrations, CHO cells *in vivo*	25, 50, 76 µg/l	–	(193)
Chromosomal aberrations (rat, liver S-9, aroclor1254), CHO cells *in vivo*	303, 352,402 µg/l	+	(193)
Drosophila melanogaster, sex-linked recessive lethal mutation		–	(140)
UDS, human diploid fibroblasts *in vitro*		–	(66)
Histidine reverse gene mutation, Ames assay		–	(146)
Mitotic recombination or gene conversion		–	(44)
Micronucleus test, chromosome aberrations		–	(194)
Micronucleus test, mice(oral) bone marrow cells *in vivo*	120, 240 mg/l in diet for 2 weeks	+	(192)
Micronucleus test, mice (injection) bone marrow cells *in vivo*	200, 300 mg/kg b.w.		(192)
Micronucleus test, human peripheral lymphocytes *in vivo*	20, 50, 75, 100 µg/l	+	(195)
Micronucleus test, rat peripheral blood lymphocytes *in vivo*	0, 25, 50, 100, 150 mg/kg b.w.	–	(196)
Micronucleus test, rat peripheral blood polychromatic and normochromatic erythrocytes *in vivo*	150 mg/kg b.w.	+	(196)
Long-term carcinogenesis assay. Rats	0, 2,000, 4,000 mg/l in diet	–	(84, 85)
Long-term carcinogenesis assay. Mice	0, 8,000, 16,000 mg/l in diet	–	(122, 123)
Carcinogenicity studies *in vivo*		–	(141)
34. Mebendazole (31431-39-7)			
Salmonella typhimurium (rat, liver, S-9, aroclor 1254), TA100, TA98	0.5–5, 0.5–5 µg/plate	–	(165)
	0.01–10 µg/plate	+ +	
Salmonella typhimurium (none), TA100, TA98	0.01–10 µg/plate	–	(165)
Forward and reverse gene mutation, body fluid assay, *Salmonella typhimurium,* host-mediated assay		+	(197)
Genotoxicity in a diploid mitotic recombination or gene mutation; genotoxicity in a haploid yeast reversion assay; gene conversion assay (strain D5 of *Saccharomyces cerevisiae*)		–	(198)
35. Mefloquine (53230-10-7)			
Bacterial mutation (Ames)		–	(47)
Cytogenetics *in vivo*		–	(47)
Long-term carcinogenesis assay, mice	30 mg/kg/day	–	(75, 199)
36. Metronidazole (443-48-1)			
Salmonella typhimurium, forward and reversegene mutation, host-mediated assay		+	(197)
Salmonella typhimurium, forward and reverse gene mutation, body fluid assay		+	(200)
Salmonella typhimurium, TA98, TA100, TA1535, TA1537, and TA1538		+	(201)
Salmonella typhimurium, TA100	25–1,000 µg/plate	+	(202)
	300 µg/plate	+	(203)
	50–200 µg/plate	+	(147)
	1–66 µg/plate	+	(204)
	50–12,800 µg/plate	+	(205)
Salmonella typhimurium, TA97, TA100, TA102, TA98	50–200 µg/plate	+	(147)
Salmonella typhimurium, TA1538, TA1537, TA100, TA98, TA1535		–	(206)
E. coli, none	0.01–0.5 mg/ml	+	(101)
	25–1,000 µg/l	+	(207)
E. coli (rat, liver, S-9, Aroclor 1254)	25–1,000 µg/l, 25–500 µg/l	–	(207)
E. coli WP2 uvra, Tryptophan reverse gene mutation	With dose response	+	(101)
Comet assay in human lymphocytes	292.1 mg/ml	+	(148)
Chromosome aberration (CA) *in vivo*	10, 20, 40 mg/kg b.w.	+	(208)
Micronucleus (MN) in the bone marrow cells of Balb/c mice *in vivo*	10, 20, 40 mg/kg b.w.	+	(208)
SCE *in vivo*, non-human		NC	(209)
SCE *in vitro*, human lymphocytes	With dose response	+	(210)
Micronucleus *in vivo*, bone marrow polychromatic lymphocytes	23, 70, 160 mg/kg b.w.	+	(211)

(*Continued*)

TABLE 3 | Continued

Test system	Dose or concentration (LED or HID)	Result	Reference
Mitotic recombination or gene conversion, *Saccharomyces cerevisiae*		–	(44)
Micronucleus test *in vivo*, chromosome aberrations, mammalian polychromatic erythrocytes		–	(43)
Chromosomal aberrations *in vitro*, human lymphocytes	0.1, 1, 10, 50 µg/l	+	(119)
Forward gene mutation, *Neurospora crassa*		+	(168)
Aneuploidy, chromosome aberrations, *Neurospora crassa*		–	(168)
Neurospora crassa, human		NC	(45, 46)
Sex-linked recessive lethal gene mutation, *Drosophila melanogaster*		NC	(140)
Carcinogenicity studies *in vivo*, non-human		+	(141)
UDS and cytogenetics *in vitro*		+	(47)
Carcinogenicity studies, mouse		+	(212)
Carcinogenicity studies, rat		+	(212)
Tumor promotion studies, mouse			(213)
37. Niclosamide (50-65-7)			
Salmonella typhimurium (none), TA1978, UTH8413, TA1538, TA98; *Salmonella typhimurium* (rat, liver, S-9, aroclor 1254), TA1978, UTH8413, TA1538, TA98	1–50 µg/plate	–	(74)
Salmonella typhimurium (rat, liver, S-9, aroclor 1254), TA98 (NR), YG1020, YG1021, YG1024	0.5–15 µg/plate	–	(74)
	0.5–20 µg/plate	+	(214)
SCE *in vitro*, Human lymphocytes		+	(215)
38. Nitroscanate (19881-18-6)			
Salmonella typhimurium (none), TA98, TA98(NR), TA98(1,8-Dnp6), TA100, TA100(NR), YG1024, YG1021, TA98, TA98(1,8-Dnp6), TA100	1–160 µg/plate	+	(216)
	20–160 µg/plate	–	
	20–320 µg/plate	+	
	10–80 µg/plate	+	
	10–80 µg/plate	–	
	0–9 µg/plate	+	
	0–40 µg/plate	+	
	10–320 µg/plate	+	
Salmonella typhimurium (rat, liver, S-9, aroclor 1254), TA98, TA98(NR), TA100, TA100(NR), TA98, TA98(1,8-Dnp6), TA100	10–160 µg/plate	+	(216)
	10–160 µg/plate	–	
	10–80 µg/plate	+	
	10–160 µg/plate	–	(216)
	10–320 µg/plate	+	
39. Nitroxinil (1689-89-0)			
Salmonella typhimurium (rat, liver, S-9, kanechlor 400), TA100, TA98, TA1535, TA1537, TA1538; *Salmonella typhimurium* (none), TA100, TA98, TA1535, TA1537, TA1538	0.05–5 mg/plate	–	(61)
Chromosomal aberrations *in vivo*, mouse bone marrow cells	0, 10, 20, 30, 40 mg/kg once	+	(80)
Salmonella typhimurium (none), TA1537	0–1,000 µg/plate	–	(56)
Micronucleus test *in vivo*, chromosome aberrations, mammalian polychromatic erythrocytes		–	(61)
40. Oxfendazole (53716-50-0)			
Chromosomal aberrations *in vivo*, spermatocytes and bone marrow cells	1,000 µg/kg	+	(217)
41. Pentamidine (100-33-4)			
Salmonella typhimurium, TA98, TA100, reverse mutation; *Salmonella typhimurium*, TA98, TA100 (rat, liver S-9, Phenobarbital), reverse mutation	0.01–1 µmol/plate	–	(218)
42. Permethrin (52645-53-1)			
Salmonella typhimurium, TA98, TA100	100–3,000 µg/plate	–	(107)
Salmonella typhimurium, TA98, TA100	5–1,000 µg/plate	–	(219)
Salmonella typhimurium, TA98, TA100	1–20 mg/plate	–	(220)
Salmonella typhimurium, TA98, TA100, TA97A	39–2,730 mg/l	–	(191)
Salmonella typhimurium, TA1535, TA1537, TA98, TA100, *E. coli*	1–7,500 µg/plate	–	(62)
Chinese hamster V79, rat hepatocytes	4–40 µg/l	–	(107)
UDS *in vitro*, DNA effects, human diploid fibroblasts		–	(66)
Mitotic recombination or gene conversion, *Saccharomyces cerevisiae*		–	(44)
43. Piperazine (110-85-0)			
Salmonella typhimurium (none), TA100, TA1535, TA1537, TA98, TA100; *Salmonella typhimurium* (rat, liver, S-9, aroclor 1254), TA100, TA1535, TA1537, TA98; *Salmonella typhimurium* (hamster, liver, S-9, aroclor 1254), TA100, TA1535, TA1537, TA98	33–2,167 µg/plate	–	(221)
Salmonella typhimurium (rat, liver, S-9, aroclor 1254), TA100, TA1535, TA1537, TA98			
Salmonella typhimurium (hamster, liver, S-9, aroclor 1254), TA100, TA1535, TA1537, TA98			
Salmonella typhimurium (rat, liver, S-9, PCB), TA100, TA98			(222)

(*Continued*)

TABLE 3 | Continued

Test system	Dose or concentration (LED or HID)	Result	Reference
44. Praziquantel (55268-74-1)			
Salmonella typhimurium (TA98, TA100, TA1535, TA1537, TA1538)		–	(223)
Salmonella typhimurium (none), TA1537, TA1535, TA100, TA1538, TA98; *Salmonella typhimurium* (rat, liver, S-9, kanechlor 400), TA1537, TA1535, TA100, TA1538, TA98	0–1,000 µg/plate	–	(56)
Salmonella typhimurium, forward and reverse gene mutation, host-mediated assay		+	(197)
Forward and reverse gene mutation, body fluid assay, *Salmonella typhimurium*		NC	(224)
Forward gene mutation, Schizo saccharomyces pombe		–	(225)
Sex-linked recessive lethal gene mutation, *Drosophila melanogaster*		–	(140)
Mitotic recombination or gene conversion, *Saccharomyces cerevisiae*		–	(44)
Dominant lethal test, rodents		–	(226)
Carcinogenicity studies, Hamster	0, 300 mg/kg in corn oil for 40 weeks	–	(227)
45. Pyrimethamine (58-14-10)			
Bacterial mutation (Ames)		–	(47)
Micronucleus test (MN) bone marrow in mice *in vivo*	40 mg/kg b.w.	+	(228)
The transplacental MN test in mice *in vivo*	40 mg/kg b.w.	–	(228)
Cytogenetics *in vitro*		+	(47)
DNA damage on ICR mice (oral)	50 mg/kg b.w.	+	(229)
Embryonic and maternal genotoxicity	50 mg/kg b.w.	+	(229)
Cytogenetics *in vivo*		+	(47)
DNA damage, SCGE, Comet assay in mice and rats	50, 120 mg/kg b.w., respectively	+	(230)
MLA		+	(47)
Micronucleus assay *in vitro*, cultured human lymphocytes		–	(231)
Long-term carcinogenesis assay, B6C3F1 mice (female)	1,000 mg/l in diet	–	(75, 122, 123)
Long-term carcinogenesis assay, mice (lung tumors)	25 mg/kg i.p.	+	(75, 122, 123)
Long-term carcinogenesis assay, F344 rats	400 mg/l in diet	–	(75, 122, 123)
46. Quinine (130-95-0)			
Salmonella typhimurium, TA98, TA100, reverse mutation, *Salmonella typhimurium*, TA98, TA100 (rat, liver S-9)	20–50 µg/plate	–	(78)
47. RH-5849 (112225-87-3)			
Salmonella typhimurium, TA98, TA100, TA97A, TA102, TA100 reverse mutation	5, 50, 500, 5,000 µg/plate	–	(232)
Micronuclei test in mouse bone marrow *in vivo*	42, 84, 168 mg/kg b.w.	–	(232)
Chromosome aberration, primary spermatocytes of testis	50, 100, 200 mg/kg/d for 5days	–	(232)
Micronuclei test in human peripheral lymphocytes SCE test in human peripheral lymphocytes	25, 100 mg/l	+	(176)
Comet assay, DNA damage, SCGE	5, 25, 50, 100 mg/l	+	(176)
Chromosome abnormality on sperm deformity of the earthworm	100 mg/kg dry soil	+	(179)
Micronucleus(MN) test in human lymphocytes *in vitro,* Micronucleus(MN) test in rat bone marrow *in vivo*	50 mg/ml 300 mg/kg b.w.	+	(174)
SCE in human lymphocytes	100, 200 mg/ml	+	(174)
DNA strand breaks and DNA damage		+	(177)
Micronucleus(MN) test in mouse	23, 45, 90 mg/kg b.w.	–	(172)
Chromosome aberration Primary spermatocytes of testis	38, 75, 150 mg/kg b.w.	–	(172)
48. Tetramethrin (7696-12-0)			
Salmonella typhimurium, TA98; *Salmonella typhimurium*, TA98 (rat, liver S-9, polychlorinated biphenyl)	0.1–1 mg/plate	+	(220)
Salmonella typhimurium, TA100; *Salmonella typhimurium*, TA100 (rat, liver S-9, polychlorinated biphenyl)	0.1–1 mg/plate	+	
Salmonella typhimurium, TA98, TA100; *Salmonella typhimurium*, TA98, TA100 (S9)	5–1,000 µg/plate	–	(219)
49. Thiophanate (23564-05-8)			
Salmonella typhimurium (none), TA100, TA98, TA1535, TA1537, TA97	33–10,000 µg/plate	–	(204)
Salmonella typhimurium [hamster, liver, S-9, aroclor 1254 (10% or 30%)], TA100, TA1535, TA97, TA98, TA100; *Salmonella typhimurium* [liver, S-9, aroclor 1254 (10 or 30%)], TA100, TA1535, TA97, TA98, TA1537	100–10,000 µg/plate	–	(204)
Chromosome aberrations, aneuploidy, *Aspergillus nidulans*		+	(233)
Chromosome aberrations *in vivo*, mammalian germ cells		–	(40)

(*Continued*)

TABLE 3 | Continued

Test system	Dose or concentration (LED or HID)	Result	Reference
50. Tiabendazole (148-79-8)			
Salmonella Typhimurium (none), TA100, TA98; *Salmonella typhimurium* (hamster, liver, S-9, aroclor 1254, 30%), TA100, TA98; *Salmonella typhimurium* (rat, liver, S-9, aroclor 1254, 30%), TA100, TA98	100–10,000 µg/plate	–	(60)
Salmonella typhimurium (hamster, liver, S-9, aroclor 1254, 10%), TA98	100–10,000 µg/plate	+	(60)
Salmonella typhimurium (none), TA98, TA100, TA97, TA104, *E. coli,* WP2S/PKM101	50–400 µg/l	+	(234)
Micronucleus test *in vivo*, chromosome aberrations, mammalian polychromatic erythrocytes		+	(235)
Mitotic recombination, *Aspergillus nidulans*		NC	(233)
Chromosome aberrations, *Aspergillus nidulans*, aneuploidy		+	(233)
Micronucleus (none) *in vitro*, V79 cells	0.5–700 µg/l	+	(236)
Micronucleus (none) *in vitro*, human lymphoblastoid wtk1 cells	0, 50, 100, 200 µg/l	+	(234)
Carcinogenicity studies, mouse	0, 0.8, 1.2, 1.6% in diet for 44 weeks	–	(237)
	0, 0.031, 0.125, 0.5% in diet for 78 weeks		
Long-term carcinogenesis assay, rats	0, 0.05, 0.1, 0.2, 0.4% in diet for 104 weeks	–	(238)
51. Tinidazole (19387-91-8)			
Salmonella typhimurium, TA100, reverse mutation	10–100 µg/plate	+	(239)
Salmonella typhimurium, TA100 (rat, liver S-9, aroclor 1254), reverse mutation	10–100 µg/plate	–	(239)
Salmonella typhimurium, TA98, reverse mutation	10–100 µg/plate	+	(239)
Salmonella typhimurium, TA98 (rat, liver S-9, aroclor 1254), reverse mutation	10–800 µg/plate	+	(205)
Salmonella typhimurium, UTH8414, reverse mutation	50–12,800 nmol/plate	+	
Salmonella typhimurium, TA98, TA100, reverse mutation	50–3,200 nmol/plate	+	
Salmonella typhimurium, TA100, reverse mutation		+	
Salmonella typhimurium, TA100(1,8-DNP6),YG1029, TA100 (NR), reverse mutation			
Salmonella typhimurium, TA100 (NR), TA100 (rat, liver S-9, aroclor 1254), reverse mutation			
52. Triclabendazole (68786-66-3)			
Chromosomal aberrations *in vitro*, river buffalo lymphocytes	25, 50, 100 µg/l	+	(239)
Micronucleus *in vitro*, river buffalo Lymphocytes, micronucleus formation in lymphocyte cultures of the river buffalo	25, 50, 100 µg/l	+	(239)
SCEs in lymphocyte cultures of the river buffalo	25, 50, 100 µg/ml		

The name of each drug is followed by the CAS number. For each type of assay: "+," positive response; "–," negative response; NR, not reported; NT, not tested; p.o., oral; i.p., intraperitoneal; UDS, DNA repair synthesis; MLA, gene mutation, mouse lymphoma L5178Y cells, TK locus; HGPRT, gene mutation, hgprt locus; SCE, sister chromatid exchange; MN, micronucleus; Trans., cell transformation;HID, highest ineffective dose; LED, lowest effective dose.

Pharmaceuticals without retrievable data: Amicarbalide, Abamectin, Acetarsone, Amprolium, Arecoline Hydrobromide, Artemether, Artemisinine, Artesunate, Avermectin, Azamethiphos, Amprolium Hydrochloride, Bunamidine, Carbarsone, Chiniofon, Clopidol, Clorsulon, Closantel Sodium, Cyromazine, Destomycin A, Diamphenethide, Diclazuril, Diethylcarbamazine, Diethylcarbamazine, Dihydroartemisinin, Diiodohydroxyquinoline, Diloxanide, Diminazene, Dinitolmide, Dithiazanine Iodide, Doramectin, Emetine, Epsiprantel, Ethopabate, Febantel, Fexinidazole, Fluvalinate, Hainanmycin, Halofuginone, Haloxon, Hetolin, Hexachloroparaxylene, Hydroxychloroquine, Hygromycin B, Imidocarb, Dipropionate, Isometamidium, Levamisole, Lumefantrine, Maduramicin, Malaridine, Metrifonate, Milbemycin Oxime, Monensin Sodium, Morantel, Moxidectin, Naftalofos, Naphthalophos, Nicarbazin, Nitazoxanide, Nitroquine, Oxantel, Oxibendazole, Oxinothiophos, Phanquinone, Phoxim, Piperanitrozole, Piperaquine, Primaquine, Propetamphos, Pyramine, Pyrantel, Quinapyramine, Rafoxanide, Resorantel, Robenidine, Salinomycin, Secnidazole, Semduramicin, Sodium stibogluconate, Sulfaquinoxaline, Sulfur Sublimat, Tetramisole, Thiacetarsamide, and Toltrazuril.

the number of antiparasitics in genotoxicity assays (bacterial mutagenicity, *in vitro* tests for gene mutation and for chromosomal damage, *in vivo* cytogenetic tests, and other types of genotoxicity assays). Of 136 antiparasitics examined, 52 (38.2%) had at least one genotoxicity or carcinogenicity test result, and 32 (23.5%) were tested only for either genotoxicity or carcinogenicity. Among 20 antiparasitics with results available for both genotoxicity and carcinogenicity, 16 had all the results required by the present guidelines for testing of pharmaceuticals: 8 of them—Albendazole, Coumaphos, Cypermethrin, Deltamethrin, Diazinon, Fenvalerate, Malathion and Tiabendazole—tested positive in genotoxicity assays but gave at least one negative result in carcinogenesis assays; 8 antiparasitics (Chlordimeform, Dichlorvos, Fenthion, Fipronil, Lindane, Metronidazole, Pyrimethamine, and Imidacloprid) gave positive responses in both genotoxicity and carcinogenicity. The remaining four with both genotoxicity and carcinogenicity data were not in agreement with the current guidelines: Amitraz and Praziquantel gave positive responses in genotoxicity but were non-carcinogenic; Atovaquone tested negative in genotoxicity but positive in mouse carcinogenicity; and Mefloquine produced negative responses in both genotoxicity and carcinogenicity.

Additional 32 antiparasitics were only tested in either genotoxicity or carcinogenicity. Only one (Ivermectin) had retrievable results in carcinogenicity. As for the rest, 31 antiparasitics had the data of genotoxicity. Twenty-one antiparasitics (Acriflavine, Closantel, Chloroquine, Cyfluthrin, Danex, Diaveridine, Dimetridazole, Fenbendazole, Fenchlorphos, Furapyrimidone, Furapromide, Mebendazole, Nitroscanate, Nitroxinil, Niclosamide, Oxfendazole, RH-5849, Tetramethrin, Thiophanate, Tinidazole,

TABLE 4 | Overview of genotoxicity and carcinogenicity testing of antiparasitics.

Antiparasitics with at least one genotoxicity or carcinogenicity tests results (**Table 3**)	52 (38.2%)[a]
Antiparasitics without retrievable genotoxicity or carcinogenicity data	84 (61.8%)
Antiparasitics with all genotoxicity and carcinogenicity data required by present guidelines (**Table 3**: 2, 10, 13, 15, 17, 19, 20, 24–26, 30, 32, 33, 36, 45, 50)[b]	16 (11.8%)
Antiparasitics tested not according to present guidelines	36 (26.5%)
Antiparasitics with least one genotoxicity and carcinogenicity test results (**Table 3**: 2, 3, 7, 10, 13, 15, 17, 19, 20, 24–26, 30, 32, 33, 35, 36, 44, 45, 50)	20 (14.7%)
Antiparasitics tested only for genotoxicity (**Table 3**: 1, 4–6, 8, 9, 11, 12, 14, 16, 18, 21–23, 27–29, 34, 37–43, 46–49, 51, 52)	31 (22.8%)
Antiparasitics tested only for carcinogenicity (**Table 3**: 31)	1 (0.7%)
Antiparasitics with at least one results in tests for bacterial mutagenicity (**Table 3**: 1–11, 13–22, 24–30, 32–39, 41–51)	47 (34.6%)
Antiparasitics with at least one results in tests for gene mutation in mammalian cells (**Table 3**: 1, 6, 7, 10, 17, 19, 20, 24–26, 28, 32–34, 36, 44, 45, 50)	18 (13.2%)
Antiparasitics with at least one results in *in vitro* tests for SCE, chromosomal aberrations, aneuploidy, or micronucleus in animal or human cells (**Table 3**: 6, 7, 9, 10, 13–26, 28, 30, 32, 33, 36, 37, 42, 45, 47, 49, 50, 52)	33 (24.3%)
Antiparasitics with results in *in vitro* data required by present guidelines (**Table 3**: 1–3, 13, 17–19, 24–26, 28, 30, 42, 50, 52)	15 (11.0%)
Antiparasitics with at least one results in *in vivo* tests for SCE, chromosomal aberrations, or micronucleus in animal or human cells (**Table 3**: 6–20, 24–26, 30, 32, 33, 35, 36, 39, 40, 45, 47, 49, 50)	31 (22.8%)
Antiparasitics which underwent testing for DNA damage or DNA repair synthesis (**Table 3**: 3, 10, 11, 14–16, 18–21, 24, 25, 30, 32, 33, 36, 42, 45, 47)	19 (14.0%)
Antiparasitics which underwent testing in other types of genotoxicity assays (**Table 3**: 1, 10, 15, 19, 20, 22, 24, 26, 28, 30, 32–34, 36, 42, 44, 45)	17 (12.5%)
Antiparasitics examined for genotoxicity in human cells (**Table 3**: 2, 6, 14–16, 19–21,23, 24, 26, 30, 32, 33, 36, 37, 42, 45, 47, 50)	20 (14.7%)
Antiparasitics tested for carcinogenicity in mice (**Table 3**: 2, 3, 7, 10, 13, 15, 17, 19, 20, 24–26, 30, 32, 33, 35, 36, 45, 50)	19 (14.0%)
Antiparasitics tested for carcinogenicity in rats (**Table 3**: 2, 3, 7, 10, 13, 15, 17, 19, 20, 24–26, 30–33, 35, 36, 45, 50)	20 (14.7%)
Antiparasitics tested for carcinogenicity in both mice and rats (**Table 3**: 2, 3, 7, 10, 13, 15, 17, 19, 20, 24–26, 30, 32, 33, 35, 36, 45, 50)	19 (14.0%)
Antiparasitics tested for carcinogenicity in other species (**Table 3**: 44)	1 (0.7%)

[a]*Values in parentheses indicate the percentage of the 136 antiparasitics considered.*
[b]*Number and percentage in parentheses are those of antiparasitics of* ***Table 3****.*

and Triclabendazole) gave positive responses in at least one genotoxicity assay; 10 antiparasitics (Amodiaquine, Amoscanate, Amphotericin B, Bithionol, Bromofenofos, Flubendazole, Pentamidine, Permethrin, Piperazine, and Quinine) were found to be negative in all the considered genotoxicity assays. With regard to the different types of genotoxicity assays: there were 47 antiparasitics with at least one result in tests for bacterial mutagenicity; 18 antiparasitics with at least one result in tests for gene mutation in mammalian cells; 33 antiparasitics in *in vitro* tests for SCE, chromosomal aberrations, aneuploidy, or micronucleus in animal or human cells; 15 antiparasitics with results in *in vitro* data required by present guidelines; 31 antiparasitics in *in vivo* tests for SCE, chromosomal aberrations, or micronucleus in animal or human cells; 19 antiparasitics in DNA damage or DNA repair synthesis; 17 antiparasitics in other types of genotoxicity assays; and 20 antiparasitics examined for genotoxicity in human cells. With respect to carcinogenesis assays, 19 and 20 antiparasitics were tested for carcinogenicity in mice and rats, respectively. Among the antiparasitics with both the genotoxicity and carcinogenicity data, 19 antiparasitics tested for carcinogenicity in both mice and rats and only 1 in hamsters.

Table 5 provides the number of antiparasitics tested for each type of assay, including the genotoxicity and carcinogenicity studies. The results are indicated as positive, negative and discordant. When carcinogenicity testing is considered, 57.9% of antiparasitics were tested negative in mice, and 73.7% in rats. Five antiparasitics (nos. 7, 10, 26, 32, and 36) and three antiparasitics (nos. 10, 26, and 36) were carcinogenic in mice and rats, respectively. The percentage of concordant results in carcinogenicity assays between mice and rats is 85.7% (12 out of 14) and only 2 (nos. 7 and 32) antiparasitics have discordant results: no. 32 tested positive in mice and negative in rats, while no. 7 produced the opposite result. The occurrence of discordant results between mice and rats may be the differences in species (e.g., metabolic enzymes). Ten antiparasitics were in IARC of 2B and 3 ground classifications of carcinogens: Chloroquine, Danex, and Permethrin do not have available carcinogenicity data; Deltamethrin, Fenvalerate, and Malathion tested negative in rodents while positive results were given by Chlordimeform and Metronidazole. Dichlorvos (DDVP) and Pyrimethamine have discordant results of carcinogenicity in mice and rats. To interpret the tumor findings in a carcinogenicity study and provide a perspective on the relevance of rodents to human, the mechanism and some investigations in tumor profile (trans-species, trans-sex, and multisite *versus* single species, single sex, and single site) were suggested by the guidelines (15).

Re-Evaluation of *In Vitro* Genotoxicity Results

Table 6 presents the incidence of misleading positive effects in *in vitro* cytogenicity when using the reduction in a top dose of 1 mM. Of 33 antiparasitics with at least one result in *in vitro* tests for SCE, chromosomal aberrations, or micronucleus in animal or human cells, 25 (75.8%) antiparasitics had at least one retrievable dose in *in vitro* cytogenicity assays, while 8 (24.2%) antiparasitics had no available dose. Under the current *in vitro* genotoxicity testing guidelines for dose limits, 10 (nos. 10, 14, 15, 16, 20, 21, 22, 32, 36, and 47) antiparasitics were identified as genotoxins at dose levels more than 1 mM. The re-evaluation results indicated the misleading positive response in the previous reports. Fifteen (nos. 1, 2, 3, 13, 17, 18, 19, 24, 25, 26, 28, 30, 42, 50, and 52) antiparasitics had *in vitro* genotoxicity results consistent with ICH S2 (R1).

Correlation between the Genotoxicity Assays

Table 7 provides the correlation among the different types of genotoxicity assays of antiparasitics, the numbers and percentages of antiparasitics that tested concordant and discordant between

TABLE 5 | Summary per assays type of antiparasitics with positive, negative, and discordant results.

Bacterial mutagenitity	Positive	4 (8.5%) (**Table 3**: 21, 26, 28, 29)
	Negative	29 (61.7%) (**Table 3**: 2–10, 13–15, 17, 19, 22, 24, 25, 27, 32, 33, 35, 39, 41–47, 49)
	Discordant	14 (29.8%) (**Table 3**: 1, 11, 16, 18, 20, 30, 34, 36–38, 44, 48, 50, 51)
Gene mutation in cultured mammalian cells	Positive	7 (38.9%) (**Table 3**: 1, 20, 26, 28, 32, 45, 50)
	Negative	11 (61.1%) (**Table 3**: 6, 7, 10, 19, 24, 25, 33, 34, 36, 42, 44)
	Discordant	0
In vitro cytogenetics	Positive	18 (54.5%) (**Table 3**: 1, 15, 16, 18, 19, 21, 22, 23, 26, 28, 32, 33, 36, 37, 45, 47, 50, 52)
	Negative	7 (21.2%) (**Table 3**: 3, 6, 7, 9, 13, 34, 42)
	Discordant	8 (24.2%) (**Table 3**: 2, 10, 14, 17, 20, 24, 25, 30)
In vivo cytogenetics	Positive	13 (41.9%) (**Table 3**: 11–15, 17, 19, 24–26, 32, 40, 50)
	Negative	8 (25.8%) (**Table 3**: 6–9, 18, 20, 35, 49)
	Discordant	10 (32.3%) (**Table 3**: 1, 2, 10, 16, 30, 33, 36, 39, 45, 47)
DNA lesions (*in vitro* and *in vivo*)	Positive	9 (47.4%) (**Table 3**: 3, 14, 16, 21, 30, 32, 36, 45, 47)
	Negative	3 (15.8%) (**Table 3**: 25, 33, 42)
	Discordant	7 (36.8%) (**Table 3**: 10, 15, 18–20, 24, 26)
Carcinogenesis in mice	Positive	5 (26.3%) (**Table 3**: 7, 10, 26, 32, 36)
	Negative	11 (57.9%) (**Table 3**: 2, 3, 13, 15, 17, 19, 22, 25, 30, 33, 35)
	Discordant	3 (15.8%) (**Table 3**: 20, 24, 45)
Carcinogenesis in rats	Positive	3 (15.8%) (**Table 3**: 10, 26, 36)
	Negative	14 (73.7%) (**Table 3**: 2, 3, 7, 13, 15, 17, 24, 25, 31–33, 35, 45, 50)
	Discordant	2 (10.5%) (**Table 3**: 20, 30)
Carcinogenesis in mice and rats	Discordant	2 (14.3%) (**Table 3**: 7, 32)
Carcinogenesis in mice and rats	Concordant	12 (85.7%) (**Table 3**: 2, 3, 10, 13, 15, 17, 25, 26, 33, 35, 36, 50)

The antiparasitic was considered as positive when it gave only positive results and as negative when it gave only negative or inconclusive results. Discordant indicates the number of antiparasitics, of which the results of genotoxicity assays were both positive and negative or inconclusive and he results of carcinogenicity assays performed in the same species were carcinogenic to mice or rats but not to rats or mice. In parentheses is the number of drugs in ***Table 3****.*

each other. On the whole, the degree of coincident correlation was higher than the discordant results, which ranged from 84.6% between bacterial mutagenicity and gene mutation in mammalian cells to 55.6% between gene mutation in mammalian cells and *in vivo* cytogenetics. When bacterial mutagenicity was compared with the following assays: gene mutation in mammalian cells, *in vitro* cytogenetics, *in vivo* cytogenetics and DNA lesions, 13 (nos. 3, 14, 15, 17, 19, 22, 24, 25, 32, 33, 45, 47, and 49) antiparasitics gave negative results in bacterial mutagenicity. Among these antiparasitics, there were 2 (nos. 32 and 45), 8 (nos. 15, 22, 24, 25, 32, 33, 47, and 49), 7 (nos. 14, 15, 17, 19, 24, 25, and 32) and 5 (nos. 3, 14, 32, 45, and 47) antiparasitics that tested positive in gene mutation in mammalian cells, *in vitro* cytogenetics, *in vivo* cytogenetics and DNA lesions, respectively.

The highly consistent correlation between bacterial mutagenicity and gene mutation in mammalian cells indicated that the same genetic end point tests might have the high consistency. The discordance (nos. 32 and 45) may be due to the xenobiotic metabolism in the liver and other organs between the bacteria and animals. With the comparison between *in vitro* cytogenetics and *in vivo* cytogenetics, 2 (nos. 18 and 49) antiparasitics gave positive responses in *in vitro* cytogenetics while no. 13 gave negative. These results were inconsistent with that in *in vivo* cytogenetics. With regard to the discordant results between DNA lesions and *in vitro* cytogenetics of the three (nos. 3, 19 and 33) antiparasitics, two (nos. 19 and 33) antiparasitics tested negative and no. 3 yield positive in DNA lesions, respectively. These results were opposite to that in *in vitro* cytogenetics.

A Novel Strategy for Predicting Carcinogenicity Based on the Genotoxicity Assays

Antiparasitics with both genotoxicity and carcinogenicity data are reported in **Table 8** to analyze the correlation between the results of the various types of genotoxicity and carcinogenicity. The results are marked positive or negative or inconclusive. It is obvious that the concordant and discordant results occurred in all the 15 pairs of assays considered. When carcinogenicity in mice or rats was considered, the percentage of discordant results ranged from 71.4% between *in vivo* cytogenetics and carcinogenicity in both mice and rats to 10.0% between bacterial mutagenicity and carcinogenicity in both mice and rats. The rank order of the consistency between genotoxicity and carcinogenicity was bacterial mutagenicity > DNA lesions > *in vitro* cytogenetics > gene mutation in mammalian cells > *in vivo* cytogenetics.

Table 9 showed 2 types and 10 combinations of gene-tox assays based on bacterial mutagenicity to indicate the predictivity for rodent carcinogenicity. The sequence of the predictivity was (Ames–DNA lesions) = (Ames–DNA lesions–*in vitro*) = (Ames–DNA

TABLE 6 | Re-evaluate the *in vitro* cytogenetic results according to the ICH S2 (R1).

Test system (*in vitro* cytogenetic assays)	Dose or concentration (LED or HID)	Result	Conversion unit (mM)	ICH S2 (R1), 1 mM Concordant
1. Acriflavine (1) (259.70)				
CHO, CHO-K1-BH4 (HGPRT)	0.5–4 μg/l	+	1.54×10^{-5}	Y
2. Albendazole (2) (265.33)				
MN, peripheral blood lymphocytes	10–100 μg/ml	+	0.377	Y
MN, human lymphocytes	10–100 μg/ml	+	0.377	Y
3. Amitraz (3) (293.23)				
DNA damage on hamster cells, comet assay	3.75 μg/l	+	1.28×10^{-5}	Y
4. Chlordimeform (10) (196.68)				
DNA effects (human diploid fibroblasts FL cell)	10^{-6} to 10^{-3} g/ml	+	5.08	N
5. Coumaphos (13) (362.78)				
CA *in vitro*, CHO cells (rat, liver, S-9, aroclor 1254)	100, 300, 1,000 μg/l	–	2.76×10^{-3}	Y
CA *in vitro*, CHO cells (none)	99.5, 299, 995 μg/l	–	2.7×10^{-3}	Y
6. Cyfluthrin (14) (434.29)				
CA, human peripheral blood lymphocytes	1,000, 2,000 mg/ml	+	4.61×10^{3}	N
SCE, human peripheral blood lymphocytes	500, 1,000, 2,000 mg/ml	–	4.61×10^{3}	N
MN, human peripheral blood lymphocytes	500, 1,000, 2,000 mg/ml	+	4.61×10^{3}	N
DNA damage, epithelial cells of human nasal mucosa	0.05, 0.1, 0.5, 0.75, 1.0 mg/ml	+	2.303	N
DNA damage and comet assay in fish	5.6 mg/l beta-cyfluthrin for 48 h	+	1.29×10^{-2}	Y
CA *in vitro*	500, 1,000, 2,000 μg/l	–	4.61×10^{-3}	Y
SCE in blood lymphocytes	500, 1,000, 2,000 μg/l	–	4.61×10^{-3}	Y
Mouse bone marrow cells *in vitro*	1,000 μg/l	+	2.30×10^{-3}	Y
7. Cypermethrin (15) (416.32)				
CAs, human peripheral lymphocytes	5, 10, 15, 20 mg/ml	+	48.0	N
SCE, human peripheral lymphocytes	5, 10, 15, 20 mg/ml	+	48.0	N
MN, human peripheral lymphocytes	5, 10 mg/ml	+	24.0	N
CA in highly mitotic kidney cells	0.4, 0.8,1.2 μg/l for 48 and 72 h	+	2.88×10^{-6}	Y
MN, erythrocytes of a freshwater fish	0.4, 0.8,1.2 μg/l for 48 and 72 h	+	2.88×10^{-6}	Y
Peripheral blood for MN test	20, 30, 40, 50 mg/l	+	0.120	Y
8. Danex (16) (257.45)				
UDS human cells	0.4–4,000 mmol	+	4.0×10^{3}	N
CA, V79 cell	0.04–0.8 mmol	–	8.0×10^{2}	N
9. Deltamethrin (17) (505.20)				
CA, CHO cells *in vitro*	0, 19, 38, 75, 150 μg/l	+	2.97×10^{-4}	Y
V79/6-thioguanine, Chinese hamater V79	4–40 μg/l	–	7.92×10^{-5}	Y
10. Diaveridine (18) (260.29)				
CA in cultured CHL cells	12.5, 25, 50, 100 μg/l	+	3.84×10^{-4}	Y
CA	100 μg/l,48 h	+	3.84×10^{-4}	Y
11. Diazinon (19) (304.35)				
DNA damage, human blood lymphocytes	750 μg/l	+	2.46×10^{-3}	Y
12. Dichlorvos(DDVP) (20) (220.98)				
CA *in vitro*, CHO cells	16, 50, 100, 160 μg/l	+	7.24×10^{-4}	Y
	50, 160, 500, 1,600 μg/l	+	7.24×10^{-3}	Y
	500, 750, 1,000 μg/l	+	4.53×10^{-3}	Y
CA, V79	1.25–5 μg/l	–	2.26×10^{-5}	Y
CHO, CHO-k1-bh4 (HGPRT)/6-thioguanine	50–150 μg/l	+	6.79×10^{-4}	Y
Mouse lymphoma, L5178Y (TK+/TK–)	0–0.33 μg/l, 0–0.12 μg/l,	+	1.49×10^{-6}	Y
	0–0.24 μg/ml	+	1.09×10^{-3}	Y
Mouse lymphoma, L5178Y (TK+/TK–)	6.25–200 μg/l	+	9.05×10^{-4}	Y
UDS human cells	6.5–650 mg/ml	+	2.94×10^{3}	N
UDS rat hepatocytes	0.005–1.25 mg/ml	–	5.66	N
13. Dimetridazole (21) (141.12)				
Comet assay, human lymphocytes	354.3 mg/ml	+	2.51×10^{3}	N
14. Fenbendazole (22) (299.34)				
Chromosomal damage in CHL cells	0.78 mg/ml	+	2.61	N
Cytotoxicity to 10T1/2 cells	0.04–1.60 mg/ml	+	5.35	N
Morphological transformation in mouse embryo fibroblasts	0.08–0.4 mg/ml	+	1.34	N

(*Continued*)

TABLE 6 | Continued

Test system (*in vitro* cytogenetic assays)	Dose or concentration (LED or HID)	Result	Conversion unit (mM)	ICH S2 (R1), 1 mM Concordant
15. Fenthion (24) (278.33)				
UDS, thymidine incorporation, rat hepatocytes	0, 5.0, 7.5, 10.0, 15.0, 30.0 µg/l	+	1.08×10^{-4}	Y
CA, CHO cells *in vitro*	0, 0.02, 0.04, 0.08, 0.15 µg/l	–	5.39×10^{-7}	Y
CA, human peripheral lymphocytes	0.5, 1.5, 2.5, 5.0 µg/ml	+	1.80×10^{-2}	Y
16. Fenvalerate (25) (419.90)				
Peripheral blood for MN test	25, 50, 75, 100 mg/l	+	0.238	Y
Chinese hamster V79 gene mutation	4–40 µg/l	–	9.53×10^{-5}	Y
CA, CHO-K1, *in vitro*	10, 25, 50, 100,150 µg/l	+	3.57×10^{-4}	Y
CA, CHO-K1, *in vitro*	5, 10, 25, 50 µg/l	+	1.19×10^{-4}	Y
17. Fipronil (26) (437.20)				
CA, human lymphocytes *in vitro*	0, 4.69, 9.38, 18.75, 37.5, 75, 150, 300 µg/l	+	6.86×10^{-4}	Y
SCEs, DNA damage, comet assay	0.3,0.7 µg/l	+	1.60×10^{-6}	Y
MN, human peripheral blood lymphocytes	0.3, 0.7 µg/l	+	1.60×10^{-6}	Y
Comet assay with gillsin, the fish *Rhamdia quelen*	0.05, 0.10, 0.23 µg/l	–	5.26×10^{-7}	Y
Nuclear morphological alterations	0.05, 0.10, 0.23 µg/l	–	5.26×10^{-7}	Y
CA, V79 cells, HGPRT mutations	0, 0.8, 4, 20, 100, 500 µg/l	+	1.14×10^{-3}	Y
18. Furapromide (28) (224.22)				
CA, V79 cell	10–120 µmol	+	1.20×10^{-4}	Y
19. Imidacloprid (30) (255.70)				
MN, human peripheral blood lymphocytes	0.2, 2, 20 µg/l	+	7.82×10^{-5}	Y
MN, human peripheral lymphocytes	0.1, 0.5 mg/l	+	1.96×10^{-3}	Y
SCE, human peripheral lymphocytes	0.1, 0.5 mg/l	+	1.96×10^{-3}	Y
Comet assay, DNA damage, SCGE	0.05, 0.1, 0.2, 0.5 mg/l	+	1.96×10^{-3}	Y
MN, Human lymphocytes *in vitro*	50 µg/l	+	1.96×10^{-4}	Y
SCE in human lymphocytes	Combination with metalaxyl at 100, 200 µg/l	+	7.82×10^{-4}	Y
SCE induction in human lymphocytes	0.1, 1, 5, 10, 50, 100 µg/l	–	3.91×10^{-4}	Y
20. Lindane (32) (290.82)				
Comet-forming activity in MCF-7 cells	10^{-4} g/ml	+	3.44	N
DNA damage and the risk for cancer on human tonsillar	0.5, 0.75, 1.0 mg/ml	+	34.4	N
21. Metronidazole (36) (171.16)				
Comet assay in human lymphocytes	292.1 mg/ml	+	1.71×10^{3}	N
CA *in vitro*, human lymphocytes	0.1, 1, 10, 50 µg/l	+	2.92×10^{-4}	Y
22. Permethrin (42) (391.28)				
Chinese hamster V79, rat hepatocytes	4–40 µg/l	–	1.02×10^{-4}	Y
23. RH-5849 (47) (296.40)				
MN, human peripheral lymphocytes	25, 100 mg/l	+	0.337	Y
SCE, human peripheral lymphocytes	25, 100 mg/l	+	0.337	Y
Comet assay, DNA damage, SCGE	5, 25, 50, 100 mg/l	+	0.337	Y
MN, human lymphocytes *in vitro*	50 mg/ml	+	1.69×10^{2}	N
SCE, human lymphocytes	100, 200 mg/ml	+	6.75×10^{2}	N
24. Tiabendazole (50) (210.19)				
MN (none) *in vitro*, V79 cells	0.5–700 µg/l	+	3.33×10^{-3}	Y
MN, human lymphoblastoid wtk1 cells	0, 50, 100, 200 µg/l	+	9.52×10^{-4}	Y
25. Triclabendazole (52) (359.66)				
CA *in vitro*, river buffalo lymphocytes	25, 50, 100 µg/l	+	2.78×10^{-4}	Y
MN *in vitro*, river buffalo lymphocytes	25, 50, 100 µg/l	+	2.78×10^{-4}	Y
CA in lymphocyte	25, 50, 100 µg/l	+	2.78×10^{-4}	Y
SCEs in lymphocyte	25, 50, 100 µg/ml	+	0.278	Y
MN in lymphocyte	25, 50, 100 µg/ml	+	0.278	Y

*The name of each antiparasitic is followed by the number in the **Table 1** and molecular weight. For each type of assay: "+," positive response; "–," negative response; "Y," consistent with results of the current guideline of ICH S2 (R1); "N," discordant with results of the current guideline of ICH S2 (R1); UDS, DNA repair synthesis; MN, micronucleus; MLA, gene mutation, mouse lymphoma L5178Y cells, TK locus; HGPRT, gene mutation, hgprt locus; SCE, sister chromatid exchange; Trans., cell transformation; HID, highest ineffective dose; LED, lowest effective dose; CHO, Chinese hamster ovary; CHL, Chinese hamster lung. Pharmaceuticals with in vitro cytogenetic results but without the retrievable dose: Amphotericin B, Atovaquone, Bromofenofos, Fenchlorphos, Malathion, Niclosamide, Pyrimethamine, Thiophanate.*

TABLE 7 | Correlation between the results of genotoxicity assays of antiparasitics.

Couples of assays considered	No. of drugs with	
	Concordant results	**Discordant results**
Bacterial mutagenicity—gene mutation in mammalian cells	11 (84.6%) (6, 7, 10, 19, 20, 24–26, 28, 33, 42)	2 (16.7%) (32, 45)
Bacterial mutagenicity—*in vitro* cytogenetics	12 (60.0%) (3, 6, 7, 10, 13, 16, 20, 26, 28, 30, 36, 42)	8 (40.0%) (15, 19, 22, 25, 32, 33, 47, 49)
Bacterial mutagenicity—*in vivo* cytogenetics	11 (57.9%) (1, 6–9, 16, 26, 30, 35, 36, 49)	8 (42.1%) (13–15, 17, 19, 24, 25, 32)
Bacterial mutagenicity—DNA lesions	7 (58.3%) (16, 18, 20, 21, 25, 33, 42)	5 (41.7%) (3, 14, 32, 45, 47)
Gene mutation in mammalian cells—*in vitro* cytogenetics	9 (75.0%) (1, 6, 7, 10, 26, 28, 32, 42, 50)	3 (25.0%) (19, 25, 33)
Gene mutation in mammalian cells—*in vivo* cytogenetics	5 (55.6%) (6, 7, 26, 32, 50)	4 (44.4%) (19, 20, 24, 25)
Gene mutation in mammalian cells—DNA lesions	5 (83.3%) (25, 32, 33, 42, 45)	1 (16.7%) (36)
in vitro cytogenetics—*in vivo* cytogenetics	13 (81.2%) (2, 6, 7, 15, 16, 19, 25, 26, 30, 32, 36, 45, 50)	3 (18.8%) (13, 18, 49)
DNA lesions—*in vitro* cytogenetics	6 (66.7%) (16, 20, 24, 32, 42, 47)	3 (33.3%) (3, 25, 33)
DNA lesions—*in vivo* cytogenetics	4 (80.0%) (10, 14, 16, 32)	1 (20.0%) (25)

In these comparisons, the drug gave only positive result (s) or only negative or inconclusive result (s) in the considered assays. In parentheses are indicated the number and corresponding percentages, as well as the numbers of ***Table 3****.*

TABLE 8 | Correlation between the multiple genotoxicity and carcinogenicity in mice and rats assays of antiparasitics.

Couples of assays considered	No. of antiparasitics with	
	Concordant results	**Discordant results**
Bacterial mutagenicity—carcinogenicity in mice	11 (78.6%) (2, 3, 13, 15, 17, 19, 20, 25, 26, 33, 35)	3 (21.4%) (7, 10, 32)
Bacterial mutagenicity—carcinogenicity in rats	15 (93.75%) (2, 3, 7, 13, 15, 17, 20, 24–26, 30, 32, 33, 35, 45)	1 (6.25%) (10)
Bacterial mutagenicity—carcinogenicity in both mice and rats	9 (90.0%) (2, 3, 13, 15, 17, 20, 25, 33, 35)	1 (10.0%) (10)
Gene mutation in mammalian cells—carcinogenicity in mice	5 (55.6%) (19, 25, 26, 32, 33)	4 (44.4%) (7, 10, 36, 50)
Gene mutation in mammalian cells—carcinogenicity in rats	5 (50.0%) (7, 24, 25, 26, 33)	5 (50.0%) (10, 32, 36, 45, 50)
Gene mutation in mammalian cells—carcinogenicity in both mice and rats	3 (50.0%) (25, 26, 33)	3 (50.0%) (10, 36, 50)
In vitro cytogenetics—carcinogenicity in mice	7 (53.8%) (3, 13, 20, 24, 26, 32, 45)	6 (46.2%) (7, 15, 19, 25, 33, 50)
In vitro cytogenetics—carcinogenicity in rats	7 (58.3%) (3, 7, 13, 19, 20, 26, 30)	5 (41.7%) (15, 25, 32, 33, 50)
In vitro cytogenetics—carcinogenicity in both mice and rats	4 (50.0%) (3, 13, 20, 26)	4 (50.0%) (15, 25, 33, 50)
In vivo cytogenetics—carcinogenicity in mice	4 (36.4%) (26, 32, 35, 45)	7 (63.6%) (7, 13, 15, 17, 19, 25, 50)
In vivo cytogenetics—carcinogenicity in rats	5 (41.7%) (7, 19, 26, 30, 35)	7 (58.3%) (13, 15, 17, 24, 25, 32, 50)
In vivo cytogenetics—carcinogenicity in both mice and rats	2 (28.6%) (26, 35)	5 (71.4%) (13, 15, 17, 25, 50)
DNA lesions—carcinogenicity in mice	6 (75.0%) (20, 24, 25, 32, 33, 36)	2 (25.0%) (3, 30)
DNA lesions—carcinogenicity in rats	4 (57.1%) (20, 25, 33, 36)	3 (42.9%) (3, 32, 45)
DNA lesions—carcinogenicity in both mice and rats	4 (80.0%) (20, 25, 33, 36)	1 (20.0%) (3)

In these comparisons, the antiparasitics gave only positive results or only negative or inconclusive results in genotoxicity assay and tested positive in at least one sex of mice or rats or gave negative or inconclusive results in both species in carcinogenicity assays. The following indicated the number and corresponding percentages, as well as the numbers of drugs of ***Table 3****.*

TABLE 9 | Predictivity of multiple combinations with Ames for rodent carcinogenicity assays of antiparasitics.

Couples of assays considered	No. of antiparasitics with concordant results	Carcinogenicity		
		Concordant results	**Discordant results**	**Without results**
Ames–Gene	11 (6, 7, 10, 19, 20, 24–26, 28, 33, 42)	5 (62.5%) (19, 20, 25, 26, 33)	3 (37.5%) (7, 10, 24)	3 (6, 28, 42)
Ames–*In vitro*	16 (1, 3, 6, 7, 10, 13,16, 18, 20, 26, 28, 30, 36, 37, 42, 50)	6 (66.7%) (3, 13, 20, 26, 30, 36)	3 (33.3%) (7, 10, 50)	7 (1, 6, 16, 18, 28, 37, 42)
Ames–*In vivo*	13 (1, 6–9, 11, 16, 26, 30, 35, 36, 49, 50)	4 (66.7%) (26, 30, 35, 36)	2 (33.3%) (7, 50)	7 (1, 6, 8, 9, 11, 16, 49)
Ames–DNA	10 (16, 18, 20, 21, 25, 26, 30, 33, 36, 42)	6 (100.0%) (20, 25, 26, 30, 33, 36)	0	4 (16, 18, 21, 42)
Ames–Gene–*In vitro*	7 (6, 7, 10, 20, 26, 28, 42)	2 (50.0%) (20, 26)	2 (50.0%) (7, 10)	3 (6, 28, 42)
Ames–Gene–*In vivo*	3 (6, 7, 26)	1 (50.0%) (26)	1 (50.0%) (6)	1 (7)
Ames–Gene–DNA	5 (20, 25, 26, 33, 42)	4 (100.0%) (20, 25, 26, 33)	0	1 (42)
Ames–*In vitro*–*In vivo*	8 (1, 6, 7, 16, 26, 30, 36, 50)	3 (60.0%) (26, 30, 36)	2 (40.0%) (7, 50)	3 (1, 6, 16)
Ames–*In vitro*–DNA	7 (16, 18, 20, 26, 30, 36, 42)	4 (100.0%) (20, 26, 30, 36)	0	3 (16, 18, 42)
Ames–*In vivo*–DNA	1 (26)	1 (100.0%) (26)	0	0

Ames, bacterial mutagenicity; Gene, gene mutation in mammalian cells; In vitro, in vitro cytogenetics; In vivo, in vivo cytogenetics; DNA, DNA lesions. In these comparisons, all the combinations took the Ames as center. The antiparasitics gave only positive results or only negative or inconclusive results in genotoxicity assay, and tested positive in at least one sex of mice or rats or gave negative or inconclusive results in both species in carcinogenicity assays. The following indicated the number and corresponding percentages, as well as the numbers of antiparasitics of ***Table 3****.*

lesions–gene mutation in mammalian cells) = (Ames–*In vivo*–DNA) > (Ames–*in vitro*) = (Ames–*in vivo*) > (Ames–gene mutation in mammalian cells) > (Ames–*in vivo*–*in vitro*) > (Ames–gene mutation in mammalian cells–*in vivo*) = (Ames–gene mutation in mammalian cells–*in vitro*).

Table 10 presents the number and the percentage of antiparasitics that were classified as non-genotoxic non-carcinogens, genotoxic non-carcinogens, non-genotoxic carcinogens, and genotoxic carcinogens according to the genotoxicity assays considered. An antiparasitic was regarded as genotoxic when a positive response was given in at least one genotoxicity assay, and carcinogenic when it was tested positive in at least one rodent sex. Of the 20 antiparasitics with retrievable results of both genotoxicity and carcinogenicity, Malathion, Diazinon, Deltamethrin, Fenvalerate, Coumaphos, Tiabendazole, Albendazole, Cypermethrin, Amitraz and Praziquantel might be classified as genotoxic non-carcinogens; Fenthion, Lindane, Chlordimeform, Fipronil, Dichlorvos, Metronidazole, Pyrimethamine, and Imidacloprid can be classified as genotoxic carcinogens; Mefloquine was considered a non-genotoxic non-carcinogen, while the non-genotoxic carcinogens only contained Atovaquone, which tested negative in bacterial mutagenicity, *in vitro* and *in vivo* cytogenetic assays, but was found to induce liver tumors in mice in a long-term carcinogenesis assay (75, 122, 123).

The bacterial mutagenicity has the highest specificity but the lowest sensitivity (**Table 8**), while DNA lesions (*in vitro* and/or *in vivo*) have the highest sensitivity and a lower specificity. A test with a low specificity induced a high proportion of misleading positive results. Therefore, the combination of bacterial mutagenicity and DNA lesions has high accuracy in relation to rodent cancer, which is consistent with the above analysis results. A proportion of 5.3% of antiparasitics gave positive in bacterial mutagenicity and was classified as non-carcinogens. There were 31.6% of antiparasitics that were regarded as carcinogenic while gave a negative result in bacterial mutagenicity.

DISCUSSION

The economic importance of parasitic infections in livestock and humans has long been recognized. Meanwhile, the most important advances in antiparasitics have come from the animal health area. Although many antiparasitics have been developed and applied to control parasitism in humans and animals, genotoxicity and carcinogenicity studies have not been conducted on a large proportion of them. Since a relationship between exposure to genotoxic compounds and carcinogenesis has been established, genotoxicity tests have been proposed for all medicinal products for human use except for some compounds (e.g., anticancer) that can interact with DNA (11). Therefore, this review was to assess the extent of antiparasitics that have been tested for genotoxic and carcinogenic activity. In addition, the ability of various types of genotoxicity assays was summarized to discriminate rodent carcinogens, which benefit to analyze the relative predictivity of carcinogenicity in rodents and humans. Furthermore, it is necessary to re-evaluate *in vitro* genotoxicity according the present revised guidelines.

With regard to the genotoxicity assays, compared to the positive and discordant results, the incidence of negative responses is 61.7, 61.1, 21.2, 25.8, and 15.8% for bacterial mutagenicity, gene mutation in cultured mammalian cells, *in vitro* cytogenetics, *in vivo* cytogenetics, and DNA lesions (*in vitro* and *in vivo*), respectively. It was observed that the incidence of negative responses was higher than the positive and discordant results in bacterial mutagenicity and gene mutation in cultured mammalian cells. Kasper et al. (240) reviewed the advantages and limitations of the standard genotoxicity tests in predicting the ability and the mode of action for carcinogens, which demonstrated that a totally negative response in all the standard genotoxicity assays was sufficient to prove the non-genetic toxicity of the chemicals, while the presence of a positive response in some genotoxicity assays, particularly in Ames and *in vitro* genotoxicity studies, did not afford support for the genetic definition of the chemicals. There have been a number of experiences in the literature regarding the high correlation among the various types of genotoxicity assays with respect to carcinogens (241, 242), which suggested that a chemical that tested positive in *Salmonella* tended to yield positive responses in any other *in vitro* genotoxicity studies, for instance, chromosome aberrations (CA), SCEs, and mutations in mouse lymphoma cells (MLA) (243).

TABLE 10 | Correlation between the results of genotoxicity and carcinogenicity assays of antiparasitics.

Assay type	No. of non-genotoxic non-carcinogens	No. of genotoxic non-carcinogens	No. of non-genotoxic carcinogens	No. of genotoxic carcinogens
Ames	8 (42.1%) (2, 3, 13, 17, 19, 25, 33, 35)	1 (5.3%) (50)	6 (31.6%) (7, 10, 19, 24, 32, 45)	4 (21.1%) (20, 26, 30, 36)
Gene	2 (16.7%) (25, 33)	1 (8.3%) (50)	5 (41.7%) (7, 10, 19, 24, 36)	4 (33.3%) (20, 26, 32, 45)
In vitro	2 (11.1%) (3, 13)	6 (33.3%) (2, 15, 17, 25, 33, 50)	2 (11.1%) (7, 10)	8 (44.4%) (19, 20, 24, 26, 30, 32, 36, 45)
In vivo	1 (5.6%) (35)	7 (38.9%) (2, 13, 15, 17, 25, 33, 50)	2 (11.1%) (1, 20)	8 (44.4%) (10, 19, 24, 26, 30, 32, 36, 45)
DNA lesions	2 (15.4%) (25, 33)	2 (15.4%) (3, 15)	0	9 (69.2%) (10, 19, 20, 24, 26, 30, 32, 36, 45)

Ames, bacterial mutagenicity; Gene, gene mutation in mammalian cells; In vitro, in vitro cytogenetics; In vivo, in vivo cytogenetics. The data show the number of antiparasitics that classified as non-carcinogens and carcinogens, which were examined in each genotoxicity assay and the result was negative (non-genotoxic) and positive (genotoxic) in the same assay. In this analysis, the antiparasitics that did not increase tumor incidence in mice and/or rats of both sexes were considered as non-carcinogens, and that increased tumor incidence in at least one sex of mice or rats were considered as carcinogens. An antiparasitic was considered non-genotoxic when it gave a single negative result, and genotoxic when it gave a single positive or concordant positive result in the indicated genotoxicity assay. The following indicated the number and corresponding percentages, as well as the numbers of antiparasitics of ***Table 3****.*

A high percentage of antiparasitics tested positive in the following assays: *in vitro* cytogenetics, *in vivo* cytogenetics, and DNA lesions (*in vitro* and *in vivo*). It is worth noting that the proportion of positive responses in *in vitro* cytogenetics is higher than in other types of assays. The *in vitro* cytogenetics seems to be more sensitive to genetic substance. However, the *in vitro* assays always lead to a number of false-positive results in genotoxicity and the carcinogenicity in rodents (244, 245). It was learned from the literature that the massive positive results only occurred at high levels of concentration. Recent surveys for *in vitro* cytogenetics were taken from compilations such as that of Müller et al. (246), Kirkland and Müller (247), Müller and Kasper (248), and Hilliard et al. (249). The conclusion was that the highest testing concentrations might lead to an increase in the emergence of misleading, toxicity-related positive results. In cytotoxicity and chromosome aberrations *in vitro*, Galloway (250) found that the positive response in genetic toxicology was caused by the cytotoxicity rather than the true drug or DNA interactions. Parry et al. (251) examined 24 carcinogens that gave positive results in *in vitro* genotoxicity at 1–10 mM, yet almost half of them were not mechanistically genotoxic carcinogens or had carcinogenic effects only in excessive doses. In the present review, we re-evaluate the *in vitro* genotoxicty according to current ICH S2 (R1) guidance. We find that the percentage of antiparasitics in agreement with the current ICH S2 (R1) guidance for *in vitro* genotoxicity data acceptance was 15 (45.5%). Thus, it is essential to re-evaluate *in vitro* genotoxicty that conducted prior to the update guideline of ICH S2 (R1) to provide a comprehensive assessment of the genotoxic effects.

Misleading positive results were found not only in *in vitro* but also in *in vivo* genotoxic assays. Increasing experience suggested that the occurrence of a positive response in rats and mice micronucleus tests was not the consequence of intrinsic genotoxicity but drug-related disturbances in the physiology (252), such as lysosomal damage, ATP depletion or impairment of mitochondrial function and the release of DNA endonucleases. However, at the time of writing, there has still been no amendment to the guidelines requirements of *in vivo* genotoxicity for dose limitations and toxicity to avoid irrelevant physiological responses. Furthermore, there is no consensus as to the highest testing concentration in *in vitro* genotoxicity assays. The method for the detection of toxicity has greatly changed in recent years, and the limitations of dose and toxicity in genotoxicity testing in OECD and ICH should be adjusted to adapt to the new changes. The standard genotoxicity system also needs to identify the cytotoxicity and genotoxicity clearly.

There are many explanations that could account for the existence of different results in the various types of genetic tests. The differences are the following: the detection of the genetic end point; the xenobiotic metabolism between bacterial mutagenicity and mammalian cells; the effective dose between *in vitro* and *in vivo*, especially the *in vivo* decomposition; the relative sensitivities of various genotoxicity assays to genetic damage; the metabolic activation pathway and metabolizing enzymes among species. *In vivo* activity, which is designed to study the mechanisms of mutagenicity in the potential target organs of rodents, is the best method to confirm the differences in cytogenetics between *in vivo* and *in vitro*. Except for the irrelevant biological reaction at high doses, it is also accepted that the metabolic activation process and metabolites could induce genetic toxicity. Some evidence suggested that the genetic toxicity of compounds may be prototypes or metabolites. For the drugs that are theoretically nitrosatable in the presence of amine, the interaction resulted in the formation of genotoxic–carcinogenic N-nitroso compounds (253). However, the current standard of genotoxicity assays cannot distinguish whether the positive results are derived from the drugs or their metabolites directly.

In **Table 7**, the percentage of concordant results between bacterial mutagenicity and carcinogenicity in both mice and rats is 90.0%, which is higher than any other correlation pairs. The same conclusion was drawn by Snyder and Green (19) in a review of the genotoxicity of marketed pharmaceuticals. Data from 467 marketed drugs were collected and no combination of gene-tox assays provided a higher predictivity of rodent carcinogenesis than the bacterial mutagenicity test itself (19). In two studies conducted by Zeiger, one identified 172 chemicals that gave negative or equivocal results in 2-year rodent assays, yet 38 (22.1%) chemicals produced positive results in *Salmonella* (243). Another found that among 158 drugs that tested negative in carcinogenicity assays, 33 (21%) were *Salmonella* mutagens (254). However, a chemical that tested negative in *Salmonella* testing cannot be regarded as a non-carcinogenicity because the percentage of rodent carcinogens that are not mutagenic is about 50% (254). It was also reported that the predictivity for rodent carcinogenicity of bacterial mutagenicity ranged from approximately 77 to 98% (254, 255). The remaining 2–23% was classified as non-carcinogen with positive result in bacterial mutagenicity, which demonstrated the flaw and insufficiency on the prediction carcinogenicity of bacterial mutagenicity.

Therefore, it requires efforts to overcome the deficiencies of bacterial mutagenicity and improve the predictivity for carcinogenicity. We try to find which genotoxicity assay(s) considered could enhance the prediction of bacterial mutagenicity to rodent carcinogenicity. Our approach has many differences and improvement compared to Snyder and Green (19), who examined only five combinations of gene-tox assays, such as Ames–*in vitro* cytogenetics, Ames–*in vivo* cytogenetics, *In vitro* cytogenetics–*in vivo* cytogenetics, MLA–*in vivo* cytogenetics, and MLA–*in vitro* cytogenetics (19). These combinations have no DNA lesions tests and no taking bacterial mutagenicity as center. A review suggested that DNA lesion alone could contribute to the prediction of carcinogenicity in mice (255). In the present article, as shown in **Table 8**, DNA lesion testing can significantly increase the predictivity of Ames from 90 to 100%, suggesting that the combination of DNA lesions and bacterial mutagenicity obtained higher prediction of carcinogenicity.

There are three types of DNA lesions: (a) the formation of DNA adducts; (b) DNA repair synthesis (UDS); and (c) the induction of DNA strand breaks and cross-links. An analysis of correlations between the induction of DNA lesions and carcinogenic activity was conducted in 2010 (256). It noted that the carcinogenic activity of some drugs can be correctly predicted by DNA lesion assays, yet neglected in the standard 3-test battery. Thus, DNA lesion assays were considered the best

supplement for the standard 3-test battery. The occurrence of the highest predictivity in a combination of bacterial mutagenicity and DNA lesions in our review suggested a close relationship between genotoxicity and carcinogenic activity. The bacterial mutagenicity test was often used to measure the ability of a drug to cause mutations rather than a definitive test of the carcinogens. The *in vivo* DNA lesion tests can detect the chemicals that reach the appropriate target with an effective dose to convert into a permanent mutation by reacting with DNA. In a few cases, the mutation escaped monitoring to survive and subsequently, carcinogenicity was generated through a loss of restriction of cell division. The *in vivo* DNA lesions can identify this "survived mutation." Thus, the combination of bacterial mutagenicity and DNA lesions showed a higher and more accurate predictivity of carcinogenicity.

The correlation between the results of genotoxicity and carcinogenicity assays of antiparasitics was indicated in **Table 9**. Among the antiparasitics that were classified as genotoxic carcinogens, 69.2% tested positive in *in vitro* and/or *in vivo* DNA lesions exhibiting a greater sensitivity to carcinogens than any other types of genotoxicity assays. Eight out of 19 (42.1%) antiparasitics gave negative results in bacterial mutagenicity and were identified as non-carcinogens. Sensitivity and specificity are commonly used to describe the capability of *in vitro* genotoxicity assays (257). Sensitivity is defined as the percentage of genotoxic carcinogens that produced positive results in the considered test, and specificity is regarded as the ratio of non-carcinogens that gave negative responses. The ability of a battery of three *in vitro* genotoxicity tests to discriminate between rodent carcinogens and non-carcinogens was made by Kirkland et al. to increase the specificity of a valid test (258). The conclusion was that the "profile" of the genotoxicity results, such as the concentration, the level of toxicity and magnitude of response, provided a body of evidence to predict the carcinogenic results (259).

The rodent bioassays were useful and relevant for predicting risks of human cancers (260). The epigenetic changes with a loss of restriction of cell division (261) and the DNA oxidative stress damage were likely to produce cancer. Trosko and Upham found that the changes in gene expression caused by cell communication systems play a key role in the imbalance of cell proliferation, differentiation, and apoptosis, eventually promoting the tumor process (262). A large number of rodent tumor findings were found not relevant for humans (262) recently. It is worth noting that traditional carcinogenicity studies are largely not predictive of human cancer risk, therefore the well-suited approaches were proposed, for instance, the genetically modified animal models (15), and *in vitro* carcinogenicity screening assays based on gene expression profiling (16, 263). From the perspective of prospects, a more useful and accurate method to predict the carcinogenicity in humans is very urgent.

Herein, 136 antiparasitics were collected from both human and veterinary pharmacopeia. Due to the design of toxicity and the highest concentration in *in vitro* genetic toxicity tests have changed enormously in current guidelines, the reliably of old data were evaluated and as low as 45.5%. For a larger proportion of antiparasitics, whose genotoxicity and/or carcinogenicity results were not retrievable, the retesting based on revised guidelines should be done to make a safety assessment of human health. The combination of DNA lesions and bacterial mutagenicity is more accurate for predicting carcinogenicity than bacterial mutagenicity alone or together with any other genotoxicity testing. Development of this method for predicting carcinogens should be applied to reduce the misleading hazard alerts of the new and effective drugs.

AUTHOR CONTRIBUTIONS

ZY conceived the idea. XW analyzed and discussed data. QL analyzed and discussed data and wrote the article. ZL performed and revised the experiments. AI and FZ revised the article. All the authors discussed the results and contributed to the final manuscript.

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Experiences in Tick Control by Acaricide in the Traditional Cattle Sector in Zambia and Burkina Faso: Possible Environmental and Public Health Implications

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Livestock, especially cattle, play a paramount role in agriculture production systems, particularly in poor countries throughout the world. Ticks and tick-borne diseases (TBDs) have an important impact on livestock and agriculture production in sub-Saharan Africa. The authors review the most common methods used for the control of ticks and TBDs. Special emphasis is given to the direct application of acaricides to the host animals. The possible environmental and public health adverse effects (i.e., risks for the workers, residues in the environment and in food products of animal origin) are mentioned. The authors present two case studies, describing different field experiences in controlling ticks in two African countries. In Zambia (Southern Africa), a strategic dipping regime was used to control *Rhipicephalus appendiculatus* ticks, vectors of theileriosis, a deadly disease affecting cattle in the traditional livestock sector in Southern Province. The dipping regime adopted allowed to reduce the tick challenge and cattle mortally rate and, at the same time, to employ less acaricide as compared to the intensive dipping used so far, without disrupting the building-up of enzootic stability. In Burkina Faso (West Africa), where dipping was never used for tick control, an acaricide footbath was employed as an alternative method to the traditional technique used locally (portable manual sprayers). This was developed from field observations on the invasion/attachment process of the *Amblyomma variegatum* ticks – vector of cowdriosis – on the animal hosts, leading to a control method aimed to kill ticks temporarily attached to the interdigital areas before their permanent attachment to the predilection sites. This innovative method has been overall accepted by the local farmers. It has the advantage of greatly reducing costs of treatments and has a minimal environmental impact, making footbath a sustainable and replicable method, adoptable also in other West African countries. Although the two methods described, developed in very different contexts, are not comparable – if public health and environmental implications are taken into account, if a balance among efficacy of the control method(s), cost-effectiveness and sustainability is reached – a way forward for the implementation of a One Health strategy can be set.

Keywords: tick control, acaricides, public health, environmental impact

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INTRODUCTION

Livestock play an essential contribution to the livelihood of agriculture-based societies throughout the poor countries of the world (1). The sustainable livelihoods framework places great emphasis on five capital assets as a source of livelihood (namely natural, social, human, physical, and financial capital). Besides the livelihoods of the livestock owners, livestock contribute to hired caretakers, vendors, workers in related industries, as well as the consumers. Livestock, especially cattle, play a paramount role within smallholder dairy, crop-livestock and livestock-dependent systems, especially in poor countries. Most – if not all – of these production systems are at risk from ticks and tick-borne diseases (TBDs). Loss of an animal or reduction of its productivity can, in turn, affect more than one type of capital assets (1).

Tick-borne diseases affect 80% of the world's cattle population and are widely distributed throughout the continents, particularly in the tropics and subtropics (2). It is in fact widely accepted that tick-borne haemoparasitic diseases are – and will likely continue to be – among the most important cattle diseases in the world, with higher impact in tropical and sub-tropical countries. It has been estimated that the annual global costs associated with ticks and TBDs in cattle amounted to between US$ 13.9 billion and US$ 18.7 billion (2). Ticks and TBDs represent an important proportion of all animal diseases affecting the livelihood of poor farmers in tropical countries (1). This is particularly true in Africa, where other serious vector-borne diseases (e.g., tsetse-transmitted trypanosomiasis, Rift valley Fever, etc.) occur in the same areas where the livestock population is already heavily affected by ticks and TBDs.

Briefly, the complex of vector-borne diseases, and in particular TBDs, constrains directly or indirectly the improvement and the growth of the whole livestock industry in Africa, which is of fundamental importance to rural people, by sustaining not only their food supply but also their daily income and other agricultural activities (1). More precisely, the epidemiological pattern and the risks are different according to the geographical areas (3): (i) in East, Central, and Southern Africa, where theileriosis due to *Theileria parva* is present (see below) and where European settlers introduced European cattle breeds, tick control measures have been implemented since the beginning of the twentieth century by the authorities. Thousands of dip-tanks (DTs) were thus built and cattle were regularly treated to prevent diseases transmission; (ii) in Western Africa, European farmers never introduced cattle breeds highly susceptible to the TBDs present in the areas. Local cattle did not suffer high losses due to these diseases, tsetse and trypanosomiasis being by far more prominent. Neither regional nor national tick control programmes were implemented (3). However, as the main tick species present in Central and Western Africa is *Amblyomma variegatum*, which is responsible for important direct losses, farmers were used to limiting cattle infestation by manual removal, and more recently by use of acaricide chemicals.

Ticks are thus responsible for indirect losses due to TBDs (reduction of production and mortality) but also for direct losses caused by their attachment to animal hides and blood sucking activity, leading sometimes to wounds, udder damages, weakness, and death of calves insufficiently fed by infested dams (4). Some particular tick species are also responsible for paralysis or "sweating sickness" due to the injection of toxins (1, 2).

According to Minjauw and McLeod (1), modified from McCosker (5), the major TBDs or TBDs complexes, which have a particularly severe effect on cattle, can be classified into four groups according to the tick vector species:

i. *Boophilus,* now *Rhipicephalus* (*Boophilus*) spp. are vectors of species of *Babesia* and *Anaplasma* (responsible of the so-called babesiosis–anaplasmosis complex). Worldwide, anaplasmosis and babesiosis constitute the most widely distributed TBDs, having a particularly severe effect on imported (exotic) high-grade dairy and beef cattle. In 2005, the so-called blue tick, *Rhipicephalus microplus*, was introduced in Ivory Coast and Benin through the importation of cattle from Brazil (6). It is now recognized that the tick has colonized the sub-region, including neighboring countries, such as Burkina Faso, Togo, and Mali (7), inducing interactions between native and invasive cattle ticks species which have been recently studied (8). Since its introduction in West Africa, *R. microplus*, which is known to be the main vector of *Babesia bovis*, has become a major problem in traditional farms because the introduced tick populations are suspected to be resistant to acaricides, even to those of the last generations (see below).
ii. *Hyalomma* spp. are responsible for the transmission of the protozoan *Theileria annulata* which causes tropical (or Mediterranean) theileriosis. It occurs mainly in areas beyond the geographical regions concerned by this review (i.e., Maghreb region and Asia), where it mainly affects exotic cross-bred animals belonging to smallholders and peri-urban dairy producers. Local cattle breeds and buffalo are much more resistant.
iii. *Amblyomma* spp. are responsible for the transmission of the rickettsia *Ehrlichia* (*Cowdria*) *ruminantium*, which causes heartwater, a fatal disease which affects mainly sheep and goats, but also exotic cattle throughout sub-Saharan Africa. *Amblyomma* spp. also transmit the protozoan *Theileria mutans*, which causes a mild theileriosis, and it is responsible (adult *A. variegatum* ticks) for the worsening of cutaneous lesions due to the ubiquitous actinomycete *Dermatophilus congolensis*, which causes significant losses in West and Central-Southern Africa (9–11).
iv. *Rhipicephalus* spp. (in particular the *Rhipicephalus appendiculatus–zambesiensis* complex) are responsible for transmitting the protozoan *T. parva* which causes East Coast fever (ECF), a devastating disease in 11 countries of Eastern, Central, and Southern Africa, responsible for major losses in both small- and large-scale production systems. For more detailed information on this deadly disease, it is suggested to consult the comprehensive work by Norval et al. (12).

According to Walker (13), "*the acaricidal treatment of livestock remains the most conveniently effective way to reduce production losses from tick parasitosis and tick-borne pathogens, despite repeated predictions over many decades that this is an*

unsustainable" method. This statement should however take into account the conclusions of the FAO expert consultation held in Rome in 1989 which indicated that TBDs control should be based on enzootic stability which means that in the majority of the traditional systems, particularly in areas where *T. parva* is absent, very little or even nothing needs to be done to control ticks (14). Enzootic stability, initially mentioned by Mahoney and Ross (15) to describe the epidemiology of babesiosis in Australia, is defined as the condition where the infection of all animals occurs within the period during which young calves are protected by various mechanisms (passively acquired and non-specific factors). These animals can thus develop active immunity without showing symptoms of infection and are later on immune when infected again. When they, in turn, breed, such immune cows transmit passive immunity to their offspring. The maintenance of this enzootic stability is possible only when tick infestation is high enough to allow regular infection of dams and rapid infection of calves, within the early months of their life. In such a situation, tick control should take care not to disrupt the early and regular transmission of the pathogen *via* the ticks (14, 16).

The most widely used method for the effective control of ticks is the direct application of acaricides to host animals by using the following options, as described by Minjauw and McLeod (1) and by George et al. (17):

i. *dip-tanks*: dipping is an efficient, practical, and convenient mean of applying acaricide to a herd of livestock. However, it requires some permanent infrastructures to be maintained, the DT itself (with roofs, crush, and holding pens, etc.) which is expensive to build and to operate; the average capacity of a DT varies from 8,000–10,000 to 20,000–25,000 L and the amount of acaricide needed is high (generally more than 10 L of active ingredient); it requires specially trained personnel to ensure proper management (e.g., initial charging, timely and accurate replenishment of both water and acaricide, and accurate recording of the animals dipped).
   A detailed description of this method, including the design, construction, and management of DTs, is provided in the FAO field manual (18). Later in this paper, the method is described as an example of technical cooperation project for tick control by using "strategic dipping" regime (see par. Case Study 1: Field Experiences in Controlling Theileriosis by Dipping in the Traditional Livestock Sector in Southern Province of Zambia).
   An alternative method to the traditional dipping (i.e., immerging the whole animal body in a dipwash solution) was conceived in Burkina Faso from field observations carried out on the invasion/attachment process of *A. variegatum* adult ticks on cattle (19); this led to a control method aimed at killing ticks before their permanent attachment to the predilection sites using an acaricide footbath (20); it is important to note that the average capacity of a footbath is about 200 L, which is about 100 times less than a DT. Some photos and drawings on the design and construction of the footbath are provided in a technical fiche by Stachurski (21). A detailed description of this method will be given later in this paper as an example of research-development project applied to sustainable tick control [see par. Case Study 2: Footbath Acaricide Treatment, an Innovative Method to Control *Amblyomma variegatum* Ticks in the Peri-urban (Semi-Intensive) Cattle Production System in West Africa];
ii. *spray races*: they are more expensive and difficult to maintain than DTs as various several mechanical parts (e.g., engine, pumps, nozzles, etc.) are required, and this has restricted their use mainly to commercial farmers in most developing countries;
iii. *hand-spray*: it is the most widely method used by small-scale farmers for treating livestock with acaricides, but it is also potentially the least effective. As the farmers prepare and use themselves the aqueous formulation of acaricide, the concentration of the chemical may be inadequate (too low) or the amount used to treat each cattle may be insufficient (this is usually done in order to spare money);
iv. *pour-ons and spot-ons*: these are solutions or suspensions of acaricides to be poured along the back line of a treated animal, which spread and disperse over the whole hair/skin. These formulations are expensive, but have the advantage of not requiring water or costly equipment for their application. As the products used in pour-ons are synthetic pyrethroids (see below), they also have a long residual effect and protect animals against both ticks and biting flies. However, it should be pointed out that, sometimes, the pour-ons do not spread enough throughout the body surface to correctly control the ticks attached to the lower parts of the body;
v. *hand-dressing*: this procedure involves applying acaricide to the preferred host attachment sites according to tick species (i.e., ears, udder, scrotum, perianal region, neck). As the procedure is time consuming, hand-dressing can be considered in cases where the tick burden is low and there are only a few animals to treat.

There are different classes of acaricides, among which the most commonly available and recommended (1, 17, 22) are the following:

i. organophosphates (e.g., chlorphenvinphos, coumaphos, diazinon, dioxathion) and carbamates (e.g., carbaryl): these compounds are generally highly effective at low concentrations and are stable in DTs. However, organophosphates tend to accumulate in tissues or milk and are therefore not recommended for lactating cows;
ii. pyrethroids, mainly synthetic pyrethroids: highly effective group of acaricides that includes permethrin, decamethrin, deltamethrin, cyhalothrin, cyfluthrin and flumethrin. They typically show prolonged residual activity (at least 7–10 days) and have the additional advantage of being effective against biting flies. They are therefore used extensively in areas where trypanosomiasis is prevalent (mainly to control tsetse flies);
iii. amidines, which are compounds showing less prolonged residual activity (4–5 days), but no residues are found in meat or milk. The only amidine compound commercialized for tick control is amitraz.

In addition to the acaricides as such, there are also other chemical compounds to be used for tick control: macrocyclic lactones and benzoylphenylureas. The former ones (i.e., ivermectin, moxidectin, doramectin, etc.) are active against a variety of endo- and ecto-parasites besides ticks, and can be administered orally, by subcutaneous injection or pour-on application. However, these products are expensive and residues can occur in the milk and meat of treated animals for several weeks after application. The latter ones (benzoylphenylureas) are growth regulators and do not kill the ticks but disrupt their development, stopping the molting process. The best-known product, difluorobenzoylurea (Fluazuron®) is applied to cattle as a pour-on, acts in a systemic way but has a long residual life in tissue and milk. These products are very effective against one-host ticks such as *Rhipicephalus (Boophilus) microplus* and may be a solution where resistance to other acaricides is high (1).

Although chemicals are an important part of efforts to control ticks on cattle, they are expensive and can be detrimental to the environment and dangerous for the consumers if the recommended withdrawal periods for food of animal origins are not respected: therefore, the use of acaricides should be minimized and integrated with alternative tick control approaches (1, 2, 23). Depending on the abundance and importance of the various tick species, control strategies/treatment regimes such as seasonal treatments at the peak of tick activity (strategic or threshold tick control) or intensive dipping/spraying at the beginning of the tick season, may be sufficient to avoid economic losses due to ticks and TBDs. Effective control of TBDs is best achieved through a combination of tick control, prevention of disease through vaccination – when available – and treatment of clinical cases, where control fails (2).

Alternative non-chemical tick control methods, such as use of predators and parasites of ticks, pasture spelling (i.e., leaving pastures unstocked to break the tick's life-cycle), anti-tick plants, tick-resistant cattle, and vaccination with tick antigens are available, but are not commonly used, and sometimes not always successfully employed (24–26).

The main methods for ticks and TBDs control are on the international research agenda for many years and have been reviewed by various authors; an integrated use of the tools available is recommended with a broader view to link TBDs control to the control of other parasitic diseases (1, 2, 26, 27).

The continuous use of chemical control to limit the harmful effects of ticks has led to the development of acaricide resistance in ticks, as it is the case with most chemicals for pest control. This is observed in particular with *R. microplus* because of the biology of this species: it is a one-host tick, accomplishing its whole parasitic cycle on the same animal within 21 days which allows the completion of 3–5 generations annually. It is therefore subject to more important selection pressure (17, 28–30). In the '90s, populations of *R. microplus* resistant to amidine (amitraz) were identified in Australia and South America, where ticks resistant to macrocyclic lactones were also found since 2000 (17, 30). In Africa, more precisely in Southern and Eastern Africa, one-host ticks (*R. microplus* and *Rhipicephalus decoloratus*) resistant to the majority of the different classes of "old acaricides" (but not to amitraz and macrocyclic lactones) have also been described (17, 22, 30). On the contrary, very few resistant three-host tick populations (*Amblyomma*, *Hyalomma*, *Rhipicephalus* spp. other than the former *Boophilus*) have been described in Africa (17, 30).

In West Africa, investigations carried out with *Rhipicephalus geigyi* in 2005 showed that even this one-host tick does not presently exhibit resistance to the acaricides under usage (31). At that time, acaricide resistance was not an issue of great concern; farmers continued to apply available acaricides to successfully control *A. variegatum* infestation during the main infestation period, the rainy season. Since the introduction of *R. microplus*, farmers were somewhat destabilized: in contrast to what they used to experience with *A. variegatum* infestation, *R. microplus* infest animal all along the year despite all kind of control means they may apply. Such alarming situation brought to suspect acaricide resistance in field tick populations (7). Preliminary laboratory bioassays on field tick population collected in Burkina Faso and Benin (i.e., testing almost all combinations of field isolates and acaricides) showed a strong resistance, mainly with pyrethroid such as deltamethrin and cypermethrin, in *B. microplus* populations as compared to what was observed for *Boophilus geigyi* (32).

Nowadays, the use of synthetic acaricides is still one of the primary methods of tick control, and therefore, it would be imperative to develop strategies to preserve their efficacy (30, 33). Negative aspects of the use of acaricide chemicals, besides their high direct costs, are the selection of resistant tick populations, the risk of jeopardize enzootic stability, the production losses due to handling of the animals and to the withdrawal periods, the public health implications due to toxicity, and environmental impact. In addition to that, some authors have claimed that systematic chemical control could reveal to be a non-cost-effective strategy, unless a complex set of variables (i.e., local epidemiological situation, infrastructural, and institutional constraints, etc.) are taken into account and carefully appraised (22, 34), which led some authors to suggest the strategic and threshold tick control regimes previously mentioned.

CASE STUDY 1: FIELD EXPERIENCES IN CONTROLLING THEILERIOSIS BY DIPPING IN THE TRADITIONAL LIVESTOCK SECTOR IN SOUTHERN PROVINCE OF ZAMBIA

The information and data reported hereunder (i.e., the set of activities described in this section: case study 1) are based on the publications, papers, and project reports by Ghirotti et al. (35); Camoni et al. (36); De Meneghi et al. (37); Scorziello et al. (38); and De Meneghi et al. (39) to which reference will be made throughout the text.

The role played by cattle in the traditional husbandry sector is of paramount importance in Zambia. National cattle herd accounts for 2.7 million head, of which 2.2 belong to the traditional agriculture system, characterized by subsistence crops, communal grazing of livestock, and cattle transhumance.

Southern province is the most important area for agriculture and livestock production in the country: it accounts for half of the national herd.

Tsetse-transmitted trypanosomiasis and theileriosis (ECF) are the two most important diseases of cattle in Zambia and constitute a major constraint to the development and productivity of the traditional cattle sector in Zambian farming.

Theileriosis had emerged as the single most important cause of mortality of cattle in Zambia: in Southern Province alone, some 30,000 head of cattle died between 1981 and 1987 (39, 40). Theileriosis due to *T. parva* is usually a fatal disease in cattle, especially in naïve adult animals and in calves. It is mainly characterized by pyrexia, lymph nodes swelling, lacrimation, anorexia and emaciation, dyspnea and pulmonary edema, digestive disturbances, abomasal ulceration, enlargement of the spleen, and lymphoid infiltration of kidneys (12).

Due to repeated outbreaks of this deadly disease and the risk of diffusion throughout the country, the Department of Veterinary and Tsetse Control Services (DVTCS), Ministry of Agriculture of Zambia, requested support and technical assistance to the Italian Ministry of Foreign Affairs, General Directorate for Development Cooperation. Hence the Animal Health Program in the Republic of Zambia (AHP), a bilateral project between the Ministry of Agriculture of Zambia and the Ministry of Foreign Affairs of Italy, was initiated and jointly implemented by the Istituto Superiore di Sanità (Higher National Health Institute of Italy) and the DVTCS. One of the main activities of the project – which started in 1987 and ended in 1992 – was the control of theileriosis in Southern Province through regular immersion of cattle in DTs containing an acaricide leading to control the main tick vector (39).

The project area included all Southern Province of Zambia, located at 25°10′–28°50′E and 15°14′–18°00′S. It covers an area of about 83,000 km^2 and is divided in seven administrative districts. Elevation varies from 770 to 1,050 m ASL in the *valley* area, and from 1,050 to 1,400 m ASL in the *plateau* area. Annual average rainfall ranges from 500–600 mm in the *valley* to 800–900 mm in the *plateau*, with a rainfall peak in December–January. Vegetation varies in *valley* and *plateau* areas, from *mopane* to *miombo* woodland and thorny shrubs, interspersed with generally poor pasture grassland. There are three main seasons: a dry-hot period (September–November), a warm-wet period (December–April), and a cool-dry period (May–August). Climate and vegetation greatly influence the seasonality, abundance, and distribution patterns of ticks (40).

There were about 130 communal DTs distributed in the Southern province, and all operating under the AHP assistance. Farmers/livestock keepers were required to dip their animals at the DTs at predetermined intervals according to a strategic dipping regime: at 1-week interval during the high risk season (from November–December to March–April), taking into account the rain pattern and the abundance of adults ticks. From May to October, dipping was discontinued in order to allow cattle to be exposed to the mild nymphal challenge during the dry period: this allowed not completely interrupting parasite–host contacts and thus not jeopardizing the establishment of enzootic stability (39).

The acaricide, provided and distributed under the project assistance to the traditional farmers, was chlorfenvinphos, an organophosphorous compound (30% active ingredient, EC formulation). Chlorfenvinphos is a non-flammable liquid, miscible with organic solvents; it is also a lipophilic substance that may be detected in fats (e.g., milk). The degradation of chlorfenvinphos in the soil is within the range of 4–30 weeks according to the type of soil, temperature, and light. Degradation in water varies according to ph values and temperature. It is transformed in photochemical reaction. In man and animals, chlorfenvinphos is an inhibitor of cholinesterase activity, and its action occurs at both peripheral and central nervous system levels. It is toxic by inhalation, ingestion, and skin contact. Dermal exposure is the main route of pesticide absorption for workers (i.e., DTs supervisors, livestock keepers), even though inhalation is also considered important. Acute intoxication may vary from mild to severe, according to length and method of exposure and the quantity of the substance absorbed. Diagnosis of the intoxication may be difficult in mild cases when only miosis, nausea, headache, vomiting, weakness, and giddiness are observed. Severe intoxication is characterized by sudden tremors, generalized convulsions; death may occur from respiratory or cardiac failure. Chronic intoxications are rare because organophosphorus compounds are in general not highly cumulative and because, in mammals, the metabolites are usually eliminated within a few days. Nevertheless, peripheral delayed neuropathy associated with exposure to organophosphorus compounds has been observed. The severe poisoning that results from the rapid absorption of the chemical by the respiratory tract and through the skin requires that special attention is paid to protective clothing. Atropine sulphate is the antidote to be used in case of organophosphorus acute intoxication (36).

The various procedures for dipping and the general practices during DT management activities include transport, storage, mixing, and immersion of animals and final disposal of the acaricide: during these activities, vet staff and livestock owners may be at risk of exposure to harmful levels of pesticide at each stage, because of mismanagement and improper handling or accidents. An aspect which is often overlooked is the likely re-use of plastic pesticide containers by local people to store drinks and foodstuffs (36, 38).

Since acute pesticide poisoning is a serious problem in developing countries, and organophosphorus compounds seem to be one of the major causes, the AHP deemed it very important to deal with public health, occupational, and environmental health aspects related to the use of acaricide. The interventions planned and carried out by the project in the period under review were inspired by a One-Health approach *ante litteram*. Several activities aimed at preventing health and environmental hazards connected with the use of the acaricide at the DTs were planned and implemented following different phases: (i) collecting information; (ii) identifying resources; (iii) defining objectives and implementing related actions, included a feasibility study *in loco* (38).

The interventions carried out within the project framework did include an integrated set of activities which have been described in various publications, scientific papers, and project reports (35–39) to which reference can be made for more detailed

information. An account of the most important and significant activities carried out within the project framework is given hereunder; data and information provided throughout this section are solely based on the papers, publications, and reports mentioned above, therefore bibliographic quotes will not be reiterated:

i. assessment of the occupational hazards (i.e., ways and modalities of exposure of workers to the acaricide) as well as environmental hazards, by using an *ad hoc* questionnaire to ascertain procedures in the working environment (i.e., safety of the DTs operators, safe disposal of empty containers, accidents at work, environmental pollution, etc.);
ii. provision of protective equipment (e.g., plastic aprons, rubber gloves, face masks, etc.) for distribution to DTs supervisors;
iii. distribution of atropine sulphate vials to all the District Veterinary Offices and District Hospitals in Southern Province to be used in case of organophosphorus acute intoxication;
iv. training activities and conferences/seminars: on-the-job training courses/workshops on DT management, health and environmental risks related to pesticide handling were organized for all DTs supervisors and field veterinary assistants working in the project area; national seminars on DT management, ticks and theileriosis control were organized for livestock officers and veterinarians in theileriosis affected areas of Zambia;
v. health promotion and health education activities: instruction leaflets on dip management (written in the local Tonga language) distributed to traditional farmers; meetings held with groups of farmers to explain the basic principles of dip management, dipping policy and the risks related to the use of acaricides; organization of a radio programme on dipping and on the related risks, broadcasted in the local Tonga language in collaboration with the National Farming Information Service (NFIS) (radio is a popular mass medium: several radio programmes in English and the major local languages are broadcasted daily all over the country, and in particular health education programmes have been developed by the Provincial Health Officers in collaboration with veterinary and agriculture extension officers); a drama group technique was used for our radio programme in order to deliver the messages in small scattered villages, as such technique is an usual communication channel in the local culture; furthermore, a TV series on agriculture ("*Lima Time*"), produced by NFIS in English language, broadcasted an episode on theileriosis and its control and prevention. Personal observations showed a good audience level and acceptability of the radio and TV messages among local people; in particular the TV programme seemed to be enthusiastically received, even though the number of TV sets is quite limited, especially in rural areas.
vi. Field research applied to public and environmental health: in order to investigate on the presence of acaricide residues in milk from dipped cattle under local field conditions, milk samples from traditional cattle herds were collected before dipping and at fixed intervals after dipping; in addition, samples were also obtained from the local milk collection depots; besides – as the use/re-use of empty acaricide tins was reportedly quite common in most villages of the project area – water samples stored in empty acaricide tins were collected (before and after washing with fresh water and/or with detergent) to evaluate the actual risk of re-using empty containers for storage of drinks and foodstuffs; our study demonstrated that milk collected and consumed 18–24 h after dipping appears to be safe for human consumption, according to the recommended international residues limit values, whereas acaricide residues in water stored in empty acaricide tins (although washed several times with fresh water and/or with detergent) were found to be well above the recommended safe levels, thus confirming the risks related to the re-use of plastic containers and, at the same time, providing useful information for health education activities.

To conclude, it should be stressed that although most of the risks for public health related to dipping management practices can be greatly reduced by using appropriate information/training activities, and/or by providing protective equipment, etc., there are other practices for which the impact on environmental health is not easy to prevent or to reduce significantly: for instance, when the dipwash from the DTs has to be removed at the end of the dipping season – especially if the pollution level is high (i.e., excess of dung and/or mud in the dipwash) – the option to pour it on fallow land near the DTs, to be degraded by the sunlight, is not acceptable; a solution – although not always possible and not completely safe for preventing environmental impact – could be to temporarily stock the dipwash in make shift decantation pits nearby, and then pour the dipwash on fallow land only after the active ingredient has been completely degraded by the sunlight and decanted in the pit.

CASE STUDY 2: FOOTBATH ACARICIDE TREATMENT, AN INNOVATIVE METHOD TO CONTROL *AMBLYOMMA VARIEGATUM* TICKS IN THE PERI-URBAN (SEMI-INTENSIVE) CATTLE PRODUCTION SYSTEMS IN WEST AFRICA

In Burkina Faso, as in most of Western African countries, traditional, extensive, and low-input cattle systems based on rearing of local breeds, account for most of the cattle production. The semi-intensive farming system, where exotic breeds are used to improve animal production, in particular dairy production, remains marginal: the corresponding farms, located mainly in urban and peri-urban areas, represent only 5% of the total cattle production (31). In West Africa, *A. variegatum*, more precisely the adults of this species, is the most harmful tick impairing animal growth and leading to sometimes very serious wounds (41, 42). Because the udder is one of the tick predilection sites, these wounds can result in the complete destruction of one or more teats (43). These lesions lead to an important reduction in the milk yield of dams and, consequently, to lower growth rates and higher mortality in their off-springs (4, 44). This tick

exacerbates dermatophilosis cutaneous lesions (9) which are also observed on local breeds although they are less sensitive than the exotic introduced ones; besides, it transmits *Ehrlichia ruminantium*, the causative agent of cowdriosis (45). Studies have however shown that local cattle breeds benefit from a certain degree of enzootic stability to this disease, which is not the case for local small ruminants or for introduced cattle (46).

The tick control practices of traditional farmers in West Africa are thus aim mainly to limit infestation by *A. variegatum* adults, which are active during the rainy season, particularly during the first months of this period (41). Farmers in low-input systems, who used to remove these ticks manually, are now increasingly using acaricides, generally applied by manual sprayers, to control the ticks. As their income is very low, the products are frequently misused: inadequate volume is sprayed, between-treatment intervals are excessive, cheaper chemicals of uncertain origin because bought on unmonitored markets are used, and acaricides are replaced with agricultural pesticides such as those supplied for the treatment of cotton fields (20, 31).

During field studies carried out in the late '90s in Cameroon and Burkina Faso within the framework of research-development projects implemented by CIRAD (*Centre de Coopération Internationale en Recherche Agronomique pour le Développement*) and CIRDES, it was observed that *A. variegatum* adults do not attach to their predilection sites (udder, chests, inguinal area, etc.) as soon as they infest cattle: they first attach, not very strongly, to the interdigital areas where they remain as long as the hosts are walking and grazing (19). Ticks reach the predilection sites when the animals lie down to rest, an important proportion of them moving from one animal to another (19). As, during the rainy season, cattle are traditionally brought to graze in the savannah for about 9 h a day, they have very little time to rest or lie down (47); consequently, ticks move to the predilection sites mainly during the night and about 90% of the ticks captured on the pasture are still attached to the feet when the animals return to night paddocks in the evening (19).

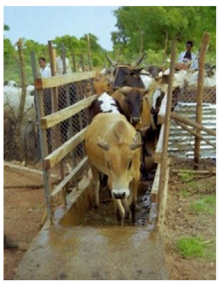
Methods to treat cattle feet in order to eliminate the captured ticks and prevent them to attach to the body were looked for (43). The first attempt consisted in localized application of a flumethrin formulation at mornings, using a manual sprayer, on cattle confined in a crush-pen. The results of this trial were not optimal, important volumes of acaricide formulation being used and tick infestation on animals increasing despite treatment, partially due to the fact that ticks could move from untreated control cattle to sprayed cattle during the night, when all animals were kept together in the kraal. A footbath was then built and allowed to obtain much better results (20). Using various pyrethroids (flumethrin, alphacypermethrin and deltamethrin), cattle treatment carried out during the peak infestation period of adult ticks (i.e., from mid-May to the end of July) proved to be efficient in preventing the ticks from attaching to the predilection sites. The method was appreciated by traditional livestock farmers, essentially because it is not time consuming (once animals are familiar with the footbath, 120 animals can be treated in less than 15 min) and because it requires only about 200 mL aqueous acaricide formulation per animal at each passage, thus greatly limiting the risk of acaricide spreading in the environment. The cost of the acaricide required to treat one animal during the peak infestation period was assessed at about 130 FCFA or 0.20 €. Of course, the cost of the installation itself was not insignificant (about 330,000 FCFA or 500 €) and could not likely be afforded by a single traditional farmer. Therefore, it was suggested that the installation should be built and used by all cattle owners of a given village, more precisely by all farmers whose herds were kept for the night less than 2 km from the footbath.

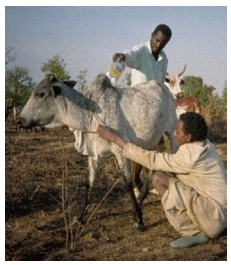
Other studies showed afterward that this control method could also kill tsetse flies, at least the species present in Burkina Faso, since the legs are the most targeted parts of the body for blood meals of *Glossina tachinoides* and *Glossina palpalis* (48). Therefore, footbath treatment of cattle can not only decrease tick infestation of treated cattle but also reduce the incidence of trypanosomiasis in cattle (49, 50). Besides, as important malaria vectors, such as *Anopheles arabiensis*, feed on cattle as well as on humans and since more than 90% of these mosquitoes feed on the legs of cattle (51), such targeted cattle treatment could also have great impact on mosquito populations and contribute to malaria control of people living near cattle herds.

From 2000 to 2007, more than 50 footbaths were established in Burkina Faso, among which the majority have been installed by development projects. A few farmers even built their own footbath after noting the efficiency of the method. Experience acquired in Burkina Faso indicates that, despite scientific evidence of the efficacy of acaricide footbaths to control *A. variegatum*, a large-scale application of this tick control measure is not obvious. The acceptability of the acaricide footbath depends on farm organization and/or farming systems. Farmers working in semi-intensive and modern systems around Ouagadougou and Bobo-Dioulasso (Burkina Faso) tended to use more easily the acaricide footbath. In contrast, farmers working in the traditional husbandry sector, which is based on extensive and nomad grazing, faced some difficulties in incorporating footbath usage into their usual practices. Such difficulties persisted even for the traditional farmers that were organized within farmers' groups (*associations d'éleveurs*). This may partly result from the paradoxical situation where, on the one hand, acaricide footbaths are necessarily fixed installations while, on the other hand, cattle transhumance is need – according the traditional husbandry system – for finding suitable pastures all year around. Moreover, it is noteworthy that any experience of tick control failure using the acaricide footbath would further enhance the unwillingness of the traditional livestock keepers to accept this tick control measure because of the efforts already experienced in adapting its use to their usual traditional practices.

A sociological study was carried out at that time in Ouagadougou and Bobo-Dioulasso to assess the adoption of this innovative tick control method (52). Authors studied the process and level of the adoption of the technology with 72 farmers. Variables describing the breeding system, the implementation and perception of the method and the knowledge of the epidemiological system were used to discriminate three clusters of farmers that were then compared using indicators of adoption. The first cluster corresponded to "modern" farmers who adopted the technique very well. The more traditional herders were discriminated into two clusters, one of which had a good adoption level, whereas the second

TABLE 1 | Comparative score attributed to the tick control methods described in case study 1 and case study 2: major advantages and disadvantages.



Control method	Dip-tank (case study 1)	Footbath (case study 2)	Portable manual sprayer[a]	Pour-on[a]
Initial investment	20 0000 US$	400 US$	80 US$	0 US$
Cost for the whole rainy season (per cattle head)	1.5 US$	0.2–0.25 US$	0.15–0.25 US$	3–5.5 US$
Usefulness to treat one/few animal(s)	*	*	**	***
Usefulness to treat many animals or more than one herd	***	**	*	**
Environmental implications/ hazards 1. volume of product to be used	***	*	**	*
Environmental implications/ hazards 2. risk of spilling/pouring/ dispersal on fallow land	***	*	***	from * to *** (depending on product used)
Public health implications/hazards 1. risk for the operators	**	*	***	*
Public health implications/hazards 2. residues in foods of animal origin	***	**	*	*

[a]*Other (most) common tick control methods used under field conditions in the study areas.*
*Key-legend of the score attributed: * low level; ** medium; *** high level*

failed to adopt the method. The economic benefit and the farmers' knowledge appeared to have a low impact on the adoption level, whereas some modern practices (cattle breed, regular use of metallic pen, number of individual facilities) as well as social parameters (individual/collective management, kind of socio-technical network) appeared determinant. The level of technical support had also a great influence on the adoption level. What can be learned from this study is that farmers in basically traditional systems with herds in movement during wet season are not suitable for footbath implementation. However, it is expected that good results can be achieved with groups of farmers engaged in innovation (semi-intensive peri-urban production systems) with good leadership.

CONCLUSION AND DISCUSSION

As it has been pointed out in the Section "Introduction," the control of ticks and the diseases they transmit is a very complex issue. A single solution does not exist: different livestock production systems, multifaceted epidemiological patterns and diverse socioeconomic contexts are only some of the many aspects to be taken into account when tackling one of the most important constraints for animal health and production, especially in the so-called developing countries. Over the decades, the initial approach of the most widely used method for tick control – chemical treatment – significantly changed: from intensive acaricide control, aimed to "eradicate" the ticks, it was changed to more ecologically and economically sustainable acaricide control methods, such as strategic, threshold regimes. Actually, the need to reduce the costs for ticks and TBDs control and to avoid the development of acaricide resistance, and – at the same time – the consciousness and willingness to limit possible public health risks, has progressively induced the veterinary authorities, researchers, policy makers, as well as the stakeholders – including livestock breeders – to start applying an integrated control approach/package which takes into account the different options/strategies for ticks and TBDs control (2, 23, 26).

The two first hand experiences on tick control presented here – although carried out in different periods (late '80s–early '90s in Zambia, and late '90s–early '00s in Burkina Faso) and not comparable (the two areas greatly differ from the ecological, epidemiological, geographical, and socioeconomic points of view) – are a "photograph" of two different contexts where the tick control methods and strategies implemented have in common an "embryo" of attention and awareness for the possible environmental impacts for public health risks due to the use of acaricides.

As already pointed out, although the two methods cannot be compared and analyzed by using a quantitative method, the authors attempted to attribute a qualitative/semi-quantitative score by comparing the most important and relevant *pros* and

cons of the two methods: (i) usefulness to treat one animal or many animals/one herd; (ii) overall costs (i.e., initial investment and treatment on a yearly basis/per cattle head), hence economic sustainability; (iii) environmental and public health implications and/or hazards (i.e., risk of spilling/pouring/dispersal of acaricide, risk for the operators, residues in foods of animal origin, etc.) (see **Table 1**).

Unfortunately, the authors cannot report first hand any updated and follow-up information on the two projects (in Zambia and Burkina Faso) where they used to work before (both projects have been now terminated/discontinued, and no published follow-up information have been found).

As regards the case study on strategic dipping in Southern Zambia, it should be added that during the last operational period of the Italian project, a FAO project was started in Monze district (Southern province) with the aim to vaccinate cattle against theileriosis by the "infection-and-treatment" method (Muguga cocktail) (53). This was a second phase of a larger FAO regional pilot project [an earlier vaccination trial was carried out in selected areas of Southern province (54)]. The vaccination strategy required that vaccinated cattle had to be exposed to *T. parva*-infected ticks in order to allow natural post-vaccination boosters, and this created some problems/misunderstanding/lack of trustfulness in those livestock farmers who were used to apply the strategic dipping under the Italian project. Actually the "infection-and-treatment" method was a more ecologically sound method for theileriosis control as compared to dipping, and it is an important component of the so-called "integrated ticks and TBDs control package," which was – and still is – strongly advocated and promoted internationally (2, 23, 26). When the FAO project was interrupted, the vaccination was discontinued for some years until when a new project was re-initiated under a Belgian funded technical assistance programme (a local *T. parva* stabilate/strain – not the Muguga cocktail – was then used for the infection-and-treatment vaccine) (55). Changes in theileriosis control strategies, project activities being interrupted/discontinued, intervention of different donors, and technical assistance agencies are factors which may induce cattle farmers to lose confidence in the control method(s) adopted, thus raising the need for assessing the acceptance of ECF immunization and/or other method(s) by evaluating the perception of farmers (56). The same ECF vaccination method promoted by FAO in Zambia was also used in selected cattle breeding areas in Tanzania during late '90s-early 2000s, under a FAO-funded project (57). Interestingly, in this case, after the FAO project assistance stopped, the vaccination was successfully continued for many years in Northern Tanzania, on a self-sustained commercial basis (58).

As regards the case study of the footbath treatment developed in Burkina Faso, after the initial demonstration of the efficacy of the method to limit *A. variegatum* adult ticks infestation, various development projects were convinced of its interest and proposed this method to farmer organizations of Burkina Faso and neighboring countries: a project, supported by CORAF/WECARD (West and Central African Council for Agricultural Research and Development) and funded by Australian CSIRO (Commonwealth Scientific and Industrial Research Organization), planned to transfer the footbath technology to other countries, targeting at first state farms in Mali (Madina Diassa) and Benin (Kpinnou) where the farmers could assess and learn the method. In Benin, where *R. microplus* tick is well established, the objective was also to check whether treatment with footbath could have any effect on *R. microplus* infestation. Unsurprisingly, the study showed that footbath treatment gave a positive result on *A. variegatum*, but was not effective against *R. microplus* because larvae of this one-host tick directly attach to the head and body of the cattle without temporary attachment to interdigital areas. As already mentioned, the socioeconomic studies carried out some few years after the introduction of this control method in Burkina Faso (52) revealed that peri-urban dairy farmers easily adopted the technique whereas traditional herders did so only if there was technical support to help them during the first months/year of use. This has to be taken into account for the potential next steps of method dissemination. On the other hand, the fact that the footbath can simultaneously reduce tick infestation and limit tsetse-transmitted trypanosomiasis (both animal and human form) could help for further acceptance of this control method. As mentioned earlier, there are a couple of examples where the treatment of cattle with insecticide/acaricide has led to indirect control effect on vectors of human diseases: in Chad, a field experience showed that treating cattle with footbath insecticide treatment has a positive effect in reducing tsetse density, hence protecting people – besides cattle – from tsetse and trypanosomes infection (50); in Ethiopia, cattle treatment with insecticide had also allowed to reduce malaria transmission by interfering with *Anopheles arabiensis* behavior and survival (51).

As a conclusion, such experiences of strategic use of acaricides/insecticides to control livestock diseases having also indirect action on vectors of human diseases are good examples of effective research-development projects whose results can be applicable at field level for integrated and sustainable disease control in poor resources countries. Once the possible public health and environmental implications of the control measures chosen have been taken into due account, and a balance has been reached among the efficacy of the control method(s), its cost-effectiveness, and sustainability, a new path can be set toward the implementation of a One Health strategy, which envisages an integrated approach for animal, human and ecosystem health.

AUTHOR CONTRIBUTIONS

DM: conception of the study carried out in Zambia, making analysis and interpretation of data of the study; FS and HA: conception of the study carried out in Burkina Faso, making analysis and interpretation of data of the study; DM, FS, and HA: drafting, reviewing, and finalizing the final version of the manuscript.

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Pesticides in drinking water – the Brazilian monitoring program

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Brazil is the world largest pesticide consumer; therefore, it is important to monitor the levels of these chemicals in the water used by population. The Ministry of Health coordinates the National Drinking Water Quality Surveillance Program (Vigiagua) with the objective to monitor water quality. Water quality data are introduced in the program by state and municipal health secretariats using a database called Sisagua (Information System of Water Quality Monitoring). Brazilian drinking water norm (Ordinance 2914/2011 from Ministry of Health) includes 27 pesticide active ingredients that need to be monitored every 6 months. This number represents <10% of current active ingredients approved for use in the country. In this work, we analyzed data compiled in Sisagua database in a qualitative and quantitative way. From 2007 to 2010, approximately 169,000 pesticide analytical results were prepared and evaluated, although approximately 980,000 would be expected if all municipalities registered their analyses. This shows that only 9–17% of municipalities registered their data in Sisagua. In this dataset, we observed non-compliance with the minimum sampling number required by the norm, lack of information about detection and quantification limits, insufficient standardization in expression of results, and several inconsistencies, leading to low credibility of pesticide data provided by the system. Therefore, it is not possible to evaluate exposure of total Brazilian population to pesticides via drinking water using the current national database system Sisagua. Lessons learned from this study could provide insights into the monitoring and reporting of pesticide residues in drinking water worldwide.

Keywords: drinking water criteria, drinking water standards, pesticide risk, Sisagua, Vigiagua

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INTRODUCTION

In 1997, at Mar del Plata, the Action Plan from the United Nations Water Conference recognized water as a right for the first time and, in 2010, the same organization stated that a sufficient and safe supply of water is essential for the realization of many other human rights (1). Since the 70s, the global population has nearly doubled, while the urban population almost tripled, in similar amount as the number of people using drinking water sources (2, 3). To serve public health, economic and human rights necessities, monitoring programs are used to track global, regional, and national progress on access to drinking water and sanitation (4). The lack of data regarding the occurrence of contaminants in waters inhibits the prioritization of substances to be regulated and the establishment of criteria for drinking water in relation to the risks associated with drinking water consumption (5). The selection of compounds to be regulated is not easy and quantity, physical and chemical properties, occurrence and potential hazard to non-target species need to be considered, for example (6).

Generally, when pesticide is applied following Good Agricultural Practices (GAP), the acceptable Maximum Residue Limits (MRLs) are not exceeded (7, 8). When a pesticide is approved, these maximum residue levels must not present risk to human health. However, the misuse of pesticides can occur and concentrations above the MRL can be found in crops (7, 8). Pesticide residues also can reach surface and groundwater, and consequently expose humans via drinking water. The contamination of water bodies can occur by leaching processes from plants and soil followed by rainwater drainage in rural and urban environments, as well as from sewage discharges, because of pesticides uses in pets and gardens.

To establish drinking water standards for chemical substances, a Chemical Risk Quantitative Assessment methodology has been used. The steps are hazard identification, exposure assessment, dose-response evaluation, and risk characterization (9, 10). The World Health Organization (WHO) and the Organization for Food and Agriculture of the United Nations (FAO) have established acceptable daily intake levels (ADIs) of pesticide residues. ADIs are values that indicate the maximum daily intake of a substance that does not represent risk to human health throughout the individual's life. Therefore, a pesticide ADI is usually obtained from its NOEL or NOAEL (no-observed-effect-level, or no-observed-adverse-effect-level), estimated from toxicity studies with laboratory animals with appropriate safety factors (varying from 10 to 10,000) (11, 12). However, the additional risk due to simultaneous exposure to several substances and different forms of exposure (i.e., drinking water, plant and animal foods consumption, dermal exposure, etc.) cannot be disregarded because synergism can occur (13, 14).

Many factors are involved in the establishment of a drinking water standard. Neto made a comparison in 2010 between the patterns of Brazilian drinking water criteria, international guidelines, and other countries data, finding a great variability in the way of establishing these values (15). The United States, for example, when establishing their criteria, take into account the potential adverse effects of contaminants on human health, the frequency and level of occurrence in public water supply systems, the available treatment technologies, and if the cost of regulation of the substance will represent a significant opportunity to reduce risks to public health (16). Otherwise, the values defined in Directive 98/83/EC adopted by the Member States of the European Community are not based on the chemical's toxicological properties, differently from WHO guidelines and those of other countries, but in the assumption that these substances must not be present in the drinking water, using a pragmatic cut off value of 0.1 μg/L for single pesticide and 0.5 μg/L for the sum of those present (17). Australia has a default value for pesticides, which is the quantification limit of the analytical method, therefore the quality criteria is not based on the toxicological properties of the substances, unless the analytical quantification limit is too high (18, 19). Therefore, setting a drinking water standard is not an easy task and includes not only scientifically but also economic, technological, and political factors.

With regard to the approach used by the United States, there are water monitoring programs to verify the occurrence of regulated and non-regulated compounds. This information is used to help in the definition of new priority contaminants that will be listed in the Drinking Water Contaminant Candidate List. This list will be used by the US EPA to define the need of the inclusion of new compounds in the drinking water standard. Their regulatory infrastructure is based on good practice analytical methods, laboratory certification, treatment technology (to identify and/or develop high quality, cost-effective treatment technologies to meet regulation), a periodical review of standards, the National Contaminant Occurrence Database, and the non-regulated contaminant candidates. This list is divided as follows: substances that are priorities for additional research, those that need additional occurrence data, and those that are priorities for consideration in rulemaking (20). This Non-regulated Contaminants Monitoring Program could guide developing countries such as Brazil for the inclusion of priority compounds in a drinking water norm.

Since 2008, Brazil is leading the global consumption of agrochemicals, a position previously occupied by the United States (1, 2). In addition to protecting crops from pests, diseases, and weeds, pesticides also pose a risk to human health and the environment through contamination of food, agricultural soil, surface, and ground water. Brazilian consumption of pesticides reached around 496,000 tons of active ingredients in 2013 according to the last report available (1, 2). Suitable chemical analytical methods are needed for the detection of pesticides and emerging contaminants. Recently, a method for quantifying several pesticide residues in water was developed and used to test drinking water samples from 9 cities, and surface waters from 13 rivers of the State of São Paulo, Brazil after 1 year of sampling collection (21). This was not the first time that difenoconazol, epoxiconazole, tebuconazole, atrazine, azoxystrobin, carbendazim, and fipronil were detected in Brazilian water bodies (21–24). One of the rivers that is the main source of drinking water to the city of Campinas have been studied for several years for the presence of emerging contaminants (5, 6, 21, 23) and endrocrine-active compounds (25). Recently, an *in vivo* study conducted with drinking water samples from this river showed evidence of endocrine disruption in prepubertal female rats (26).

Currently, in Brazil, there are 380 active ingredients authorized by the Ministry of Agriculture for pesticides used on crops and 1,670 formulated plant protection products on the market (27). Pesticide registration is regulated by Decree No. 4074/2002. It is a shared responsibility of the Ministry of Agriculture, Livestock and Supply (MAPA), Ministry of the Environment (MMA), and Ministry of Health (MH). The Ministry of Health is responsible for the analysis of the health aspects of the registration procedure and also for monitoring pesticides in food (among other activities). One of its departments, the Brazilian Health Surveillance Agency (ANVISA) coordinates the Pesticide Residues Analysis Program in Food (PARA). For example, in 2010, 28% of the samples were found unsatisfactory because of the presence of unauthorized pesticide residues or authorized ones above the MRLs (28).

Drinking water quality is not regulated by ANVISA but by the General Coordination of Health Surveillance (CGVAM) from Health Surveillance Secretariat (SVS), also sectors of the MH. The drinking water norm that is in place is the Ordinance No. 2914/11 and it defines standards and procedures related to the control and surveillance of water quality. CGVAM also coordinates the

National Monitoring Water Quality for Human Consumption Program (Vigiagua), a monitoring water quality program that operates through the Monitoring Information on Water Quality for Human Consumption System (Sisagua). Sisagua compiles the data that is included in the database. The drinking water suppliers are responsible for the quality control of drinking water; however, the water quality surveillance activity is a task of CGVAM, in collaboration with state and municipal secretariats (7).The latter are responsible for the inclusion of the data in the Sisagua database. In summary, the norm indicates that the data on the drinking water quality needs to be provided to MH through Sisagua, and then, the public health authorities are able to verify if the water consumed by the population complies with the current regulation, including with regard to the risks it may pose to human health.

The water quality Ordinance MH No. 2914/2011 regulates 64 chemical substances, of which 27 are pesticides monitored every six months and with data insertion in Sisagua. **Table 1** shows the regulated pesticides and their Maximum Allowed Concentrations (MAC).

The aim of this study was to evaluate the monitoring of pesticides data from the National Monitoring Water Quality for Human Consumption Program (Vigiagua), available on the Monitoring Information on Water Quality for Human Consumption System (Sisagua). Therefore, in this paper, we will critically evaluate the inclusion, compilation process, and assessment of pesticides data in the drinking water database available from the Vigiagua federal program.

MATERIALS AND METHODS

Water Quality Control

Quality control of drinking water in Brazil is assured through the evaluation of several parameters, which include microbiological, physical–chemical, and pesticides analyses (for details, please see Ordinance MH No. 2914/2011). The laboratories must perform their analyses under quality control systems, e.g., ISO17025 (30). Unfortunately, no information on the analytical methods applied was available in the Sisagua dataset.

Vigiagua Pesticides Data Analyses

CGVAM/MH provided the monitoring data set corresponding to the years 2007–2010 because the Sisagua dataset is not publicly available. The Brazilian drinking water ordinance states that analysis of pesticides must be performed in the water produced by the Drinking Water Treatment Plant (DWTP). If a sample presents a result not in compliance with the norm, the same pesticides should be then analyzed in the respective distribution network. As a consequence, limited data on the distribution network were retrieved, and therefore, only data from DWTPs were considered in our analyses.

We excluded invalid results in our data analysis after we observed different types of inconsistencies in the data set and reported them in number of non-valid results. Pesticide active ingredients in drinking water were reported by region, state, state capitals, and other municipalities (31). The verification of

TABLE 1 | Pesticides regulated by Brazilian Ordinance MH No. 2914/2011 and their maximum allowed concentrations (MAC) (29).

Pesticide (active ingredient)	CAS registry number	MAC (μg/L)	Pesticide (active ingredient)	CAS registry number	MAC (μg/L)
2,4-D + 2,4,5 T	94-75-7 93-76-5	30	Lindane (γ HCH)	58-89-9	2
Alachlor	15972-60-8	20	Mancozeb	8018-01-7	180
Aldicarb + aldicarbsulfone + aldicarbsulfoxide	116-06-3 1646-88-4 1646-87-3	10	Methamidophos	10265-92-6	12
Aldrin + dieldrin	309-00-2 60-57-1	0.03	Metolachlor	51218-45-2	10
Atrazine	1912-24-9	2	Molinate	2212-67-1	6
Carbendazim + benomil	10605-21-7 17804-35-2	120	Parathion-methyl	298-00-0	9
Carbofuran	1563-66-2	7	Pendimethalin	40487-42-1	20
Chlordane	5103-74-2	0.2	Permethrin	52645-53-1	20
Chlorpyrifos + chlorpyrifos – oxon	2921-88-2 5598-15-2	30	Profenophos	41198-08-7	60
DDT + DDD + DDE	50-29-3 72-54-8 72-55-9	1	Simazine	122-34-9	2
Diuron	330-54-1	90	Tebuconazole	107534-96-3	180
Endosulfan (α, β, and salt)	115-29-7 959-98-8 33213-65-9 1031-07-8	20	Terbuphos	13071-79-9	1.2
Endrin	72-20-8	0.6	Trifluralin	1582-09-8	20
Glyphosate + AMPA	1071-83-6 1066-51-9	500			

compliance with the drinking water standard was performed using the previous Ordinance MH No. 518/04, because during the period of this research it was the norm in place. When the information was reported as below certain value, we assumed that this was the limit of quantification and, if this was above the maximum allowed concentrations, the sample was considered non-compliant with the norm.

Evaluation of Pesticides Under the Current Ordinance MH No. 2914/2011

A survey was conducted on the best-selling active ingredients in Brazil to assess whether the regulated pesticides in the current drinking water were representative. The survey was based on the marketing data from ANVISA (from 2nd half of 2010 and 1st half of 2011), the Agrofit system (System of Phytosanitary Pesticides from the MAPA) and the most recent Pesticides Trading Report, released by IBAMA (Brazilian Institute of Environment and Renewable Natural Resources) (27, 32, 33). We considered only the most sold pesticide active ingredients in Brazil, from 2009 to 2012, which were used in a minimum of 1,000 tons/year. This list was compared with the Ordinance MH 2914/11, as well as with the canceled pesticides or the ones in registration revaluation (27, 33, 34). For information we consulted the monographs or toxicological reassessment files available at the official website of ANVISA. The information about the registered pesticides in Brazil was obtained in Agrofit (27, 32, 34).

Drinking Water Quality Criteria Calculation

Drinking water criteria were calculated using the ADIs publicly available in the ANVISA monographs, and the proposed WHO algorithm, applying 20% of allocation factor, 60 kg of body weight and 2 L of water consumption per person per day (10, 32, 35, 36).

RESULTS

Pesticide Active Ingredients Consumed in Brazil

The pesticide active ingredients most consumed in Brazil from 2009 to 2012 were glyphosate, mineral oil, 2,4-D, atrazine, sulfur, methamidophos, vegetable oil, carbendazim, acephate, mancozeb, and diuron. **Table 2** shows data on the substances whose sales were more than 1,000 tons in each reporting year, accounting for more than 80% of total sales (33).

Vigiagua Data Analysis

Participation Assessment of Municipalities by State and Region

Geographically, the Brazilian states are grouped in regions for statistical interpretations, common public service management systems and implementation of public policies of the federal and state governments. Currently, there are five official regions: Midwest, Northeast, North, Southeast, and South. Area, population and Gross Domestic Product (GDP) are presented in **Table 3**. The North and Midwest regions have the largest areas, but the smallest population density and the lowest GDP, and it is where the federal district is located. The Northeast region has the third highest GDP; the Southeast has the highest GDP with the highest population density and it is where the two most populous cities are located: São Paulo, with 11 million inhabitants and Rio de Janeiro with 6 million. The South has the smallest area and a middle-size population, but is the second richest region in the country, and the one with the highest Human Development Index (HDI), the highest literacy rate and levels of education, health and social welfare of the country.

The data available in Sisagua comes from the municipalities (state cities) of the Center-West, Southeast, and Southern regions of Brazil. The participation of municipalities in the North and Northeast was poor and did not contribute significantly to the data in the system. **Table 4** shows the number of municipalities per state and region and the number of those that contributed pesticides data to Sisagua from 2007 to 2010 (31). We observed that the municipalities' participation increased, although not consistently, along the years.

Pesticides Data from Sisagua

Taking into account, the Canceled number of municipalities that provided data in the system, failure to comply with the minimum Brazilian drinking water norm sampling request was also observed. Assuming that all municipalities have at least one DWTP and a minimum of two samples per year analyzed, we would expect at least 979,440 records in Sisagua during the studied period. However, only 169,080 (17%) were found. Failure to comply with the minimum pesticides analysis required by of the norm is therefore observed for all regions of Brazil (**Figure 1**).

Compliance to the Ordinance

The percentage of results above the drinking water standard ranged from 0.1 to 0.4%. Of the non-compliances (414), the highest percentage was for aldrin and dieldrin (38%), chlordane (19%), heptachlor and heptachlor epoxide (16%), endrin (7%), atrazine (5%), and other pesticides (15%). The non-compliance events could be related to the compounds with the lowest standard values, which suggest the need of a review in the analytical procedures to verify if false positives are being detected.

Sisagua Data Quality

To verify if a sample is in compliance with the drinking water quality standard, a suitable analytical method power (LOQ – Limit of Quantification) is necessary. Usually a "desirable LOQ" is 30% of the established standard (38, 39). The recorded data in Sisagua did not indicate the LOD and/or LOQ (Limit of Detection and/or Limit of Quantification) or the analytical methods used. We observed that 10–30% of the reported analyses were considered as not valid, mainly because of inconsistencies in the data, such as: (a) lack of information on the LOD and the LOQ of the analytical method used; (b) typing errors, the use of unidentified acronyms, numerically unacceptable expression of results, and no standardization on the number of decimal figures for the same analytical method measurement; (c) a high number of identical results, expressed in whole numbers, for different pesticide and for the same pesticide within the same drinking

TABLE 2 | The highest volume pesticide active ingredients in Brazil from 2009 to 2012 (above 1,000 tons/year).

Pesticide (active ingredient)	2009	2010	2011	2012
2,4-D	12,116.12	19,450.29	23,116.97	32,163.99
Acephate	5,204.89	5,233.44	8,124.83	13,080.63
Ametryn	1,624.09	2,858.40	3,441.88	4,705.76
Atrazine	10,133.80	12,811.48	18,580.93	27,139.56
Azoxystrobin	–	–	–	1,634.41
Bentazone	1,017.28	1,064.48	–	–
Carbendazim	6,712.59	7,629.82	12,216.92	7,999.80
Carbofuran	–	2,178.80	–	–
Chlorothalonil	1,964.75	2,488.77	3,001.41	2,987.65
Chlorpyrifos	2,966.39	3,191.78	4,288.36	6,218.35
Cipermetrine	–	–	3,219.22	–
Ciproconazol	–	1,707.27	1,653.27	1,090.87
Clomazone	2,712.01	5,255.42	6,171.87	4,731.45
Copper hydroxide	1,047.75	2,355.71	2,571.59	2,566.66
Copper oxychloride	3,152.99	3,364.24	3,706.01	3,854.88
Cymoxanil	1,189.55	–	–	–
Diuron	2,147.97	6,123.86	6,978.62	8,502.78
Endosulfan[a]	2,980.42	6,083.34	3,631.37	–
Etefom	–	–	1,244.48	1,554.26
Fipronil	–	–	–	1,068.60
Fluazinam	–		1,028.86	–
Flutriafol	–	–	–	1,044.19
Glyphosate	118,484.57	127,585.92	128,514.31	186,483.39
Glyphosate, isopropylamine salt	–	6,531.37	3,383.68	1,293.79
Hexazinone	–	1,155.16	1,560.75	2,009.96
Imidacloprid	1,399.15	2,441.11	5,074.00	5,476.11
Malathion	1,057.67	1,464.41	2,334.28	4,147.18
Mancozeb	3,590.35	6,917.62	7,290.18	7,134.82
Methamidophos[b]	10,774.80	17,661.77	12,838.84	–
Methomyl	–	3,350.53	4,247.09	6,376.02
Mineral oil	32,634.09	40,967.83	44,561.90	36,962.20
MSMA – monosodium methyl arsenate	1,399.88	1,672.78	1,515.11	1,778.80
Paraquat dichloride	1,977.19	3,113.24	4,275.38	5,249.54
Parathion-methyl	2,691.33	1,743.90	1,225.79	1,763.44
Picloram	–	–	1,485.90	1,625.86
Serricornim	–	–	–	3,612.38
Simazine	–	–	1,025.82	–
Sulfur	11,514.80	12,343.12	14,133.51	9,678.46
Tebuconazole	2,676.88	2,066.78	1,441.43	1,430.00
Tebuthiuron	–	2,041.97	3,195.36	3,650.86
Thiophanate methyl	3,754.32	4,472.94	4,947.79	4,800.58
Trifluralin	–	1,380.68	1,824.04	1,467.41
Vegetal oil	13,422.60	8,488.43	7,758.19	7,770.64
Total	260,348.23 (86.7%)[c]	327,196.66 (85.1%)[c]	355,609.94 (84.2%)[c]	413,055.28 (86.4%)[c]
Other active ingredients	40,001.47	57,304.62	66,632.32	64,737.16
Total of sales	300,349.70	384,501.28	422,242.26	477,792.44

[a]*Canceled by ANVISA in 2013.*

[b]*Canceled by ANVISA (Brazilian Health Surveillance Agency) in 2012.*

[c]*% related to the total of pesticides sold in the country. Pesticides with more than 5,000 ton sales in 2012 are highlighted in bold.*

water provider; (d) results expressed as less than a value that was actually, above the standard established by the norm; and (e) several results reported as "not detected" preventing us from verifying compliance of the sample with the norm because of lack of information on the LOD/LOQ of the analytical method used. **Table 5** summarizes the available data and the results considered as valid.

Drinking Water Criteria for the Pesticides with an ADI Established by ANVISA

From the 380 active ingredients approved as pesticides, 210 have ADIs established by ANVISA, and among them 13 are listed in the current drinking water norm (29). For 170 pesticides that do not have established ADIs by ANVISA, 60 of these active ingredients are of biological origin (pheromones, live bait, biological

TABLE 3 | Geo-economic characteristics of Brazilian states by region.

Region	Area (km²)	% of national territory	Population	% of population	GDP US$ thousands (2012)
North	3,869,638	45.2	17,231,027	8.50	115,691,500
Northeast	1,556,001	18.2	56,186,190	27.71	297,691,000
South	600,316	6.8	29,016,114	14.31	350,177,339
Southeast	927,286	10.9	85,115,623	41.9	1,194,091,133
Midwest	1,612,077	18.86	15,219,608	7.51	215,231,500

Data from IBGE – (Brazilian Institute of Geography and Statistics) (2014) (37); GDP: Gross Domestic Product (estimated in US dollars).

TABLE 4 | Number of Brazilian municipalities by state and region and the number that recorded data in Sisagua (2007–2010).

Region	State	Number of municipalities	Number of municipalities with results in Sisagua			
			2007	2008	2009	2010
Midwest	Distrito Federal	1	–	–	1	–
	Goiás	246	1	31	15	77
	Mato Grosso do Sul	78	8	26	24	29
	Mato Grosso	141	1	7	14	20
Subtotal		466	10	64	54	126
Northeast	Alagoas	102	–	–	–	–
	Bahia	417	–	24	15	6
	Ceará	184	–	47	4	1
	Maranhão	217	–	–	–	–
	Paraíba	223	–	–	–	–
	Pernambuco	185	–	–	–	1
	Piauí	224	–	1	–	–
	Rio Grande do Norte	167	1	–	–	1
	Sergipe	75	–	2	3	3
Subtotal		1,794	1	74	22	12
North	Acre	22	–	–	–	–
	Amazonas	62	–	1	–	–
	Amapá	16	–	–	–	–
	Pará	143	–	–	–	–
	Rondônia	52	–	–	–	–
	Roraima	15	–	–	–	–
	Tocantins	139	–	1	3	12
Subtotal		449	–	2	3	12
Southeast	Espírito Santo	78	2	6	5	4
	Minas Gerais	853	72	245	181	246
	Rio de Janeiro	92	–	7	4	9
	São Paulo	645	31	42	32	201
Subtotal		1,668	105	300	222	460
South	Paraná	399	347	352	270	252
	Rio Grande do Sul	496	61	83	42	4
	Santa Catarina	293	3	39	31	73
Subtotal		1,188	411	474	343	329
Total		5,565	527	914	644	939

insecticides, plant extracts, among others) (32). Thus, there are 110 active ingredients without an established ADI.

Because of the lack of readily available water quality criteria for several pesticides, these values were calculated for 197 pesticides that are not listed in the current Brazilian drinking water norm. For water quality standards, please see Table S1 in Supplementary Material.

After calculating the drinking water criteria (Table S1 in Supplementary Material) according to the WHO and ANVISA ADIs, we identified some discrepancies in relation to the Brazilian norm standard currently in use. We found, for example, that our calculated value for glyphosate, the most consumed pesticides in Brazil, was 252 mg/L, while the standard established in the current norm is 500 mg/L. For aldicarb, carbofuran, chlorpyrifos, 2,4-D, parathion-methyl, permethrin, and trifluralin, the calculated values are all greater than those in the norm (Table S2 in Supplementary Material). It seems that ADIs different from the ANVISA ones were used in the Brazilian norm[1] or

[1]http://portalsaude.saude.gov.br/images/pdf/2014/julho/24/Documento-Base-de-elaboracao-da-Portaria-MS-2914.pdf

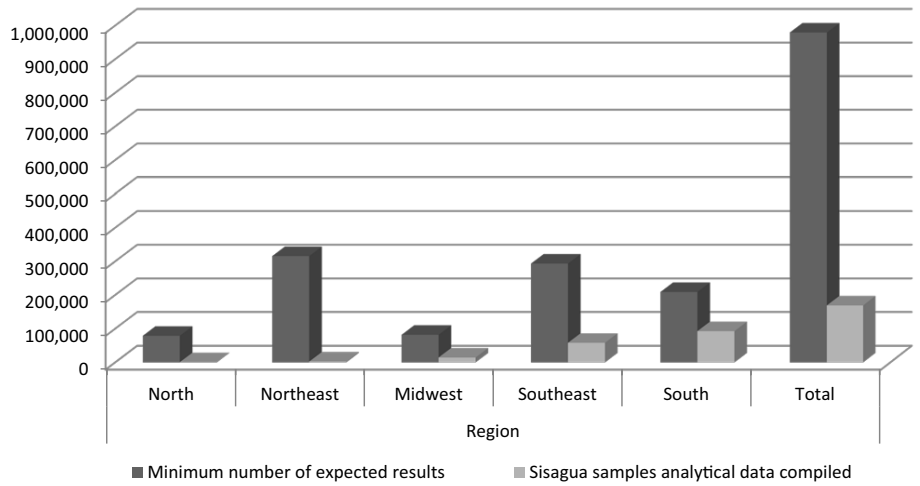

FIGURE 1 | The expected and actual number of pesticides analyses informed by each Brazilian region in Sisagua from 2007 to 2010.

TABLE 5 | Number of pesticides analysis results after Sisagua data selection for the period (2007–2010).

Number of records in Sisagua	2007	2008	2009	2010	Total
Reported	34,900	52,561	30,818	50,801	169,080
Reported as not detected	2,727	7,742	5,249	10,183	25,901
Considered as non-valid	9,757	7,324	3,186	5,954	26,221
Considered as valid	25,143	45,237	27,632	44,847	142,859

See text for clarification of categories.

different allocation factors were applied in the calculations. The values for carbendazim, mancozeb, profenophos, tebuconazole, and terbuphos were identical, indicating that the federal norm applied the same ADI from ANVISA (Table S2 in Supplementary Material). For aldicarb and DDT, DDD and DDE, the criteria suggested by WHO were used. For diuron and mancozeb, the Health Canada ADI was used (15.6 and 30 μg/kg bw, respectively). For the latter, the ADI is the same as the one published by ANVISA. For 2,4-D, alachlor, aldrin/,dieldrin, atrazine, chlordane, endosulfan, endrin, lindane, metolachlor, molinate, pendimenthalin, permethrin, simazine, and trifluralin, the calculation of how the criteria were established was not reported and it appears that the values adopted were from WHO guidelines. For glyphosate, the value used was the same as the previous version of the norm, which was based on a previous WHO report. However, the WHO no longer provides a guideline value for glyphosate using the rationale that this substance would occur in drinking water at concentrations well below those of health concern (10). In this scenario, a new Maximum Allowed Concentration value could be calculated using the ADI set by ANVISA (0.042 μg/kg bw).

DISCUSSION AND CONCLUSION

A review of the actual exposure of the population to pesticides via drinking water is only possible with a complete and consistent dataset comprising a comprehensive period of study. The Monitoring Information on Water Quality for Human Consumption System (Sisagua) in Brazil is a management tool used by Vigiagua for monitoring the quality of drinking water (40, 41). Therefore, it is of fundamental importance to verify if the analyzed samples are in compliance with the Drinking Water Norm. As described here, several inconsistencies on the monitoring data were identified, and could be attributed to insufficient standardization of the expression of the analytical results, as well as difficulties of the health sector to critically evaluate the data informed by the water suppliers. However, part of this deficiency may also be due to the lack of information about the LOD and LOQ values and the analytical methods used. In 2012, a new Vigiagua form was launched with the requirement to include LOD and LOQ information. Currently, the system is under a redesign process to be adjusted with the new requirements of the MH Ordinance No. 2914/11 (41). This renovated system will be of high importance to the Health sector in the critical evaluation and validation of monitoring data, and will support enforcement actions.

Since the first water quality norm was published in 1977, the number of regulated pesticides has increased (29, 42), reflecting the increasing concern on the use of pesticides in the country. Although the norm lists fewer than 10% of the authorized pesticide active ingredients in Brazil, the current drinking water Ordinance has been assertive on the choice of parameters, including the most widely consumed in the country. It is possible that the established minimum sampling number per year (one sample every six months) is not sufficient considering the consumption and conditions of use of certain pesticides, as well as the differences in each region of the country. The main concern, however, is not on what should or should not be regulated, but whether and how the Ordinance is being enforced. We observed an urgent need for action for the Vigiagua program to work with the health sector to make an effort to have complete pesticides information in the dataset.

Although the Ordinance MH No. 2914/11 included the main active ingredients that have been used in Brazil at the time the norm was issued, important pesticides were left out, such as clomazone, ametryn, tebuthiurom, malathion, picloram, and paraquat dichloride, among others (27, 43, 44). It is important to emphasize that approximately 30% of the 27 pesticides in the current Ordinance are no longer authorized for use in Brazil. Among those that have been canceled are aldrin/dieldrin, chlordane, DDT, endrin, and lindane. Aldicarb, methamidophos, and endosulfan were canceled recently. Most of these substances are persistent organic pollutants (POPs), known as bio magnifier contaminants, and often are monitored and detected in several countries; therefore, they should stay in the norm. However, Sisagua monitoring data suggests that there are some analytical shortcomings in their analyses.

According to Umbuzeiro, the monitoring only of regulated substances usually is not sufficient to ensure the protection of the exposed population (45). There are several other pesticides sometimes used in specific regions that must be analyzed in the drinking water. However, considering the inability to regulate all pesticides with potential occurrence in drinking water, it is necessary that each state or region identify their priority compounds and include them in regional monitoring programs. Another important limitation for the establishment of Brazilian drinking water standards is that several ANVISA monographs, does not inform the ADI values, although in this work we were able to obtain data and offer interim drinking water quality criteria for 197 substances (46). But this approach was not possible for about 110 pesticide active ingredients due to the lack of their ADIs in the ANIVSA monographs.

We also suggest that an allocation factor used for food risk analysis should be used in ANVISA monographs. It would help to determine the proper allocation factor to be used in drinking water criteria as well. This choice is usually guided by physical and chemical properties of the active ingredients. Another important consideration is that, even non-food crop substances should be considered for inclusion in the drinking water norm because they may end up in water bodies too, as verified elsewhere (21, 23, 47–54).

In our study, we observed important differences in ADI reference values between ANVISA monographs and the current Drinking Water Ordinance 2914/11 (e.g., glyphosate, and others; Table S2 in Supplementary Material). Therefore, one intention of the proposed list of drinking water criteria for 197 pesticides is to offer calculated values based on ANVISA's ADIs to the next revision of the Ordinance. The allocation factor can be discussed and altered if necessary, always in agreement with the food risk assessors, to make sure that no more than 100% of the ADI is used in the water and food reference calculations.

The effective dissemination of water quality information to consumers via Sisagua and by the water suppliers would be also an important form of social control, which could lead to a request to increase the number of monitoring data in Sisagua and to improve the data quality of the system (55). In Europe, for example, there is web-based service called Water Information System for Europe (WISE) provided by a web-portal entry to water related information, with comprehensive information of the quality of inland and marine waters. For users from EU institutions or other environmental administrations, WISE provides input to thematic assessments in the context of EU water related policies; for water professionals and scientists, WISE facilitates access to reference documents and thematic data, which can be downloaded for further analyses; and for the general public, WISE illustrates a wide span of water related information by visualizations on interactive maps, graphs and indicators (56).

There is no doubt that monitoring of pesticides in water is a complex activity which starts with the sampling plan and priority substances that will be analyzed. Chemical analyzes are expensive, require modern equipment and labor skills. As advised by WHO, it is necessary to discuss and assess whether the sampling procedures are appropriately selected, especially sampling sites and sample preservation (10). Therefore, the evaluation and validation of the data needs to occur systematically, with effective actions to improve the information quality. A constant interaction with the water supplier through guidance, reporting and monitoring is also important. In conclusion to our work, we observed that monitoring data of Sisagua during the study period does not assess the exposure of the population to pesticides via the drinking water, especially because of inconsistent and/or absence of data.

The strengths and pitfalls of the Vigiagua program presented in this study represent what was observed during the database evaluation and should not be viewed as a criticism, but as an opportunity for improvement. We believe that the provided information can enhance the awareness and highlight the importance of monitoring toxic chemicals in drinking water as well as in the source waters. The majority of elements highlighted in this study may be relevant in a similar scenario in other developing countries when considering the need to respond to the world's future drinking water situation. The expectation of this study is to positively mobilize different social actors to the issue, to describe, characterize and identify knowledge gaps and, in particular, to protect the health of people and the planet.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fpubh.2015.00246

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Risk Factors for Non-communicable Diseases in Vietnam: A Focus on Pesticides

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Agent Orange, which was used in southern Vietnam, is confirmed the main source of dioxin exposure in Vietnam. Since early 1990s, agriculture of Vietnam has attained advances under intensive cultivation. Both production and yields per crop have increased significantly at the farm level, but the quantity of pesticides used in agriculture also increased in the absence of regulations and good practices. Illegal business of pesticides with false labels, as well as marketing of expired or poor quality products in stores without license are popular in Vietnam. Misuse and improper use in agriculture in Vietnam has led to a variety of problems, such as environmental pollution (including food producing animals) and adverse health impact on animals and humans. Open dumpsites worsen the general scenario. Similar to the environmental exposure, human exposure to DDT in Vietnam was ranked among the highest worldwide, with recognized effects. Exposed communities have to face birth defects, health disorders and non-communicable diseases (NCDs), from metabolic syndrome, asthma, infertility and other reproductive disorders through to diabetes, obesity, cardiovascular and neurodegenerative diseases, and cancer. A common feature of many chronic disorders and NCDs is metabolic disruption: environmental chemical factors disturb cellular homeostasis, thus affecting the ability of the body to restore a functional internal environment. Among these, endocrine disrupting pesticides can interfere with the action of hormones including metabolic hormones, and are likely to represent the main concern for developmentally-induced NCDs. Since pesticides are often persistent and bio-accumulate in the food chain through the living environment of food-producing organisms, this paper discusses relevant aspects of risk assessment, risk communication and risk management.

Keywords: Vietnam, pesticides, one health, exposure, metabolic syndrome, risk analysis

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EPIDEMIOLOGICAL TRANSITION IN VIETNAM

The mortality rates of Vietnam is no high as other economically developing countries in Southeastern Asia, and mortality indicators suggest that Vietnam is experiencing an epidemiological transition (Huong, 2006; WHO, 2012, 2014). The WHO (World Health Organization) country profile reports the non-communicable diseases (NCDs) accounting for 75% of all deaths in Vietnam (WHO, 2012). Changes in Vietnamese population follow two main drivers (Hoa et al., 2012):

I Geographic distribution and social characteristics due to socioeconomic development and rapid urbanization.
II Demography, because of declining birth rate, longer life span and the transition in causes of death.

In 2002 the Vietnam's Prime Minister ratified with Decision 77/2002/QD-TTg for the first time that Vietnam would have a National Target Program for the prevention and control of NCDs. Besides other factors like genetics, changes in diet, physical exercise, stress, and aging, nutritional status and exposure to environmental dietary chemical factors, especially during development, affect health (Frazzoli et al., 2009). With regards to nutrition security, the Vietnamese Food based dietary guidelines (FBDGs) have been developed in Vietnam in 1995 and revised every 5 years (Hop et al., 2011).

As far as food safety is concerned, besides foods of vegetable origin, one should consider how with the development of semi-intensive rearing and the small food-processing industry, toxicants exposure transmittable by farm animal to humans may represent a new aspect of foodborne zoonoses (Frazzoli and Mantovani, 2010). Indeed, the development of an intensive rearing and processing industry introduces new and multiple risk factors, with the use of chemicals, as well as biological and pharmacological aids in animal productions, that call for risk assessment and management (Mantovani et al., 2015). In this scenario, the control programs of foods should develop around a food safety framework, based on new production chains, new exposure patterns, and disease scenarios. In fact, developmental exposure to environmental chemical factors may increase the risk of adverse pregnancy outcomes and impact on the programming of neurologic, metabolic, immune and reproductive functions, with major consequences on the risk of a variety of health disorders and NCDs in adult life, from metabolic syndrome, asthma, infertility and other reproductive disorders including puberty disturbances through to diabetes, obesity, cardiovascular and neurodegenerative diseases, and cancer (Costa et al., 2008; Van Der Mark et al., 2012; Mostafalou and Abdollahi, 2013; Tomar et al., 2013; Chevalier and Fénichel, 2014; Fucic and Mantovani, 2014; Jaacks and Staimez, 2015; Khalil et al., 2015). In this frame, according to the International Academy of Ecology and Environmental Sciences (Wenjun et al., 2011), the outbreak of cancer and other serious chronic diseases on a global scale (but especially in economically developing countries) is related to the rising environmental pollution with pesticides, accounting for 5–6%. Chronic effects of particular relevance for pesticides are:

a) Neurobehavioral development (organophosphorus compounds, but also pyrethroids and neonicotinoids) and Parkinson's disease (European Food Safety Authority, 2016);
b) Cancer: older genotoxic poisons that are still used; emerging research reveals implication in childhood leukemia (European Food Safety Authority, 2016);
c) Infertility and other reproductive problems (EDC);
d) Thyroid, and *in utero* and childhood development; many pesticides are thyrostatic EDC (European Food Safety Authority, 2014);
e) Link with metabolic syndrome, especially for EDC but also e.g., for substances that cause oxidative stress.

In this paper, we review the present situation of the risk of pesticides exposure in Vietnam (**Figure 1**) and reflect on possible actions for effective risk management.

PESTICIDES IN VIETNAM

Vietnam is known mainly as an agricultural country and one of the biggest rice exporting countries in the world (Thuong Hien, 2014). Agriculture in the north is concentrated in the lowland areas of the Red river delta and along the central coast southward. About 15% of the land in the north is arable, and 14% of it is already under intensive cultivation. The Mekong delta, one of the biggest rice-producing regions of the world, is also the dominant agricultural region of the South Vietnam.

Pesticides is a broad term that includes products such as biocides, which are intended for uses other than plant protection to control pests and disease carriers, such as insects, rats, and mice. Biocides are applied in several points of the food chain, from feeds to feed and food stores, farm animal barns, etc. Several chemicals used as pesticides, like organophosphorus or pyrethroid insecticides, may have a wide range of uses, either in agriculture to protect plants from diseases and infestations, as well as on farm animals, pets, buildings, gardens, and public places. However, the term "pesticides" is commonly understood as a synonym of plant protection drugs and include herbicides, fungicides, insecticides, acaricides, plant growth regulators, and repellents.

Pesticides have been frequently used in Vietnam in significant amounts to reduce crop losses and enhance agricultural (in particular rice) yields, with beneficial effect on food security. However, continuous misuse of pesticides in agriculture poses serious risks to both the ecosystem and human health. Until recently, the estimation of environmental burden of persistent organic pollutants (POPs), particularly organic chlorinated insecticides, in Vietnam has not been clear. The systematic inventory of toxic synthetic chemicals is lacking due to limited survey activities. In particular, studies on organochlorines and organophosphates have been performed while no information is available on recently used pesticides (particularly carbamates, pyrethroids, and triazoles). In general, a survey on agriculture (Berg, 2001) showed that ∼50% of the pesticides used in Vietnam were insecticides and 25% were herbicides. In recent years, although the frequency of insecticide applications has been decreased, Vietnamese farmers have increased herbicide (paraquat is one of the most popular herbicides) or fungicide spraying due to increased demand of rice production. Particularly, a survey showed that more than 22% of the interviewed used pesticides three times for each crop (Berg, 2001). Besides pesticides use in rice cultivation, vegetables are also sprayed with pesticides by farmers in Vietnam, as an effective tool to maintain productivity and ensure the look of products.

The misuse of most pesticides and the lack of control by the authorities do pose a threat to human health and

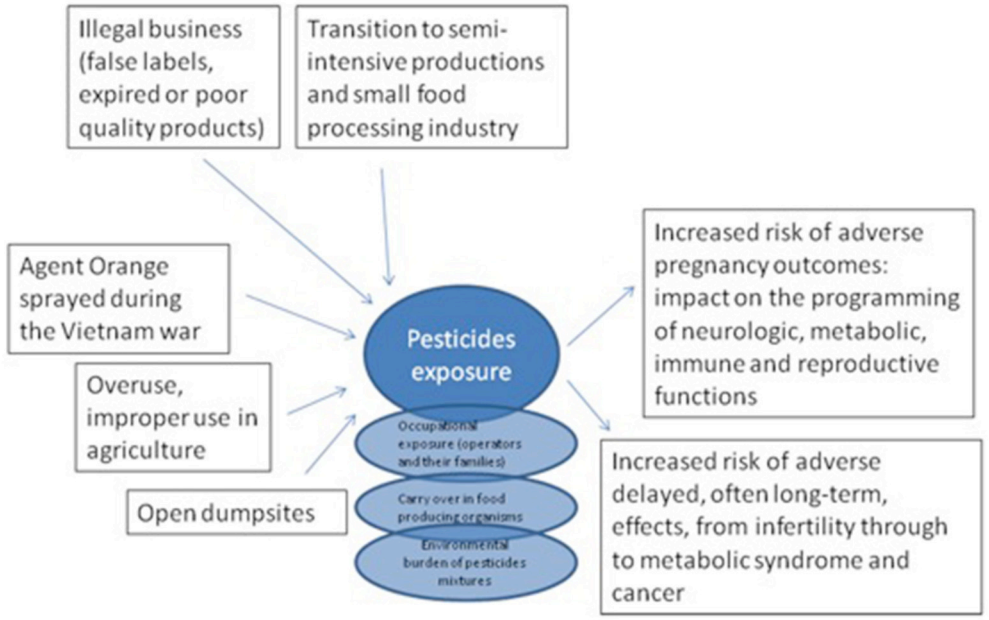

FIGURE 1 | Pesticides exposure sources and their long-term effect in Vietnam.

the environment. Overuse and improper use of pesticides in agricultural areas of Vietnam has led to a variety of problems, such as residue of pesticides in environmental matrices (soil, surrounding water and even sediments of river, and canal systems), and adverse health impact on animals and humans.

According to the Treatment Department of the Ministry of Health, there were over 3,000 cases of pesticide poisoning, nearly 3000 victims and over 100 people died in the first half of 2011. According to Sarter et al. (2012), between 2002 and 2010, 10.4% of poisoning outbreaks in Vietnam are due to chemicals and, in particular, pesticides' residues.

Besides substances intended for use in agriculture, the POPs exposure scenario is amplified by the exposure to hazardous environmental contaminants such as polychlorinated biphenyls (PCBs), whose environmental accumulation may be associated to oils imported from former Soviet Union, China, Romania and from electrical equipment like transformers (Frazzoli et al., 2010). Other possible sources of PCBs in Vietnam could be the weapons which were extensively used during the Indo-China war. The information about the usage of PCBs in Vietnam is still obscure, and it is necessary to get more data on pesticides residue in Vietnam.

An additional and remarkable pollution source of PCB, DDT in Vietnam and other Asia developing countries is open dump-site systems (Minh et al., 2008).

Within the general population, Vietnamese farmers and their families and agricultural communities are the population groups generally more directly exposed to pesticides. Communities in rural areas have frequency of direct and indirect exposure to pesticides that is higher than communities living in the city, due to the activities in the paddy fields and other exposure pathways, such as contact with contaminated clothes at home. Rural women have to face various problems such as infectious outbreak or NCDs due to lack of safe water and exposure to the hazards of water sources polluted with fertilizers, pesticides, and so forth (Hussein, 2011).

PESTICIDES MARKET

In Vietnam, around 40,000 tons of plant protection drugs are produced by 97 chemical factories per year, and these amount of pesticides is distributed to 20,000 pesticide sales agents. Besides its national production, Vietnam annually imports about 100,000 tons of plant protection drugs and their materials with total importing value of 7 million USD (Hong, 2015). Of these, 80% (of which 45–47% are herbicides) are imported from China. Pesticides are 23% of total imported products. According to the MARD (Ministry of Agriculture and Rural Development), imported plant protection chemicals in 2005 was 20,000 tons, then the imported amount of pesticides in 2014 has increased up to 50,000 tons (Ha, 2015).

Vietnam has a long history of bulky presence of highly persistent pesticides; nearly 9,000 tons of dichlorodiphenyltrichloroethane (DDT) and hexachlorobenzene (HCB) were imported per year from former Soviet Union. In addition, it was counted that more than 24,000 tons of DDT was moved into Vietnam territory from 1955 to 1990 (Minh et al., 2008). According to Minh et al. (2007), during 1986–1990, ∼800 tons have been used.

From the early 1990s, the pesticide market has changed dramatically in Vietnam. The numbers of pesticides producing companies have grown quickly. New retailers have come into

business and the market is overwhelmed by the annual increase of pesticide trade names. In particular, from 1999 to 2008 the number of active ingredients has almost doubled, while the number of trade names has increased 3.6 times. A report of the Ministry of industry and Trade shows 43 toxic chemicals in 286 commercial drug names (Ha, 2015). Highly toxic pesticides in Vietnam are abused and the control from authority agencies is lacking.

Quantity of category II pesticides increased from 18.4 to 40.6% (Van Hoi et al., 2009, 2013). The increasing number of pesticide trade names of category II is associated with an increasing use of category II pesticides by farmers. Around 100 pesticide trade names corresponding to more than 50 different active ingredients, from more than 20 chemical groups, were used in surveyed areas (Toan et al., 2013). According to a survey among chemical groups used by respondent farmers in the Mekong river delta, the most commonly used pesticides were conazole fungicides followed by pyrethroid insecticides and biopesticides. Organophosphate pesticides, profenofos, and chlorpyrifos ethyl were listed in the "frequently used" group, whereas chlorinated phenoxy herbicides and amide pesticides were attributed to the "commonly used" group (Van Hoi et al., 2013). This originated from outbreaks of insects in recent years, especially in 2006 with brown hopper. Half of the used pesticides belonged to the WHO categories of II and III (moderately and slightly hazardous, respectively). Organophosphates and, to a lesser extent, organochlorine pesticides were still applied by the farmers, with their active ingredients falling into category II (Van Hoi et al., 2013). During the past two decades, the frequency of application of organochlorine and organophosphate compounds have decreased gradually while the application of pyrethroid and carbamate insecticides have become more regular (Van Hoi et al., 2013). According to WHO's category of hazardousness, even if some pesticides were banned or restricted in economically developed countries, they were still applied in developing countries including Vietnam, thus creating serious health problems and environmental contamination (Donald, 2001). Vietnamese farmers still use some hazardous pesticides even though they are banned, probably due to the availability of stocks, cheaper price and effectiveness for pests (Dung and Dung, 2003).

Selling pesticides in Vietnam also requires regulation. Although the Government, MARD, and the People's Committee of provinces have issued regulations on the business of pesticides, particularly on substances used on vegetables, the illegal business could be found everywhere. In 2007, a total of 13,664 commercial pesticides stores were checked and there were 2,030 cases of violations, mainly due to inadequate business conditions (857 cases), false labels mark (333 cases), expired products and (302 cases) poor quality products (Ha, 2015). In addition, the number of stores without licenses for business and professional practice certificates remains high and popular, e.g., 16.5% in Hanoi and 25.0% in Thai Binh. Overall, this scenario is alarming for the pesticide marketing practices, and the business management systems require implementing strict regulations.

PESTICIDES ENVIRONMENTAL BURDEN: THE NORTH AND THE SOUTH

During 1986–1990 the use of such large quantity of pesticides caused a high presence of residues in the environment and associated severe outcomes in both humans and animals. The use of certain pesticides also entrained the pollution by dioxins. In the past, the main source of dioxin in Vietnam has been the Agent Orange (prepared from phenoxy herbicide with small amounts of the highly toxic dioxin congener) and other defoliant herbicides sprayed in southern Vietnam during the Vietnam War (Ngo et al., 2006; Đỗ and Kim, 2009). After 1960, American government had sprayed more than 45 million liters of Agent Orange in 10 years (Minh et al., 2008) and no one made sure that this pollution had broken up completely in the sprayed areas.

From the early 1990s, agriculture of Vietnam has attained advances under intensive cultivation. Both production and yields per crop have increased significantly at the farm level, but on the other hand a corresponding increase in the quantity of pesticides used in the absence of regulation is plausible.

In the Mekong delta (south of Vietnam), pesticides are used much more than in Song Hong (Red river) Delta (north of Vietnam) because the area and rice farming in the south is bigger than in the north (Dung and Dung, 2003). Rice farmers used organophosphate and organochlorine insecticides, but the trend to use pyrethroids has rapidly increased here. It is reported that 64 different active ingredients were used in rice cultivation in Can Tho and Tien Giang Provinces of Vietnam (Van Hoi et al., 2013). People used such hazardous pesticides also for fruit gardens. Even though some types of pesticides were banned according to their toxicity (organochloride and organophosphate compounds), some of them (e.g., methylparathion and endosulfan) have still been used in the Mekong delta (Van Hoi et al., 2013). In the Mekong delta, rice cultivation combined with fish farming in rice paddy fields is popular. According to Berg (2001), pesticides were used much more in the paddy field without fish while they were used less in the paddy field combined with fish culturing due to the effect of pesticides on fish farming.

Difference between the north and the south of Vietnam was also in terms of:

- Expenditures; the expenditures on pesticides of farmers in the Mekong delta (39.3 USD per ha) was remarkable higher than in the Red river delta (22.3 USD per ha);
- Frequency of application; the frequency of application was greater in the Mekong delta (on average, pesticides are applied 5.3 times per crop season) than in the Song Hong Delta (3 times per crop), although very high applications of pesticides could be seen in most rice farming regions of the whole country.

Severe pesticide contamination from various sources has been described. Comprehensive monitoring surveys of Minh et al. (2007) showed that POP contamination of air, water and sediment in Vietnam was rather higher than in developed countries such as Japan.

According to a survey of Hoai et al. (2010), all sediment samples from sewage rivers system in Hanoi (such as To lich, Kim Nguu, Nhue, Lu, Set, and Yen So lake) were positive with DDT, PCB, hexachlorocyclohexane (HCH), and HCB. According to Nhan et al. (1998), chlorinated pesticides, PCBs and DDT were detected in sediment samples but also in biota, especially in mollusks living in fresh water canal in Hanoi region with high levels (Nhan et al., 1998). This study also showed that in densely populated areas DDTs were detected at the highest concentration while in rural areas it was detected at lower concentration (Nhan et al., 2001). These findings suggest that the DDT could have been used to control mosquito and other insects in overpopulated areas. Another study in the largest paddy rice showed that water samples were positive to polar compounds e.g., diazinon and fenotrothion, while many samples of sediments and biota were positive to many kinds of non-polar chlorinated compounds like DDT, HCH, endosulfan and PCBs (Hoai et al., 2010). Vietnam is a country with higher level of OC residue than other countries in fish, mussels and birds (Minh et al., 2007; Hoai et al., 2010).

Interestingly, a study on pesticides concentration in wildlife in 1997 revealed that the concentration of DDT in the migratory birds is lower than in the resident ones in Vietnam (Minh et al., 2002). In particular, a relationships exist between places with elevated DDT and rate of exposure to DDT in resident birds from North Vietnam.

According to Minh et al. (2004), pesticide compounds, particularly HCH, were detected in human breast milk in Vietnam, with significant differences between the north (Hanoi) and the south (Ho Chi Minh City). This suggests recent high background levels of HCH, as found in a variety of environmental samples in the Hanoi compared to Ho Chi Minh City (Minh et al., 2004). It may be due to possible import from China, one of the high HCH users, and differences in climate between Hanoi and Ho Chi Minh City. In fact, the Mekong River delta in the southern Vietnam is characterized by the typical tropical climate with high temperature and heavy rainfall. Rapid volatilization of highly volatile HCH isomers may therefore be enhanced in the environment of southern Vietnam, resulting in lower residues in various environmental and human samples. Similar to the environmental exposure, human exposure to DDT in Vietnam was very high and ranked among the highest respect to developing countries and developed nations (Donald, 2001; Minh et al., 2004; Carvalho et al., 2008).

Based on the survey carried out in the Anh Son district, Nghe An province, in 2011, the spread of chemicals in soil and groundwater has been described and calculated (Pham, 2011). Pesticide residues have been dispersed into the environment, with serious health effects on generations of animals and humans.

POTENTIAL LONG-TERM HEALTH RISKS OF UNREGULATED USE OF PESTICIDES

The prevention of NCDs finds one strategic step in the prevention of environmental risk factors for homeostatic imbalances and metabolic disruption (Mostafalou and Abdollahi, 2013; Heindel et al., 2015). Lifestyle factors such as decreased physical activity and energy rich diet, together with a genetic predisposition, are known as main factors in the onset of metabolic dysregulation and metabolic syndrome and related obesity, diabetes, and cardiovascular risks (Kirkley and Sargis, 2014). On the other side, the limited success in reversing such morbidities by focusing solely on nutrition, physical exercise or drug therapies fosters the hypothesis of a significant contribution from environmental chemical factors (Heindel et al., 2015).

Pre- and post-natal metabolic programming is largely dependent on endocrine homeostasis (Le Magueresse-Battistoni et al., 2016; De Long and Holloway, 2017). Endocrine disrupting chemicals (EDCs), including many pesticides, can interfere with the action of hormones including metabolic hormones, and are likely to play a role as risk factors in the onset of metabolic syndrome (Heindel et al., 2015).

According to the Developmental Origins of Health and Disease hypothesis, *in utero* development is a sequence of "critical/most sensitive windows" in development, during which stressors can alter gene expression, possibly by interacting with the epigenome, protein levels, cell numbers, differentiation and/or arrangement in tissues to make changes in their functions (Heindel and Vom Saal, 2009). In some case, these changes may persist after the stressor is gone (the functional change to be expressed as a phenotype) as well as increase the susceptibility to "second hits" during childhood, adolescence or adulthood, e.g., amount of fat, sugar added in the diet, stress or infection; these developmental hits ultimately lead to increased risk of a variety of NCDs later in life (Heindel et al., 2015; Russ and Howard, 2016). When epigenetic mechanisms are altered, adverse phenotypes may persist until at least the third generation (transgenerational predisposition, from grand-mother to grand-children), thus highlighting the urgent implementation of sustainable food safety policies, i.e., health protection of the generations to come by ensuring the safety of foods today (Frazzoli et al., 2009). There is strong experimental evidence, as well as increasing epidemiological evidence, that prenatal exposures to EDCs (e.g., some groups of plasticizers and pesticides) during development does impact the programming of reproductive as well as neurologic, metabolic, immune functions, and on the maturation of target tissues (Dang et al., 2007). Thus, EDC may affect human development in two ways; they may increase the risk for adverse pregnancy outcomes (e.g., fetal loss, intrauterine growth restriction, preterm birth, birth defects of the genito-urinary tract) as well as exert delayed, often long-term, effects including puberty disturbances, infertility and other reproductive disorders, neurobehavioral deficits, increased predisposition to asthma and obesity, and certain adult cancers such as testicular cancers (Latini et al., 2010).

Growing scientific evidence points to infancy, childhood and puberty as potentially sensitive developmental windows for adverse long-term effects on brain, skeletal, metabolic functions, immune system (Dietert, 2014), and for cancer predisposition (Maranghi and Mantovani, 2012). It should also considered how age imparts a growing body burden of bio-accumulating EDCs (Frazzoli et al., 2009), which may worsen the risk and/or the severity of adult health disorders or diseases, such as metabolic syndrome and related cardiovascular disease, diabetes, and

cancers. Indeed, menopause and aging might represent further windows of enhanced susceptibility to acute, or chronic exposure to metabolic disruptors; however, further research is required to characterize the hazards.

While EDCs are likely to represent the main concern for developmentally-induced NCDs, attention should be given to other chemicals and toxicological targets as well. Oxidative stress, mitochondrial dysfunction, interactions with nutrients (vitamins, essential elements) leading to lipid/glucose dysmetabolism and epigenetic changes in target tissues are events related to an increased risk of metabolic syndrome and/or related diseases (e.g., type 2 diabetes) and/or related pathogenic pathways (e.g., chronic inflammation in the adipose tissue) (Mostafalou and Abdollahi, 2013; Lei et al., 2014). Many pollutants do impinge in such events and pathways, including many toxic elements (e.g., inorganic arsenic, cadmium) and pesticide groups (from glyphosate to paraquat to chlorpiriphos, etc.). The association of type 2 diabetes with exposure to inorganic arsenic (Sung et al., 2015), which is an important pollutant of water bodies and rice as well as an enhancer of oxidative stress and epigenetic alterations, represents a relevant example.

INTEGRATED PEST MANAGEMENT

Vietnam has adopted an "Integrated Pest Management" (IPM) in rice as a method for protecting plant which has helped, and is helping, increasing the agricultural productivity (Dung and Dung, 2003). It integrates practices for economic control of pests based on a large scale approach. With the aim of suppressing pest populations under the economic injury level (EIL), IPM was defined as "*the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms*" by the UN's Food and Agriculture Organization (FAO) (FAO, 2011). In March 1989 Vietnam became a participant in the FAO; from that time IPM system was started but only after 1992 Vietnam officially took a part in such network. In 1994, rice farmers were provided with a more efficient service after the setting up of the rice IPM program (Dung and Dung, 2003). Coordinated by the *International Rice Research Institute,* IPM network transferred knowledge directly to farmers, and helped farmers in increasing their ability in pest management and raise yield and production of rice (Van Mede et al., 2001).

The IPM program in Vietnam run into two direction: (a) training of trainers and (b) field schools. According to the survey reported by Dung and Dung (2003), more than 1350 IPM trainers have undergone training. After this training, the network of 7,000 "farmers' field schools" were covered in more than 50 provinces, with a total of 3,000 villages involved. The members of IPM popularized IPM to other farmers and by this way the farmers participating in the IPM program lessened the usage of pesticides by nearly 75% (Plant Protection Division, 1996; Dung and Dung, 2003). The IPM farmers got output better than non-IPM farmers according to the less use of fertilizers, seeds and also pesticides. According to Berg, the amount of pesticides used by IPM farmers was half as compared to non IPM farmers. Moreover, IPM farmer also minimized the frequency of pesticides applications from 2 to 3 times per crop (Berg, 2001).

In 2002, Berg showed that the agricultural practice and pest management strategies are not the same between different farmer categories in Mekong Delta. Based on economic comparisons, the results showed that there was a significant difference about net income between IPM rice fish farmers and non IPM ones due to the different production approach (Berg, 2002). The reduction of pesticides use gave the farmers higher incomes ($58 per hectare in the winter-spring, and $35 per hectare in other crop) and, in particular, the reduction of used pesticides made 80% of the increased incomes (Huan et al., 2005).

According to Huan et al. (1999) there are two ways to abate residues of pesticide used in agricultural areas, including media campaigns and farmer field schools. Other approaches than farmer field schools, such as IPM seminars, radio, television or games aimed at educating on plant protection were evaluated as less effective to transfer this technology to farmers. Hence, it was the basic for launching a media campaign to scale up the adoption of these IPM practices at national level.

HINTS FOR RISK ANALYSIS BASED ON ONE HEALTH

Endocrine disrupting pesticides are directly employed in food production (Mantovani and Frazzoli, 2017). As already mentioned, subjects generally more exposed to risks due to pesticides are agricultural communities: the multifaceted governance scenario starts from risk perception by the users (in their workplace and home) and good pesticide application practices. Empowerment of food producers (at both back yard and commercial production levels) and reducing risks posed by unsafe use of pesticides, are absolutely necessary for minimizing direct and indirect exposure.

Improved risk perception can be obtained for instance through easy, understandable toxicological charts to explain to agriculturists and agronomists the health risks, in both the short and long term, of different pesticides or pesticide groups. The toxicological information can support the agronomists in the formulation of treatment protocols that minimize risks and optimize benefits. Good practices mitigating the contamination of foods of animal origin (meat/milk chains) should cover feed, water, and involuntary soil ingestion as ways of livestock exposure. Integrated rice—fish farming with IPM practices provides an example of feasible implementation of sustainable food productions.

Proper information and communication should be extended to the population for correct use of pesticides in domestic environment. Besides prevention measures, such as cultural controls, biological controls, and appropriate pesticide use, the

following strategies should be applied to mitigate the residues of pesticides used in agriculture (Van Hoi et al., 2013):

i) While heavy rainfall events are forecasted, pesticides should not be applied. It has been observed how the concentration of pesticides' residues in water is raised when a heavy rain occurs immediately afterpesticides application;
ii) To reduce the transport of pesticides' residues into aquatic environments, water should not be drained soon after pesticide application;
iii) The construction of wetland systems with various combinations of vegetation, sand and gravel should be considered to reduce pesticides in surface water;
iv) To avoid the pollution of surrounding environments from industrial waste discharges, variety of control measures need implementation. All source of industrial waste need to be treated before spilling into water environment based on the lesson learned from Vietnam marine life disaster in 2016, when a steel plant caused mass fish deaths (Nhat, 2017).

The acknowledgement of the effectiveness of "food chain" approach to protect health makes the "from farm to fork" model seeking for governance strategies in Vietnam, starting from the environmental burden and the environments at the food producing animals-humans and plant-humans interfaces. Pesticides are often persistent and bio-accumulate in the food chain through the living environment (e.g., pastures, feeds, fertilizers) of food-producing organisms (Mantovani, 2016). Control strategies, from analytical know how and facilities, monitoring and surveillance tools and plans, laws and regulations (for both pesticides products and residues in foods) are pivotal for health and trade.

Rapid and on-site detection methods are crucial to assess and monitor the environmental burden of pesticide residues. The analytical data should be collected, integrated and assessed using the following categories: (1) hazard identification, (2) dose-response assessment, (3) exposure assessment and (4) risk characterization.

The availability of the data throughout the country should be improved, and biomonitoring of sentinel species (Frazzoli et al., 2014) as well as registers of human and animal health (including malformations) should be foreseen by modernized prevention plans (Frazzoli et al., 2015). Registration system as optimal source of data is currently only seldom fully functional in Vietnam. Among foods, milk has specific vulnerability to contamination with specific EDCs (e.g., polychlorinated and polybrominated chemicals) and is a useful sentinel matrix: indeed, primary milk producers could gain an increasing role in the surveillance of the territory (Mantovani, 2016).

New technologies and methodologies accounting for the potential for "cocktail" effects from multiple pesticide residues will support the "mixtures approach." Indeed, multiple chemicals with different half- lives, metabolism, persistence, tissue accumulation and target sensitivities affect many aspects of metabolism (Kirkley and Sargis, 2014). Metabolic disruption is crucial to the effectiveness of prevention plans (cumulative exposure). Environmental chemical factors disturb cellular homeostasis and cause homeostatic imbalances, thus posing as a risk factor affecting the ability of the body to restore a functional internal environment.

Finally, all substances that induce a similar effect in the same organ/tissue (e.g., reduced thyroid function) should be considered in the assessment of cumulative risk, regardless of any differences in chemical structures and/or toxicity mechanisms at biochemical/molecular level. The most accurate and protective model to describe a cumulative effect is additive: indeed, substances can contribute in a summative way to the same effect, each with their potency estimated from the available toxicological data.

CONCLUSIONS

Non-communicable diseases are increasing in Vietnam also due to environmental chemical risk factors as a result of poor or ineffective management of pesticides market and use.

The design of appropriate prevention and control measures requires one health and sustainable food safety plans protecting developmental phases of (as mentioned) two generations.

The United Nations Development Program (UNDP) project on the support of capacity, coordination and knowledge sharing for the application of one health approach, has been recently (2011–2015) approved by the government of Vietnam. Lessons learned from one health interventions in biological outbreaks in Vietnam as well as current knowledge of environmental fate of pesticides make the building of one health governance mechanism strategic and feasible. The contribution of all actors, from associations of farmers, consumers, plant protection departments, control agencies/bodies, ministries, media sectors (e.g., pesticides producers, government bodies, regulatory authorities) and disciplines (e.g., environmental health, agronomy, veterinary sciences, public health) dealing with human and animal health, foods and the environment are called to protect and improve health, with positive outputs for the general public, agro-farming productions, domestic, synanthropic and wild animal populations, based on the sharing of living resources and reciprocal interconnections. Good governance mechanisms (including sustainable food safety system) can contribute to the formulation of the national strategic plan and policy for NCDs in Vietnam. These would also safeguard the chances of a healthy adulthood for fetuses and children by preventing the toxic body burden of women in childbearing age. Indeed, to pursue sustainable development in Vietnam, new governance schemes should include toxicants that are able to interfere with developing organisms, as well as the alleviation of the environmental burden of pesticides.

AUTHOR CONTRIBUTIONS

HD wrote and revised the final version. LN wrote and revised the manuscript. HT, HN, AD and VL collected articles and references. CF gave the concept of manuscript, revised the manuscript.

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REVIEW
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Contaminants in Foods of Animal Origin in Cameroon: A One Health Vision for Risk Management "*from Farm to Fork*"

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Foods of animal origin represent an important share in the diet of Cameroonian populations. Cameroon is known to be a food basket in the west and central Africa sub-region, and an important supplier of foods on the international markets. In the meantime, food importation is continuously increasing to meet the high demand of a more westernized segment of the population. Cereals, fish, sea products, eggs, honey, shrimps, chicken, and feed ingredients are important share in the international trade of agricultural products. Few controls are made on the quality and safety of these products. Certain safety standards do exist but are still yet to be enforced. Inspections done so far by regulatory authorities are partial and do not cover important hazards that require laboratory analysis. The increasing awareness of population, the burden of new types of disease, as well as the recurrence of food scandals have recently launched a scientific and population debate on the contribution of foods items, especially those of animal origin, to the toxic exposure of food producing animals and humans. This paper critically reviews the occurrence of toxicants in most consumed foods of animal origin in Cameroon. This study included the most consumed food of animal origin, identified during the national household budget survey and contributing to 8.1% of the total diet of an individual. Data evaluated suggest an important contamination by toxic metals, mycotoxins, veterinary drugs' residues, and pesticides. The current national legal framework is briefly analyzed to explore possible intervention measures in the frame of the One Health approach.

Keywords: contaminants, residues, One Health, risk management, toxicants, adult equivalent

INTRODUCTION

Cameroon is known to be the Africa in miniature. Situated at the heart of the central Africa region, the country is classified as a lower and middle income country (1). Food insecurity, malnutrition, and food intoxication remain heavy challenges. Health indicators are seen as an alert: life expectancy at birth has timidly increased in average, from 54 years (1985), to 56 years in 2015 (2). Exclusive breastfeeding is estimated at 20%. Prevalence of severe or moderate underweight and stunted children under 5 years are at 15 and 33%, respectively (3). According to the same references, known causes

of deaths of under-5 children are: pneumonia 15%, malaria 12%, diarrhea 11%, HIV/AIDS 3%, and congenital diseases 2%. It remains important to note that up to 22% causes of infant deaths remain unidentified and unknown (2). These figures are subjects of hot discussions among health professionals and within the population. In addition, the emergence of non-communicable diseases (NCD), from cancer to hypertension, diabetes, and congenital malformations (4–6) throw some light on some risk factors. Hazards in foods of animal origin are gradually considered as public health threats in Africa: if inappropriately produced and handled, foods may be vectors of various toxic contaminants. For instance, the Yaoundé cancer diseases registry estimates the cancer incidence rate according to age group at 107 new cases per 100,000 inhabitants, with 42% male and 58% female (7). Contribution of food contaminants in the occurrence of these cases is still controversial. They however recognized that many causes of cancer have been so far underlined by international bodies, including eating of red meat and processed meats, consumption of low fiber diets, absence of breast feeding, obesity, increase of adult height, and practice of sedentary lifestyles (8).

Specific lifestyle risks factors like the decrease in physical activity, and consumption of energy-dense diets, associated with genetic predisposition, are also well-known criteria in the onset of metabolic syndrome and related comorbidities (obesity, diabetes, and cardiovascular diseases). On the other side, the limited success in reversing such morbidity cases by focusing uniquely on nutrition, exercise or drug therapies again fosters the hypothesis of a significant contribution from environmental factors like chemical pollutants. Because pre- and postnatal metabolic programming is largely dependent on endocrine homeostasis, endocrine disrupting chemicals are suggested to play a role as risk factors in the onset of metabolic syndrome (9): the burden of neoplastic and infectious diseases has been related to the rising environmental pollution (10), especially in economically developing countries.

The ongoing concerns in Cameroon underlines the need for collaborative research on perceptions, practices, and behaviors of actors at all levels of food chains, in order to identify diseases' risk factors and their interplay in diseases appearance (11). This paper reviews some findings regarding contaminants in most consumed foods of animal origin in Cameroon. This gives a picture of the actual situation, as well as orientations to better investigate sources of contamination and assess population health risk.

MOST CONSUMED FOODS OF ANIMAL ORIGINS

Foods of animal origin constitute a significant share of the Cameroonian diet. Food consumption data have been estimated at national level during the second Cameroonian Household Budget Survey (HBS/ECAM II) in 2001 (12). The data are expressed for consumers only and per adult equivalent (AE). This survey revealed that consumption of animal products covered up to 8.1% of the total diet including fish (52 g/day per AE), beef, poultry and eggs (17 g/day per AE), and milk and dairy products (10 g/day per AE). In particular, smoked fish is the most consumed food of animal origin (22.4 g cooked/day per), followed by mackerel (18.3 g cooked/day) barrel fish (10.9 g cooked/day), poultry (9.81 g cooked/day), eggs (7.15 g cooked/day), and beef with bone (11.1 g cooked/day). Fresh water fish (12.4 g/day), sea fish (10.4 g/day), evaporated sweetened full-cream milk (7.2 g/day), and shrimps (1.3 g/day) are also consumed.

Milk and dairy products are also widely consumed in all 10 regions of the countries. Milk products are imported and also locally produced. In Northern and Eastern regions where animal breeding is the dominant agricultural activity, a lot of traditional milk and milky products are produced for daily consumption.

Honey for its properties is commonly used to replace sugar in various food preparations, especially during breakfast and during formulation of traditional medicine especially for infants.

With more than 250 ethnic groups, insects eating are ancient cultural habits for certain ethnic groups found in the south, center, east, and western region.

Because these foods from animal origin represent the most important source of animal proteins in the diet of an average individual, we reviewed some contaminations already reported in these food matrices. In this review, we also use a similar foods grouping to match the ones used by authorities for national household survey.

OCCURRENCE OF CONTAMINANTS IN MOST CONSUMED FOOD GROUPS AND FEEDS

Fish and Sea Foods (Mackerel, Barrel, Smoked Fish, Shrimps, Freshwater Fish)

The fishery sector plays an important role in Cameroon. It is a well appreciated source of affordable and accessible animal protein for a huge portion of the population. Cameroon produces many fish species, both from the industrial fleet and artisanal operators. Cameroon has been exporting fish products to the European Union market. The main export from Cameroon in 2010 was shrimp, it was banned because of insufficient hygiene conditions and inappropriate official control on products destined for the export market (13). An audit mission report from the EU food and veterinary office identified some lapses in official control (deliverance of health certificate, laboratory analyses, and water quality). Journalists from the "green news infos" reported findings by Ntaryike in 2016 (14) from a study done in the Douala coastal borders, on the high contamination of fish with mercury. Moreover, traditional methods for fish drying constitute additional sources of contamination. The national newspaper "Cameroon tribune" (15) reported that some local fishermen use toxic chemicals in order to improve their catch, while those who smoke fish use plastics and worn-out car tyres. Ahmed et al. (16) studied the influence of smoke and traditional drying on the quality of three fish species coming from the Lagdo Lake. All fish samples analyzed were found to be of poor microbiological quality. *Escherichia coli*, fecal streptococci, *Staphylococcus aureus*, sulfite-reducing clostridia, and molds were detected at levels above recommended standards.

Gimou et al. (12) estimated the average intake of some toxic metals by Yaounde population. Aluminum intake from

fish was estimated to be in average at 11.4 μg/kg body weight/day, 0.155 μg/kg body weight/day was found for cadmium, and 0.963 μg/kg body weight/day for lead. The same study showed that fish was among the major contributing food to population exposure to aluminum, with boiled "dried and smoked fish and shrimps" representing up to 15% of the total exposure. Boiled fish "mackerel" accounted for 7% of cadmium exposure in the whole population. Fish was found to be the food group containing in average the highest quantity of total arsenic (1.20 mg/kg), therefore accounting for up to 71% of total arsenic exposure in the population; boiled mackerel fish alone constituted 33% of this exposure level, followed by smoked fish and shrimps 24%. Arsenic in fish is usually mainly present in the organic form, with limited toxicity effects. Moreover, in water bodies, the inorganic mercury is methylated to methylmercury (MeHg). This methylated form is the most toxic organic form which is capable to bioaccumulate in marine organisms, and biomagnifies through the entire food chain. Fish is by far the most important dietary vehicle of MeHg (17). The total amount of mercury was quantified only in the fish group, being one of the two food groups of outdoor meals that contribute to population exposure to MeHg (18). Smoked fish and shrimps represented 6% of lead exposure, and 6% of nickel exposure. Vanadium was also found to be present in fish products at a concentration of 0.167 mg/kg. Fish products were identified as the main vector of exposure of Yaounde population to vanadium with 43% for the total exposure share. The very first Cameroonian Total Diet Study (19) revealed that pesticides residues were not detected in fish products.

Poultry

Poultry meat consumption accounted for 9.81 g/day/AE. With regard to the quality and safety of this product, the issue had been raised since the year 2000 and an important concern was the ban of the importation of frozen chicken. Nzouankeu et al. (20) evaluated the prevalence of pathogenic microorganism (*E. coli*, *Campylobacter*, and *Salmonella*) in imported frozen chickens. One hundred and fifty chickens were collected from eight retail markets in Yaoundé and were examined for the presence of these microorganisms, using standard bacteriological procedures. Out of the 150 chickens, 90% were contaminated with *Campylobacter* (68.9% *C. coli* and 31.1% *C. jejuni*). All the chickens showed the presence of *E. coli*. Among the 150 isolates obtained, 11.3% were enteropathogenic *E. coli*. Furthermore, 103 *Salmonella* strains were also discovered in 90 chickens. 45.6% of *Salmonella* Enteritidis and 28.1% *Salmonella* Hadar were found to be the most common serotypes present. Multiple contamination was found in 94.6% chickens, of which 83 (i.e., 55.3%) were concurrently contaminated with *Campylobacter*, *E. coli*, and *Salmonella*. Aflatoxin B1 has also been detected in gizzard and chicken muscle (21). In the second Cameroonian Total Diet Study, Gimou et al. (22) found out that poultry meat contains cadmium at a concentration of 0.019 mg/kg.

Eggs

Average consumption of boiled eggs by the whole population is estimated to be 3.86 g/day (19). Other consumption methods like frying with other ingredients and swallowing whole eggs were not considered. Moundipa et al. (23), determined the presence of aflatoxin in eggs collected from different poultry farms, in different agro-ecological zones of the country, polled together to make one composite sample for laboratory analysis. 45.2% of the eggs were found to have detectable level of Aflatoxins (AFB_1, AFB_2, and AFM_1). In addition, they found out that the forest zone had the highest toxin contamination. The level of cadmium has been estimated at 0.019 mg/kg (22).

Beef and Pork Meat

Cooked beef with and without bone represents 11.1 and 8.61 g/day/AE, respectively. Meat inspection and control remains insufficient all over the country. The hygiene conditions of slaughter houses constitute crucial point to guarantee quality of the final product. In Cameroon, only two modern slaughter houses exist in Douala and Yaounde, others are traditional. A classification of traditional slaughterhouses and butcher shops based on microbiological characteristics of beef was conducted in the Northern part of Cameroon by Afnabi et al. (24). They collected 125 samples. Microbiological analyses showed significant contamination of carcasses in slaughterhouses, with average concentrations of 4.03 ± 0.8, 2.26 ± 0.8, 0.37 ± 0.55, and 2.2 ± 1.02 log cfu/cm^2, respectively, for mesophilic aerobic bacteria, coagulase-positive staphylococci, anaerobic sulfur-reducing bacteria, and thermo tolerant coliforms. In a previous study, Afnabi et al. (25) administered questionnaires to a number of 469 assistant butchers, from 15 traditional slaughter houses in the area of the study. The objective was to evaluate their perception of basic rules of hygiene. Their conclusion was that, whatever the types of slaughterhouses (traditional) found in the northern part of Cameroon, the hygiene practices were mainly linked to the poor know—how and management of personnel, as well as during production (treatment process of carcasses).

Fonkem et al. (26) assessed the microbiological quality of a traditional dried meat called "Kilishi." Seventy nine samples of Kilishi were collected at various selling points. The results showed that the quality of Kilishi was highly affected by the location of the production and the season. The total counts (colony-forming unit/gram) of bacterial, mold, and yeast were lower than recommended accepted limit, as well as the total viable bacterial counts of micro-organisms in meat at the point of consumption.

Pork meat consumption is rapidly growing among all classes in the society. It is eaten in certain regions of the country as traditional food, but its consumption is also urbanized. It is eaten in restaurant and out on the street as vended foods. Pork meat has become a major source of protein and fats. Yannick et al. (27) analyzed the bacteriological profile of pork meat prepared and sold along commercial streets of Nkwen and Bambili in the North-west region. Eleven (duplicate) pork samples were randomly collected and analyzed for bacteria. 100% of the pork meat samples confirmed the presence of bacterial pathogens: *S. aureus* (81.8%); followed by *Klebsiella pneumoniae* (72.7%), *Escherichia coli* (54.4%), *Salmonella* spp. (45.4%), *Proteus vulgaris* (27%), and *Shigella* spp. (9%). Djoulde et al. (28) carried out a study on street-vended meat samples purchased from street food sellers in five major towns from Soudano–Sahelian zone of northern Cameroon. The total aerobic microflora, *S. aureus*, *Bacillus cereus*,

Salmonella, *Escherichia coli* type 01 non-0157:H + *Escherichia coli* strain, yeast, and molds were checked. The mean aerobic counts and *E. coli* in roasted beef meat, fried pork meat, and roasted chicken for all street-vended samples collected from mobile and stationary food sellers were not significantly different from one to another. However, all the counts were as much as the permitted level of count (3.0 log10/g) for cooked foods. Based on the relatively low bacterial counts, the quality and safety of street-vended meat products analyzed in this study was considered to be acceptable. Meats from slaughtering houses are inspected by certified inspectors before being sent to the market. Unfortunately, there is no regulation or standard specifying the parameters that have to be checked and verified; currently, inspection consists of physical checking for any abnormalities and very few laboratory test (e.g., temperature, pH). It is therefore difficult to gain information on toxicological risk. Practices at risks are however well known, such as improper use of veterinary drugs for animal treatment, feed quality, and bad hygienic conditions from slaughtering to markets points. Meat transport is an important point for all sorts of contamination. No quality control is done once the meat is at the market.

Milk and Dairy Products (Sweetened Full-Cream Milk, Industrial Yogurt)

Milk and dairy products are consumed in different forms across the 10 regions of Cameroon: evaporated full-cream milk, powdered full-cream milk, and other local traditional forms such as *Kossam* (milk in peul language), *lebol* (traditional fermented milk), and *Kindirmou* (traditional butter). The northern regions of Cameroon (Far North, North, Adamaoua) are the main producers of milk. According to the processing type, the specific terms used are as follows: *Biraadam* for the raw, fresh, non-fermented, unskimmed milk; "*Kindirmu*" for thick milk, this is ordinary milk, heated and coagulated; "*Penndiidam*" for fermented milk made from skimmed "*Biraadam*," heated and fermented and "*Dakéré*" for a mixture of fermented milk and cassava semolina; yogurt. There are two types of yogurt, i.e., the factory made yogurt and the semi-manufactured type marketed under the label Kossam (29). The traditional production process of "*kossam*" was described by Djoulde et al. (30) as in **Figure 1**.

Milk and dairy products are widely used for infant feeding, whereas the average consumption of milk products by the population according the mentioned total diet study is up to 7 g/day per adults. Dietary exposures to trace elements were calculated from these same studies. Milk and dairy products were found to be the food groups containing most calcium on average (4,161 mg/kg), the second for potassium (4,750 mg/kg). If these products are appreciated for their high nutritive content, they are also known as potential carriers of various contaminants. Production conditions and application of improper procedures during milking and processing greatly affect the quality and safety of these products (31). Local and traditional milk factory are most vulnerable to diverse and massive contamination with public health importance. "*Lebol*" and "*Kindirmou*" are two local dairy products mostly consumed in the northern part of the country. Edima et al. (29) investigated two production sites of these products in the Adamawa Region. Questionnaires were administered to farmers; additional observations and microbiology analyses were also carried out. Good hygienic practices for the essentials were not respected and ignored by producers. Both "*Lebol*" and "*Kindirmou*" products were contaminated with yeast/mold germs.

Moundipa et al. (23) indicated the amount of aflatoxin in milk. They detected aflatoxin M1 in 15.9% of cow raw milk at levels up to 0.525 µg/L. Levels of antibiotics residues contamination in raw milk were assessed in Ngaoundere (Adamawa Region). The veterinary doctor reported the main use of three antibiotics (oxytetracyclin, penicillin, and streptomycin) in cow health; 27% of milk samples collected in various farms of the locality was found to be contaminated with one or more antibiotic residues. Antibiotics of the beta-lactams and/or tetracycline families (penicillin, oxytetracyclin) were suspected to be possible sources of contamination for 53.85% of milk samples, while antibiotics residues of macrolide and/or aminoglycoside (streptomycin) were detected in 15.38% of the samples (32).

Aflatoxins are known to be toxics and have been proved to be a cause of human liver cancers. In high doses, they are also causes of deaths from aflatoxicosis (33). Aflatoxin M1 was found in milk (21) and can be transmitted to unborn baby through breast milk (34) and potentially cow milk. Cow's milk in Africa is known to be a major food for young children. This stresses the importance of AFB1 monitoring in milk, dairy products, and in food products of animal origin as a whole. Moundipa et al. (23) detected aflatoxin metabolites in urine from children suffering from kwashiorkor and marasmic diseases (45.5%), and in the body fluids (sera) of 63.9% of primary liver cancers patients. However, the combination of these risk factors could not justify the increase in incidence and prevalence of malnutrition and cancer in Cameroon. As management measure, a cost-effective animal health-milk safety scheme should be established in the complex, multifaceted scenario of dairy production chain in Africa (35).

Honey

Honey is a sugary substance, produced from the nectar of certain flowers by the worker bees. It is a complex mixture that can present large variations in their composition and characteristics depending on their botanical and geographical situation (36). The consumption of honey is constantly growing locally because of its high nutritional value and therapeutic claims in the treatment of various diseases. Yeast and spore forming microbes are useful indicators of the sanitary and commercial quality of honey. Cameroon is listed among the recognized non EU-countries which are allowed to export honey in the European Union (37). In the heart of the "Oku mountain" in the North-west region of Cameroon, the best honey in the world is produced: "The white Honey of Oku." The Oku Mountain provides a unique ecosystem for the production of this honey. The Oku honey is one of the three African products to have received in 2013 the label "Geographical Protected Indication" by the African Organization for Intellectual Property. This calls for a more stringent residue monitoring plan for the analysis of antibiotics residues, sulfonamides, pesticides, and heavy metals to meet standards. In Cameroon, common

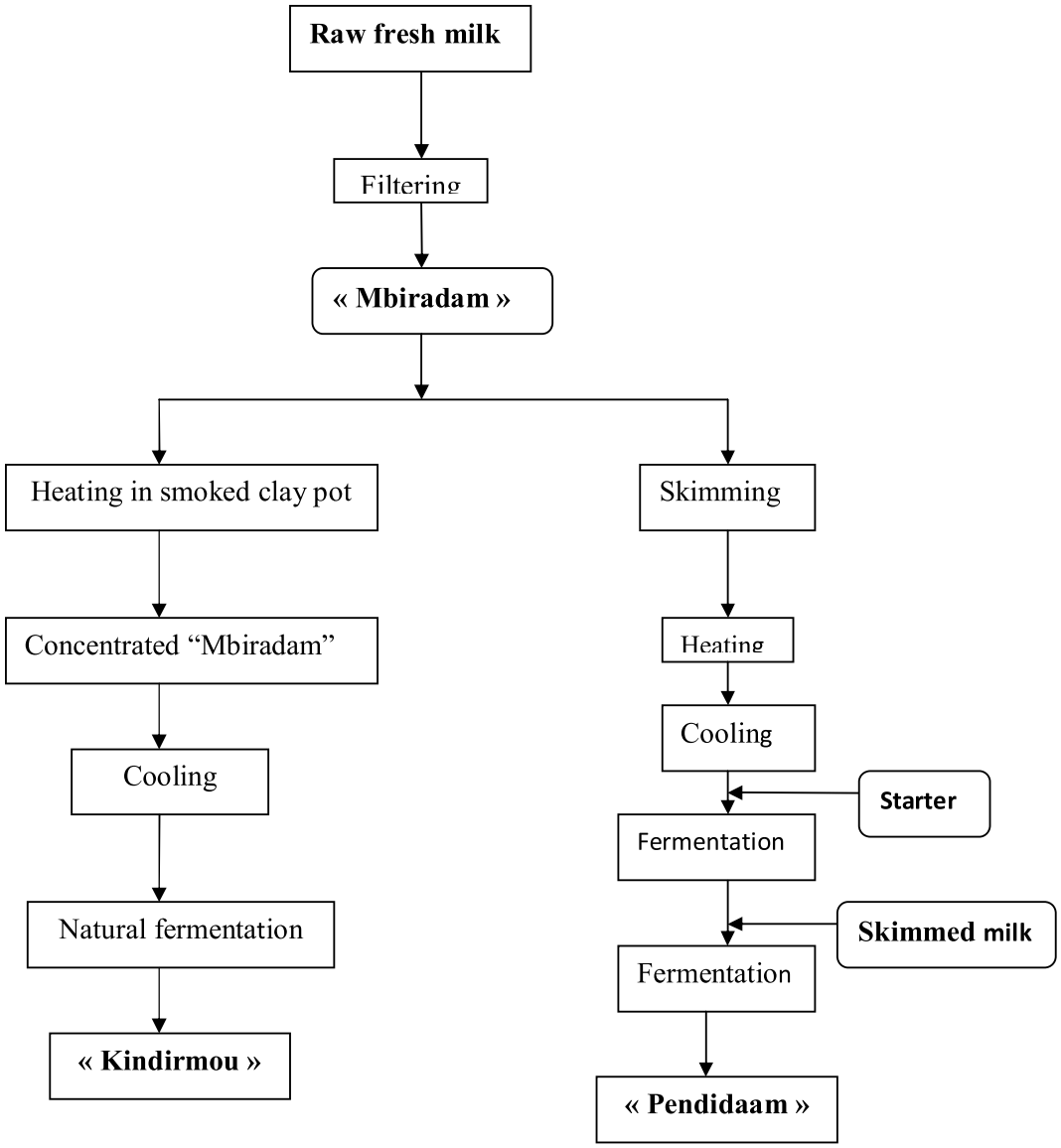

FIGURE 1 | Artisanal process production of local milk "Kossam" (Mbiradam, Kindirmou, Pendidaam) by Djoulde et al. (30).

practices for profit reasons are to dilute pure honey with a little amount of water before selling. This is believed to affect the quality of the products, and also it shelf-life.

The increasing numbers of consumer's awareness on foods risks, coupled to trade globalization, are driving the honey markets. The global production worldwide is constantly increasing since 2000 (37). Honey can be polluted *via* different sources of contamination. In Cameroon, some concerns are related to the use of pesticides, antibiotics, and microorganisms. Pesticides are known to be used worldwide to control certain bee diseases and pests in apiculture. However, in most instances, their handling and administration are uncontrolled and can be applied without approved protocols.

The use of such chemicals inside a beehive can therefore cause direct contamination of honey. Moreover, use of pesticides in agriculture is a common practice to increase productivity. Therefore, pesticides' residues detected include acaricides, organic acids, insecticides, fungicides, herbicides, and bactericides (38). In addition, non-respect of good phytosanitary practices can cause contamination to the environment, animals, and humans. Apiarists make use of antibiotics in the hive to treat bacterial diseases. As a result, traces of drugs can be found in the honey itself. Residues of oxytetracycline and chloramphenicol have been found above accepted regulatory standards set for honey (39). Same authors indicate that other antibiotics are also used: erythromycin, lincomycin, monensin, streptomycin, and enrofloxacin. Presence of antibiotics' residues is most often the result of improper management and bad beekeeping practices. Drugs' residues have already been found to be above regulatory standards (39).

In 2007, Tchoumboue et al. determined characteristics (physicochemical and microbiological) of honey collected from the West region (Sudano-Guinean zone). They bought 43 honey samples from the local markets and directly collected 7 additional samples from the bee research farm of the University of Dschang to be used as reference honey sample. More than 73.47% of honey samples bought in local markets were also contaminated with microbes (*Bacillus* sp. and fungi). The most frequent fungi in decreasing order were *Candida*, *Aspergillus*, *Geotrichum*, and *Rhizopus* spp. Important sources of contamination are handling and adulterations, as confirmed by the absence of associated contamination in the honey harvested in bee farms where processing and handling are carried out in better hygienic conditions (40).

Insects

Insect consumption is widespread in Cameroon. A lot of studies have demonstrated that edible insects contain important levels of good quality and highly digestible proteins (41, 42). Insects are also rich sources of fat, vitamins, and minerals, in particular iron and zinc (43–45). Commonly consumed insects include termites, locusts, grasshoppers, weevils, and caterpillars (46). Examples of toxic insects are given, but often traditional cooking methods are used to remove the poisoning substance. Eating insects does not depend only on taste and nutritional value but also on cultural considerations (customs, ethnic preferences, or prohibitions) (42). Culinary treatment in which these insects undergo before consumption varies from one ethnic group to another. Regarding locusts, they are eaten raw within certain ethnic groups or they are boiled, smoked, fried in oil before consumption. In all cases, insect consumption may prove to be dangerous to human health. According to European Food Safety Agency, there are possibilities for transmission of various contaminants (chemical, microbiological, etc.) on insects during their nutrition. For example, Ene indicates that the occurrence of prions in non-processed insects is related to whether or not the substrate includes protein of human origin or ruminant origin. Some authors conclude that environment and production, as well as the substrate in use, the stage and period of harvest, and the insect species can have important impact on the occurrence of chemical and biological hazards in foods and feeds derived from insects. Therefore, related environmental hazards are expected to be comparable to other animal's production systems (47).

Feedstuffs

In Cameroon, animal feed production remains artisanal. The first national survey of animal feed factories was done in 2014 by the Ministry of Fishery, Livestock and Animal Industries (MINEPIA). This survey aimed at making an appraisal diagnostic situation of the sector. Six out of 10 regions were included for their importance in the production of at least one of the ingredients of the feeds. West, Littoral, and Center Regions represented 85% of total production (48). Traditional poultry production is the most important production systems. Chickens, pigs, ducks, and pigeons are the dominating produced species. MINEPIA (49) identified four types of feed in some rural farms in Bamenda (North-west region). Most often, feed factories proposed cereals based feeds composed usually of maize, soya beans, fish flour, minerals, concentrates, vitamins, additives, and bone powders. Feed ingredients are purchased locally or imported from various countries, and mixed together in specific proportions using artisanal grinder (50). Feed factories surveyed in Cameroon have been found to work in unhygienic conditions, thus rendering animal feeds a possible vector of toxicants. In the farm-to-fork model, animal feeds are known to be at the beginning of the food safety chain. Animal feeds are frequently contaminated by bacterial foodborne pathogens like Non-Typhi serotypes of *Salmonella enterica* (51), fungi species *Aspergillus flavus, Aspergillus niger, Aspergillus oryzae, Fusarium solani, Fusarium verticillioides, Penicillium* spp., and *Rhizopus* spp. (52, 53). Maize grains that are spoiled and different types of snacks that are consumed in the Western Highlands of Cameroon have been found to be infected by several mycotoxin producing fungi. *Fusarium* and *Aspergillus* species were isolated in the frequency ranging from 20 to 100% presence in the samples analyzed, while *Staphylococcus* and *Salmonella* species were the most isolated bacteria (54). These fungi (*Fusarium* and *Aspergillus* species) in certain conditions can produce toxic metabolites and mycotoxins. For instance, the presence of ochratoxin A in foods of animal origin may occur as a result of direct fungi contamination or indirectly *via* contaminated feeds (55, 56). The cases of fumonisins, B-trichothecenes, zearalenone, fumonisins, aflatoxins and ochratoxin A (56); the case of fumonisins, deoxynivalenol, and zearalenone have also been detected in maize sampled in Cameroon (54). Farmers and traders adopted some practices that exposed cereals grains and other feeds to mycotoxins contamination. Rodrigues et al. in 2011 underlined on (i) the use of stock seed as planting materials by farmer, (ii) delayed harvesting, (iii) heaping of harvested maize cobs on the field, (iv) broadcasting method use for planting, (v) dipping and teeth cracking method with hand to determine dryness of maize, (vi) use of wooden stalls with poor ventilation for maize storage at market centers, and (vii) temporal storage in the open air, resulting in moisture re-absorption (57). Some of these feeds contaminants are of great public health concerns, although they remain ignored and unaddressed in some countries (58). Prevalence of animal feed contamination by mycotoxins is frequently high. Kana et al. (54) sampled 201 farms products (maize, crab peanuts, poultry feed) in three different agro-ecological zones in Cameroon. They detected aflatoxins in 9% of maize samples, 100% of crab peanuts, and 93.3% of poultry feeds. There were no significant differences in the level of contaminations across all three agro-ecological zones. In a similar study, Abia et al. (59) sampled 20 poultry feeds in different farms and analyzed them for 320 fungi metabolites. Deoxynivalenol and fumonisins were dominants in samples from the West Region of Cameroon, while aflatoxins were dominants in sample from Yaounde. Average aflatoxin B1 concentration (40 μg/kg) was higher than the European Union, Codex Alimentarius, China, and USA tolerable limits. Ediage et al. (60) analyzed 420 food items (maize, peanuts, and cassava) from three agro-ecological zones and tested for the presence of 25 mycotoxins: 51% of all samples were positives to at least one mycotoxin, 74% for maize, 62% for peanuts, and 24% for manioc. Aflatoxin prevalence for all samples was 22%. Moreover, zearalenone were detected in 14% of the maize samples, but all

concentrations were below the European Union tolerated maximum level in non-processed maize products (350 μg/kg). Since several deaths of children in Africa are suspected to be caused by mycotoxins compounds (61, 62), the issue deserve serious assessment and management.

THE LEGAL OPERATING FRAMEWORK

Food control and inspections are governed by laws, and regulations competent authorities elaborate rules and standards, and then ensure enforcement. These regulations have to define the principle and scope of the law, as well as the roles of each party.

In Cameroon, a specific food law does not exist. Control of food industries is regulated by the law no. 98/015 of July 1998 on hazardous food settlements that categorize operators, modalities for inspections, and responsibilities of each party engaged in the process. This law embodies all the activities of the economic sector and are not specific to agricultural and food industries. Many others regulations are then taken from other administrations and agencies to ensure its implementation. The Prime Ministerial order no. 99/918 PM of November 1999 defined the modalities for the exploitation of hazardous settlements, including agricultural and food industries. Another order (no. 2012/382 of September 2012) creating and organizing the MINEPIA conferred to this ministry the elaboration of government policies concerning issues on food of animal origin (agreement and authorization, promotion of hygiene in animal industries, law enforcement, standards elaboration). MINEPIA is therefore responsible only for certain sectors of the food chain from farm-to-fork. This situation is also true for other administrations. Consequently, Cameroon experienced non-coordinated actions, overlapping between many actors. The Prime Minister order no. 2014/2379 PM of August 20th 2014 set modalities for the coordination of inspections and official control of enterprises susceptible to generate risks for workers and population. These legal dispositions are completed by certain standards already homologated at national level to serve as a guide. More than 20 standards concerning food of animal origin exist, with a good number of them transformed as technical regulation, to enforce and ease official control activities at the national level. Surveillance and quality assessment of these products is becoming an urgent issue for population health. The creation of a toxicovigilance system as described by Pouokam et al. (63) is a crucial step in ensuring the wholesomeness of foods of animal origin in Cameroon. Besides, setting of technical standards for periodic controls will help improve the overall quality of meat food. Since 2002, a laboratory for analysis of foods of animal origin has been constructed and partially equipped within the MINEPIA in Douala, but unfortunately it is not yet functional till date.

ONE HEALTH: CONCLUSION AND PERSPECTIVES

Foods of animal origin eaten by the Cameroonian population are found to be contaminated by microbial and chemical contaminants, and most often by a mixture of both categories (see **Table 1**).

More often, the concentration of contaminants varies with agro-ecological zones, harvesting seasons, preparation, and cooking methods (63). Compared to existing international norms, some of these contaminants exceed the legal maximum or tolerable limits. Disease risks are linked to the level of exposure to these contaminants. The association between exposure to contaminants and prevalence of certain diseases among the population remains critical but often difficult to establish. Proietti et al. (65) underlined the need to consider cultural behaviors in building reliable exposure scenarios to appreciate the level of health risk. In the first report on global burden of foodborne diseases, the WHO estimates the disease adjusted life years (DALYs) of some selected food hazards. Thirty one foodborne hazards, found to cause 32 diseases, were identified and included in the study. Examples of included hazards are aflatoxin, peanut allergens, dioxin, and cyanide in cassava. In that study, disease burden due to aflatoxins was estimated using a counterfactual approach, i.e. by estimating i) the relevant diseases fraction via the exposure estimate, ii) the carcinogenicity potency factors, and iii) by applying these factors to WHO estimates for incidence and mortality using the case of hepatocellular carcinoma. Forty % of the foodborne disease burden was recorded among children less than 5 years of age worldwide; with 18 million DALYs attributed to foodborne diarrheal disease agents. The highest burden per population was observed in Africa (60). Poor hygienic working environment and lack of official control along the food chain is an aggravating factor for contamination. Pouokam (51) audited some animal feed factories in Yaounde to assess their conformity to good hygienic practices. All feed factories failed. Their working conditions revealed a lot of weaknesses and absence a food management system. The actual legal and technical framework does not allow products surveillance and inspection to be done properly (66). With the creation of the national quality and standards agency, certain norms have been approved and transformed into technical regulations for enforcement. Unfortunately, the Ministry in charge of foods inspection is not yet fully operational. Some data on foods contamination are produced in various university laboratories and research institutions, but the absence of a coordinating body led to an under-exploitation of existing data in policy formulation. A National Public Health Observatory exists at the Ministry of Public Health, with a mandate that could allow for the overseeing of these activities, but is not yet fully operational. Another body that could be suitable to take over these actions is a national One Health program.

One Health, i.e. a science-based approach linking human health and nutrition with animal and environmental health, calls for improved collective and concerted actions across the three sectors (environment, animal, and human). Operationalizing this concept in complex health challenges like food safety requires building first on the global institutional framework (67). For instance, this calls for changes in the ongoing models of training and implementation of public health policies in African countries (68). These changes pivot on improved stakeholders' perception of implication of their work on public health as well as the identification of both actors (from field production of raw materials, to management and policy) and interactions

TABLE 1 | Summary on the contamination of some foods of animal origin in Cameroon.

Food items	Contaminant risks	Reference
Fish	Mercury	(12, 14)
	Aluminum, cadmium, lead	(12)
Smoked fish	Toxics products used for cashing and smoking	(15)
Smoked fish	*Escherichia coli,* fecal streptococci*, Staphylococcus aureus*, sulfite-reducing clostridia, and molds	(16)
Smoked fish and shrimps	Lead, nickel	(12)
Outdoors meals	Methylmercury	(18)
Fish	Pesticides residues	(19)
Frozen chicken	*E. coli*, *Campylobacter*, and *Salmonella*	(20)
Gizzard and chicken muscle	Aflatoxin B1	(21)
Poultry meat	Cadmium	(22)
Eggs	Aflatoxins B1 and B2, cadmium	(22)
Beef meat carcasses in slaughterhouses	Mesophilic aerobic bacteria, coagulase-positive staphylococci, anaerobic sulfur-reducing bacteria, thermo tolerant coliforms	
"*Kilishi*" (dried meat)	Bacterial, mold, and yeast	(26)
Pork meat (street vended)	*S. aureus, Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella* spp., *Proteus vulgaris*, and *Shigella* spp.	(27)
Street-vended meat (roasted beef meat, fried pork meat, and roasted chicken)	*S. aureus*, *Bacillus cereus*, *Salmonella*, *E. coli* type 01 non-0157:H + *E. coli* strain, yeast, and molds	(28)
Lebol (traditional fermented milk)*, Kindirmou* (traditional butter)	Yeast and molds	(29)
Raw milk (cow)	Aflatoxin M1 and penicillin, oxytetracyclin, streptomycin	(23, 32)
Honey	Pesticides residues and residues of oxytetracycline and chloramphenicol	(38, 39)
Honey	*Candida*, *Aspergillus*, *Geotrichum*, and *Rhizopus* spp.	(40)
Insects	Prions	(47)
Feedstuffs	Non-Typhi serotypes of *Salmonella enterica*	(51)
	Aspergillus flavus, Aspergillus niger, Aspergillus oryzae, Fusarium solani, Fusarium verticillioides, Penicillium spp., and *Rhizopus* spp.	(52, 53) (59, 64)
	Myctoxins (aflatoxin, deoxynivalenol, fumonisins, zearalenone)	

and dynamics among them. A One Health working framework can provide an integrated food safety risks understanding and management, from the whole ecosystem of the food system by using a web of causation approach (69, 70). The first One Health workshop was organized by Cameroon in 2011 with all stakeholders to define the national One Health strategies. Today, a coordination structure under the supervision of the Prime Minister's office has been put in place. This position helps to speed up the decision process and ensure full participation of stakeholders. There are regular meeting sessions between members of the committee including laboratories, universities, training schools, and ministries. The Cameroonian One Health strategy was launched in 2013 with the development of the program for the prevention and control of zoonotic diseases. Today, the Committee has successfully delivered two documents: the National One Health Strategy (**Figure 2**) chaired by the Prime Minister with 11 ministers as members and the National Program for the Prevention and Control of Emerging and Re-emerging Zoonosis, which is part of the implementation of the One Health strategy.

FIGURE 2 | Cameroon One Health strategy components. NPPFERZ, National Program for the Prevention and Control of Emerging Re-emerging Zoonoses.

The ongoing program covers the surveillance of diseases in wildlife, prevention and control of rabies, capacity building for the detection and risk analysis of zoonoses, and integrated rapid responses systems. The program does not take into account zoonosis from feeds and foods of animal origin, nor toxic chemicals that can both be transferred from feed and food of animal origin to human beings and from mother to the child, thus constituting "toxicant-related zoonosis" as described by Frazzoli and Mantovani (71) and Frazzoli, Bocca, and Mantovani (72).

Therefore, programs for feed and food surveillance need to be established with a more integrated understanding on the transfer and circulation of harmful microbial and chemicals agents across the three components of the One Health web (environment, animals and humans).

The One Health committee should shift from an administrative tool to a more science-based and technical body, in charge of assessing, planning, and centralizing each stakeholder contributions from the three components geared at addressing food safety risk in the entire farm-to-fork chain. Finally, Frazzoli et al. about the concept of sustainable food safety (SFS), define it as the complex of actions to "build" the healthy growth and adulthood of the new generation through proper and safe nutrition *in utero* and in early years of life (67). In this paradigm, the need for actions appears urgent in developing countries, such as Cameroon, where growth problems and preventable morbidity and mortality are still high in newborns and young children (70). As illustrative scenario, in many Cameroonian communities the whole family eats the same meal from a common pot. "Special" recipes for young children, pregnant, and/or breastfeeding mothers are not envisaged within the local eating culture. Thus, the SFS framework should address widely and highly consumed ingredients of main daily traditional recipes and diets, while nutrition of fetuses and newborns depend on the maternal diet (transgenerational diet) during both pregnancy and breastfeeding.

Africa is an emerging food producing area and aspects should be examined, namely: (i) the farming scenario and its environment; (ii) primary production role in food security and safety; (iii) risk management pillars as modern infrastructures, effective farmer organizations, and institutional systems to guarantee animal health and safety of products; (iv) feasible interventions to protect food chains from hazards (e.g., sustainable use of fertilizers, feeds, veterinary drugs, pesticides) at farmers' community level, based on good practices and risk assessment; and (v) transnational consortium as a platform for technology transfer and solution exchange (63, 70, 71, 73).

Social innovation based on the empowerment of the primary food producers emerges as crucial for sustainable and safe food production (74). Sustainable policies should be supported by the mobilization of stakeholders of One Health (35, 74).

Poverty and inequality underlie high rates of communicable diseases, and also give rise to NCD risk factors including poor and unsafe diet, driving a double burden of disease, particularly among rural communities and infants, requiring a Global Health action (75).

CONCLUSION AND RECOMMENDATIONS

Foods of animal origins constitute an important share of Cameroonian diet. Smoked and fresh fish, poultry, pork and beef meats, eggs, milk and dairy products, shrimps, honey, and insects make up the top foods items consumed between 1.3 and 22.4 g/day. Contaminants analysed and found in these food items are toxic metals (aluminum, cadmium, lead, arsenic, methylmercury, vanadium), mycotoxins (aflatoxins), veterinary drugs' residues (oxytetracyclin, streptomycin, penicillin), pesticides, and microorganisms (*Salmonella* sp., *Campylobacter* sp., *E. coli*, *S. aureus*, *Bacillus* sp.). Efforts made so far by authorities to guarantee the safety of foods remain largely ineffective and inefficient, exposing populations to hazards with potential huge health impacts. This review can serve as an initial step to evaluate and document specifics risks scenarios, as well as short- and long-term preventive actions to mitigate risks. For this purpose, the One Health approach appears as an appropriate tool to carry out situational and integrated diagnostic risk assessments studies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Transdisciplinary Project Communication and Knowledge Sharing Experiences in Tanzania and Zambia through a One Health Lens

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The project "Strengthening food and nutrition security through family poultry and crop integration in Tanzania and Zambia" brings together animal, crop, and human health specialists, economists, ecologists, social scientists, and practitioners to work with participating communities. It aims to increase poultry value chain, crop farming systems efficiency, and household food and nutrition security and thus requires understanding of, and ability to work effectively within, complex systems. In this context, communication knowledge sharing and synthesis between stakeholders from diverse backgrounds and a range of experiences, perspectives, agendas, and knowledge is a challenge. To address this situation, communication is conceived as a dialog and a participatory process bringing together all stakeholders. This process results in unanticipated and unexpected results that require a high degree of flexibility and adaptability from team members. The paper analyses the approach and aim of the communication strategy developed for the project and the challenges faced.

Keywords: communication, transdisciplinarity, one health, nutrition, participatory

INTRODUCTION

Improving food and nutrition security remains a global priority, requiring an integrated approach to achieve long-term, sustainable solutions. Stunting (or low height-for-age) indicates chronic restriction of growth and is associated with reduced cognitive capacity, poor school performance, lower income-earning potential, and lower birth weight of future offspring (1). Progress toward international development targets has been particularly poor in sub-Saharan Africa, where population growth has resulted in an increase in the overall number of people affected by undernutrition in recent decades (2). In Tanzania and Zambia, the prevalence of stunting in children under 5 years of age, a major determinant of individual development, is estimated to be 42 (3) and 40% (4), respectively, despite years of agricultural research and targeted health and nutrition programs.

Problems such as food and nutrition security and childhood undernutrition are regarded as "wicked" problems, meaning that they go beyond complexity and require transdisciplinary

approaches. Brown (5) provides a clear and succinct definition of wicked problems that explains why problem definition and clear focus are such a challenge. "A wicked problem is a complex issue that defies complete definition, for which there can be no final solution, since any resolution generates further issues, and where solutions are not true or false or good or bad, but the best that can be done at the time" (5).

Poultry-keeping and crop systems in rural communities are highly complex, including social, economic, gender, cultural, and biophysical elements (6). Creating a cohesive and coherent research and development team and leveraging the very diverse expertise and viewpoints of stakeholders, including both male and female farmers, requires an approach to communication and evaluation that is adapted to dealing with the disorder and diversity of complex systems.

This paper documents the communication and knowledge management approach and explores learnings in a transdisciplinary research project "Strengthening food and nutrition security through family poultry and crop integration in Tanzania and Zambia," which is addressing a current wicked problem.

SUSTAINABLE SOLUTIONS TO THE FOOD AND NUTRITION SECURITY CHALLENGE IN A ONE HEALTH APPROACH

Efforts among human health-related multilateral agencies have historically focused on approaches such as promotion of infant and young child feeding, micronutrient fortification, and supplementation through Ministries of Health. In contrast, agriculture-related multilateral agencies have supported increased production of agricultural commodities. The lack of interconnection and the long-term sustainability of these interventions is being questioned, because many of the rural poor are not able to access fortified foods, and increased agricultural production has tended to emphasize energy-rich and nutrient-poor staples such as hybrid maize (7). There is a need for sustainable solutions that will bring the two sectors together, that is, to improve human nutrition through improved household income and dietary diversification. Local initiatives, such as enhancing traditional livestock-crop systems, can provide a sustainable solution to the ongoing demographic challenges in Africa that are driving the need for more food, improved livelihood opportunities, and reduced migration to urban centers. In addition to bringing together the two sectors, there also needs to be a deeper, more comprehensive collaboration with non-disciplinary knowledge and expertise, ranging from policy implementers and practitioners, to the beneficiaries and families themselves.

This raises the importance not only of transdisciplinary research in the sense of going beyond the disciplines to include Edmund Husserl's "Lebenswelt" or lifeworld (8) but to a system where the whole is greater than the disciplinary parts. This requires not only disciplinary work, but "strong transdisciplinarity" with an emphasis on lack of boundaries, a consistent methodology (as opposed to methods) and a contextual and ever-changing perspective on reality (9–11).

The importance of involving a wide spectrum of disciplines in addressing complex problems such as chronic undernutrition is well-recognized (12); however, there is a need to distinguish between varying levels of integration and collaboration. Rosenfield (13) proposed a taxonomy whereby a "multidisciplinary" approach involves researchers working sequentially or in parallel within their own field to address a common problem, "interdisciplinarity" involves researchers working together but still from a disciplinary-specific basis, and "transdisciplinary" research incorporates a shared conceptual framework, which draws on various theories, concepts, and approaches.

There is considerable recognition among authors and practitioners that communication plays a crucial role in cross-disciplinary work (14) and in development projects (15–19). There are inherent issues of communication and knowledge sharing associated with transdisciplinary research. There are many differences to bridge in terms of research methods, epistemologies, work styles, assumptions as well as language (20). This is further exacerbated with the inclusion of beyond-disciplinary "Lebenswelt" stakeholders, not to mention multi-lingual and international collaborations where fundamental cultural diversities need to be bridged and included in the collective whole.

THE PROJECT AND ITS DIVERSITY

Our project "Strengthening food and nutrition security through family poultry and crop integration in Tanzania and Zambia" focuses specifically on evaluating the impact of the control of Newcastle disease (ND) in village poultry and a range of crop improvements on household food security and reducing childhood undernutrition. The project is designed to analyze and test opportunities to enhance the key role that women play in improving poultry and crop integration and efficiency to strengthen household nutrition in an ecologically sustainable manner (21).

It is a 5-year project funded by the Australian Centre for International Agricultural Research (ACIAR) and implemented by the University of Sydney (Faculty of Veterinary Science, Faculty of Agriculture and the Environment and School of Public Health) in collaboration with the Tanzanian Veterinary Laboratory Agency, the Tanzanian Ministry of Agriculture, Food Security and Cooperatives, the Tanzanian Food and Nutrition Centre, the Sokoine University of Agriculture (animal and crop health and production), Muhimbili University of Health and Allied Sciences (public health), the University of Dar es Salaam (social sciences), the Tanzanian Commission for Science and Technology, the Zambian Ministry of Fisheries and Livestock, the Zambian Ministry of Health, the National Food and Nutrition Commission of Zambia, the Tropical Diseases Research Centre, the University of Zambia (animal and crop health and production, public health and social sciences), and the Kyeema Foundation and the Royal Veterinary College in London.

This approach aspires to strong transdisciplinarity as defined above, with a strong focus on communication and synergy (as opposed to consensus). Transdisciplinarity involves crossing disciplinary and non-disciplinary knowledge boundaries to create a holistic approach. The complexity of this project requires new ways of interacting and working to transition beyond

multidisciplinary and interdisciplinary approaches. This requires a greater degree of flexibility and adaptability in terms of project processes, training, dissemination, communication, including understanding the perspectives of research team members from different disciplines and practice groups, and various research approaches.

COMMUNICATION IN A COMPLEX AND TRANSDISCIPLINARY PROJECT

The aims of communication processes within the context of this research project are to support the research objectives and associated outputs and facilitate the interaction between all participants and stakeholders. Communication allows those involved to "identify the attitudes, perceptions, and needs of each, and on that basis formulate explanations, recommendations and messages about policies and activities that best address the collective interest" (22).

The communication strategy in this context is a dynamic structure, requiring a number of iterations as the project unfolds. Communication processes are intended

- To ensure that the aims, objectives, and achievements of the project are well understood by key stakeholders as well as the scientific community, appropriate public institutions, and the wider community;
- To assist in the ongoing adaptive development of the project design and directions;
- To facilitate information sharing and collective knowledge creation;
- To facilitate effective, efficient, and participatory interactions within the transdisciplinary team, the broader project participants and specifically with male and female farmers;
- To foster an open and inclusive approach to different viewpoints and contributions; to open up possibilities and opportunities for multiple and ongoing solutions, and ensure these are taken into account in decision making processes;
- To create a safe space for all participants to share ideas and discuss and resolve issues in an equal and inclusive way;
- To support mechanisms for an iterative, reflective, and evaluative approach that enables ongoing learning and adaptation as well as identifying and learning from emergent ideas and strategies;
- To communicate the successes and learnings from the project to relevant stakeholders.

A transdisciplinary team and participatory approach have inherent advantages in addressing some of the challenges and opportunities of working with complex systems; however, the process is not devoid of problems. For communication in this context to be successful, there is a need to

- Create shared meanings without losing the richness of the various communities of practice with whom we are partnering;
- Build a framework for collective knowledge creation and sharing;
- Leverage different viewpoints, ways of knowing and perspectives to create a coherent whole;
- Engage with participants in design and implementation of the project;
- Accommodate a complex systems approach;
- Map the relationships between, and influence of, stakeholders;
- Learn from and consider different approaches, methodologies and viewpoints; and
- Be open to new practices and methodologies.

It is of utmost importance to facilitate the interaction with all the project participants during the project life. Emergent strategies arise throughout the process: unanticipated elements which can provide either opportunities or threats, but require an ongoing developmental evaluation process as well as a strategic communication approach (23). This process results in realized and unrealized strategies, as the project management team responds to this.

COMMUNICATION AS DIALOG

Brazilian educator and activist Paulo Freire's seminal work "Pedagogy of the oppressed" (24) has had a strong influence on community development and communication. Freire developed a problem-solving approach where communication is conceived as a dialog and a participatory process for social transformation. The traditional model of communication describes a one-way linear process from sources to receivers. This top-down approach, initiated by the educated, expert or intellectual (the "haves") and directed toward the uneducated or ignorants (the "have nots"), aims to have inform, educate, convince or persuade individuals.

In contrast, the model of communication for social change – as adopted by this project – is conceived as a horizontal, symmetrical relationship with a series of networks and nodes involving the sharing or exchange of information between two or more participants at all levels from the field (for example, the participants of this project's randomized controlled trial) to the international level. All participants have the potential to act on the same information, none are passive receivers. The information can be created by the action of any participant or it may originate from a third source, such as a media source or religious gathering. There is an emphasis on the role of perception and interpretation of participants' understanding, as part of a dialog or an ongoing cultural conversation. The outcomes of information processing by the participants are social perceiving, interpreting, understanding, and believing.

One important aspect in transdisciplinarity is that a broad spectrum of meanings or definitions is not only possible but essential. Early discussions within this project centered on the significance of chicken meat and eggs. Members of the research team from veterinary and public health backgrounds worked to build a shared understanding of the "quality" and "bioavailability" of protein. At the same time, there was a need to understand the widespread reluctance among farming families to eat eggs, in circumstances where chickens are scarce and represent a valuable source of cash income. Consumption of a single egg is perceived as the loss of a potential chicken. In considering the contributions of poultry to improving food and nutrition security, the aim has been to ensure that a broad, inclusive understanding of

terminology is held. Rather than trying to come up with a consensus view on this, the wide variety of perspectives and knowledge is taken into account.

To advance the communication aims within the project and build strong relationships between stakeholders, regular meetings with leaders and participants at the ward and village levels are facilitated through monthly visits to the project sites by project personnel. This is intended to ensure local stakeholders remain informed and have an opportunity to contribute to project activities and share community feedback. Meetings with district leaders are also held regularly with international, Tanzanian and Zambian project personnel.

For the project to achieve significant impacts with sustainable adoption pathways, all key national (i.e., government and private sector agricultural services in addition to national agricultural research organizations) and regional stakeholders (i.e., regional economic communities and multilateral agencies) have been closely associated with the development of the project from the very early stages and throughout the project and continue to be intimately involved with its implementation. Country Coordinating Committees (CCCs), comprising stakeholders from the agriculture, livestock, and human health sectors (including representatives from government ministries, universities and other research organizations) were established in Tanzania and Zambia during the design phase and continue to meet every 3–4 months during project implementation.

The CCCs have directed the identification of project field sites (using the criteria of high stunting rates, absence of other significant nutritional interventions and contrasting agro-ecological zones) and overall national project team composition. These committees are responsible for the in-country oversight of project implementation and the communication of key findings to senior policy makers. One of the current project activities has been the development of nutrition education materials, advocating for the consumption of eggs by pregnant and breastfeeding women as well as young children. A poster, "Eat Eggs," is being pre-tested with village residents and was discussed in the last meeting of the Tanzanian CCC in October 2015. While the general concept and text ("Eat eggs for health, strength and growth") has been approved, feedback has been received on characteristics of images used, and the poster is currently being revised to reflect this input.

A Senior Advisory Board known as the Project Coordinating Committee (PCC) has also been established to assist with broad long-term oversight and cross-sectoral coordination. The PCC meets every 6 months, alternating between Tanzania and Zambia. The ability of research findings to contribute to positive impacts will be facilitated by undertaking the research within the regulatory, financial and policy environment in which the findings are to be applied.

The project team also coordinates and collaborates with relevant human nutrition projects and programs in Tanzania and Zambia (e.g., WHO, GAIN, UNICEF, USAID, WFP) to ensure that there are no overlapping areas of nutritional interventions and to share lessons and findings. The project uses regional institutions to provide inputs and guidance as appropriate and facilitate the sharing of lessons learnt and policy findings among member states. These regional institutions include the Food, Agriculture and National Resources Directorate of the Southern African Development Community (SADC), the East African Community (EAC), the Agriculture and Food Security Division of the African Union (AU), the Interafrican Bureau for Animal Resources (IBAR), and the Pan African Veterinary Vaccine Centre (PANVAC).

A key challenge that emerged early within the project was the lack of a complex systems focus on nutritional status in local communities. The impact of seasonal dietary fluctuations and the importance of wild foods eaten by local people had not been taken into account in previous research activities and interventions, nor had information from these various activities been shared among the organizations involved. Thirdly, dietary recommendations were not tailored to be locally and seasonally specific. To address this, an additional Small Research Activity (25) was conducted to develop locally relevant and feasible dietary diversity tools and messages. Outcomes of this project have included the development of participatory tools for: (1) collecting information about current dietary patterns, (2) suggesting optimal approaches to preparing and combining foods for people of different ages and physiological stages, (3) sharing information with communities.

There has been an ongoing focus on communication linkages and knowledge exchanges throughout the project. During a workshop held to bring together national and international institutions, it was evident that most nutrition, veterinary and agricultural specialists had not interacted and shared information in the past. Colleagues well-placed to contribute to work within this field were not always aware of the prevalence and complex causes of malnutrition within the country. The practice of consuming wild or non-cultivated foods in rural areas and the need for nutrition recommendations to be region- and season-specific have generally not been taken into consideration by those in the health sector. Collaboration with others with an understanding of local ecosystems and the seasonality of agricultural activities has the potential to contribute to a deeper understanding of the "wicked" problem of chronic undernutrition.

Participatory approaches form a central part of the diverse methodology employed by the project. Research tools such as participatory rural appraisal (PRA), participatory epidemiology (26, 27), and participatory impact assessment (28) are used regularly. Using a gender-sensitive approach, these tools have been adapted to explore the roles of men and women and address issues of access, control, and benefit over resources (29–31). These tools are also based on the notion that people learn and retain information better when their own knowledge and experience is valued, and when they are able to share and analyze their experiences in a safe collective environment. For example, during interdisciplinary field team visits, male and female farmers' insights have been used to guide the research approach in identifying appropriate crops and crop varieties to improve human nutrition.

A significant challenge inherent in operating in diverse and complex systems is the degree of uncertainty, unpredictability, and unknowns, which arise in such a project, in part as a byproduct of the inherent "messiness" of complex, self-organizing systems. For example, long-distance travel schedules and meeting coordination are challenging to coordinate across a diversity of stakeholders with different timezones, timelines, and operating calendars

(including but not restricted to the obvious differences between agricultural and academic calendars). In addition, there are several larger system variables and events, which impact on such a project, such as currency fluctuations, funding cycles, weather patterns, national and international policies, and events. Clear communication strategies and practices are essential to mitigate the impacts of these challenges. For example, the project employs a wide variety of communication approaches, including a website allowing collaborative modification by project team members, facebook page, and frequent conference calls and meetings. A number of the team members had worked together on previous projects, so these existing linkages and relationships were crucial to maintain cohesiveness within the team. Considerable time and resources are devoted to maintaining these linkages within the project with considerable benefit.

DISCUSSION

Achieving an enabling environment to conduct effective transdisciplinary research is a challenging and time-intensive process. Language, priorities, assumptions, experiences, methodologies and, importantly, approaches to communication can vary substantially between contributors from different disciplinary backgrounds. The slow process that a participatory approach entails is not always well understood, and its benefits not always valued. To overcome the tendency for suspicion toward unfamiliar research methodologies and results, there is a need for researchers to have confidence in the academic rigor and scientific standing of their colleagues from different fields (14). It often takes time to appreciate the value in alternative research approaches, tools, and practices. This requires an environment for team members to express their points of view and conduct open, inclusive discussions, as well as appropriate mechanisms for integration. This "safe space" depends on strong relationships, respect, trust, and frequent communication. Members of a research team need to be open to an iterative process of ongoing learning, adaptation and the creativity to deal with unplanned situations and findings.

In particular, the ability to accept and work with uncertainties and unknowns and a degree of unpredictability is the hallmark of good transdisciplinary practice. Striving for strong transdisciplinarity and research practice is an ongoing process, and the lived experience of researcher–participants operating in such transdisciplinary projects provides valuable lessons to communicate to, and share with, other transdisciplinarians.

The role of social scientists in cross-disciplinary work has been highlighted (32), as they provide illuminating insights into human behaviors and assist the different scientific disciplines to communicate more effectively. Social anthropologists' training as acute listeners and observers means they are often aware of miscommunication before other team members and can contribute to a transdisciplinary approach (33).

Successful communication and knowledge management needs to be interwoven into the project design and implementation, not a separate area of endeavor. It should be considered an integral part of the research approach with ownership by the team in general, rather than as an optional "add on" or a separate specialist input as has often been the case in the past.

CONCLUSION

Transdisciplinarity is of fundamental importance to developing sustainable solutions to complex, wicked problems. There is a need to invest time in creating an inclusive and comprehensive communication strategy to overcome challenges, allow individuals and institutions to accept unfamiliar (and at times incompatible) views and experiences, and interact effectively with colleagues from a range of disciplinary fields. It is essential that communication is an integral part of the research design and approach rather than an external input or "add on." In addition, communication and knowledge management need to be integrated into the monitoring and evaluation planning, with clear assessment and review throughout the project. This requires commitment from not just the project team, but also from the donor agencies as well.

AUTHOR CONTRIBUTIONS

BB framed the paper. EC contributed with transdiciplinary research. ML added the health perspective. WM included aspect from experiences in Tanzania and HL expanded on the constraints in Zambia. RM addressed the barriers to communication. JB contributed with issues related to nutrition. RA gave an overall international perspective.

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Safe and Sustainable Traditional Production: The Water Buffalo in Asia

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The water buffalo (*Bubalus bubalis*) is considered an efficient converter of poor quality forages into high quality milk and meat. This species is ubiquitous, with prevalence though in Asian and Mediterranean countries. From a genetic standpoint, the species is characterized by two main subspecies: river and swamp type. The former to be found predominantly in Mediterranean countries, whereas the latter is found only in the Asia continent. At present, the majority of the total world buffalo population is distributed in Asia, holding around 97% of the available stock. There, animals are mostly fed on low quality roughages and crop residues with poor nutritive value, resulting inevitably in reduced productive and reproductive performances. A distinctive differential production system is in effect between river and swamp type buffaloes, due to a significant production capacity of the two sub-species. An overview of production systems and their sustainability in the two sub-species with an emphasis on country of origin and feed availability is presented.

Keywords: buffalo, Asia, production, sustainability, health

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INTRODUCTION

The world population is growing at a fast rate and is expected to reach 8–9 billion by the end of 2030. Therefore, the advancement in a number of scientific and technological fields linked to animal production and related biotechnologies is mandatory. This will sustain not only the growing demand for food, but more importantly, will achieve a sustainable production methodology throughout the world and its different ecological areas, social and economic systems (Mehra, 2001; Pasha, 2013). In turn, a global and efficient sustainable system ensuring the availability of food for animal and human consumption, is reflected into the sustainability of each single enterprise, both for crop and animal production. In particular, in Asia the human population has grown dramatically, fuelled largely by a declining mortality linked to a better health system and improved living conditions. The growth in human population in Asia has also been accompanied by unprecedented economic growth that has allowed increases in income and purchasing power, and changes in food preferences (Cruz, 2007). These recent developments have major impacts on the demand for animal derived products, particularly meat and milk. In this framework, the buffalo, traditionally raised in a mixed crop livestock system, has played an important role over the centuries, and especially in Asia, for the lives of millions of people, by ensuring work power and food at the end of their career as work animals. The buffalo (*Bubalus bubalis*) is represented by two sub species: swamp and river, with a diploid chromosome number of 48 and 50, respectively. This animal is a major source of food (milk and meat), power, fuel, and by-products (hides, hoof, and bones), as well as manure to be used as fertilizer, especially in developing countries. Buffaloes are

distributed worldwide, although the majority (around 97%) of the total world buffalo population is present in Asia, where countries such as India, Pakistan, and China hold most of the available stock (**Table 1**). In these countries, animals are typically fed on "Low External-Input System" (FAO, 2011) based on low-quality roughages, like agricultural crop-residues/and industrial by-products containing high fibrous materials. In fact, differently from cattle and thanks to a better rumen fermentation (Wanapat et al., 2000) and nitrogen utilization (Devendra, 2007), buffaloes possess an intrinsic natural potentiality to strive and produce in hostile environments, thanks to their ability to efficiently utilize poor quality feed resources. Nevertheless, an improper feeding regimen and food availability inevitably impacts on reproductive and productive performances, by increasing mortality rates, delay in resumption of cyclicity, longer calving interval, and reduced growth rates (Qureshi et al., 2002; Tiwari et al., 2007; Sarwar et al., 2009; Pasha and Khan, 2010). In Asia, the river buffalo represents ∼75% of the total buffalo population mostly in South and West Asia, with the remaining 25% represented by the swamp type found in South East Asia and South China (Borghese and Mazzi, 2005). According to the use made of these two subspecies across Asia, a different growth trend has been reported: (i) a positive trend in the buffalo population in milk producing countries of South Asia and (ii) a dramatic decline in many South East Asia countries where buffaloes are used mainly as draft animals. The entire population of buffaloes residing in Asia, is mostly raised by small hold farmers as an essential source of milk, meat and draft power, in a region where about 60% of the human population reside with an availability of roughly 33% of the Earth's arable land. Therefore, a major challenge for the future is to sustain the need for food of a fast growing human population, against the background of an ever decreasing unit area of arable land per person (Cruz, 2007). Although, the contribution of buffaloes to the zoo-economy of Asian countries has always been measured by the value of milk, meat, hide, and leather, it should be taken into account the hidden contribution, and its reflection in monetary terms, of this species as a source of draught power for the production of major crops such as rice, corn, sugarcane, and coconuts (**Figures 1**, **2**).

TABLE 1 | Top 11 countries in Asia for buffalo population.

Country	Buffalo heads	% of the World	% of Asia
India	110,000,000	56.38	58.07
Pakistan	34,600,000	17.73	18.27
China	23,779,811	12.19	12.55
Nepal	5,178,612	2.65	2.73
Myanmar	3,426,000	1.76	1.81
Philippines	2,844,149	1.46	1.50
Viet Nam	2,511,900	1.29	1.33
Bangladesh	1,500,000	0.77	0.79
Indonesia	1,320,600	0.68	0.70
Lao PDR	1,153,000	0.59	0.61
Thailand	1,020,088	0.52	0.54

ZOO-ECONOMICAL BACKGROUND

The recent higher wealth and income in Asian countries, reflected by the increasing demands for food of animal origin, opposed to the declining land areas for agriculture and feed production, has brought an increasing trend in production and commercialization of swine and poultry. Poultry and swine production is more attractive to producers and investors due to a faster growth rate of the animals and commercialization of the end products, thanks also to the inherent efficiency advantage over ruminants in converting quality feedstuff into edible meat. This trend has inevitable implications on the importation of feed grains, which are typically not abundantly produced in the region. Therefore, the sustainability in Asian countries of food production from non-ruminant animals will be largely affected by the availability of feed grains in the international market, as well as the prevailing commodity and transport costs. In addition, the extent of possible conversion of grain to ethanol in Asian countries may have also significant impact in the years ahead. The rise in income among urban population has also brought about a corresponding shift in food preference as demonstrated by the increasing demand for beef and milk of ruminant origin. With the reduced land area for grazing and forage production, the only immediate option to meet the growing requirements is to increase the imports of milk and

FIGURE 1 | Swamp buffalo in Vietnam (photo courtesy of Giorgio A. Presicce).

FIGURE 2 | Buffaloes are popularly used for draught purpose in Bangladesh (photo courtesy of Talukder T. Nahar).

beef. As a long-term development strategy, however, efforts in fast-growing economies in Asia have also included programs to enhance growth in their respective local dairy industry, both cattle and buffalo, with massive infusion of dairy cattle stocks from Australia and New Zealand. This approach is evidently more meaningful in most of the Asian countries that still import large amounts of milk and dairy products. The reason has to be found in the skyrocketing of prices of milk in the international market due to (i) policy and regulatory measures in some exporting countries, and (ii) to unfavorable climatic factors, that have resulted in reduced production and reduced available and commercialized milk in the international market. In addition, the rising demand for dairy animals in order to restock farms in Europe and Latin America following BSE epidemic, has been coupled to the inevitable increase of prices for heads of dairy breeds. Most of the growth in the Asian region occurs in urban areas due to continued migration of people to the cities. This results in the formation of large concentration of consumers in urban communities, and consequently an increasing commercialization of various products in peri-urban areas. Commercial size livestock operations have created new challenges due to rising concerns on the resulting impact of increased waste and pollutants to the environment. Likewise, in view of the growing livestock production in the region, there is a heightened awareness on the need to achieve disease-free status to enhance trade in livestock and livestock products (Cruz, 2007).

CONTRIBUTION OF BUFFALOES TO ASIAN ZOO-ECONOMY

Riverine and swamp buffaloes co-exist in Asia, although the riverine type is typically seen in South and South-West Asia, whereas the swamp type is more characteristics and more easily found in East and South-East Asia. These two sub-species, as already anticipated, differ largely in terms of productivity and utilization within their respective geographical location. Such significantly different productive expectancy is reflected into the production system in which the two sub-species are positioned, respectively. The swamp buffalo is usually confined into a sort of mixed farming system within small-holder families, with a reduced number of buffaloes (1–5) per family, primarily used for draft purpose and meat at the end of their career as work animals. During the1990's, a dramatic decline in swamp buffalo heads was recorded, largely as a result of a massive farm mechanization and irrigation system in rice-producing areas where the swamp buffaloes are utilized primarily as source of draft power. Other countries in Southeast Asia, like Thailand, Myanmar, Vietnam, Indonesia, Cambodia have experience the same negative trend, coupled to a different positive trend in farm mechanization (**Figure 3**).

Swamp buffaloes though, still continue to be an important asset and tool for crop production and the sustenance of small families across Southeast Asia. This is so true that, some swamp type buffaloes like the carabao, found in the Philippines, have been part of an intensive effort lately in order to improve

FIGURE 3 | Replacing swamp buffaloes with mechanization (photo courtesy of Giorgio A. Presicce).

their efficiency as work animals and, more importantly as milk and meat producers. A leading center in the Philippines, the Philippine Carabao Center (PCC) has been operating since 1993 for the improvement, propagation and promotion of the "Philippine Carabao." The efforts in such direction started much earlier though, since early 1900 until 1982, when the organization seed to become lately PCC, was the first and only at that time in Southeast Asian countries to use wide scale Artificial Insemination (AI) in water buffaloes. The PCC can be considered a leading example on how to steer energy and efforts to enhance the genetic potential of the swamp buffalo. A number of studies have been conducted in that direction. They have focused on farmer's capacity to improve the buffalo production management system and skill enhancement on dairy livestock, as well as to the acceptance, application and adoption of animal-related reproductive technologies, like AI, *in vivo* and *in vitro* embryo production, and cloning. In recent years, PCC has been participating in a number of international co-operations aiming at improving the overall productivity and exploitation of the carabao, by using the most recent technological advances for the improvement of management, reproduction, and production aspects in carabao farming. As an additional example of serious commitment to the carabao and its amelioration and preservation to become a superior swamp germplasm, the use of sexed semen has been taken into consideration and set in motion. This has been coordinated in further collaboration with Guangxi University, China (Goyagoy, 2011). Finally, as for other breeds of swamp buffaloes, the use of crossbreeding with river buffaloes is currently undergoing, in order to improve milk and meat producing capacity from F1 and F2 animals.

The river buffalo counterpart on the other hand, owing to its inherent higher milk productivity is being capitalized into emerging semi-commercial and commercial-size dairy operations around the peri-urban areas. Such buffalo farming is conducted in total confinement system, where animals are fed forages and other feedstuff produced in farms and then hauled to the dairy facilities. With regard to riverine buffaloes though, and despite their intrinsic higher productive potential, different systems of milk production are in effect across the

countries where they are mostly stocked (India and Pakistan). As for swamp, riverine buffaloes mostly belong to a smallholder system, where animals are a strong asset in the family economy and production drive. At the village level, there are usually few animals per family, and in the majority of cases, produced milk is consumed within the family itself. This is due to the inherent difficulty to reach milk market centers and larger cities. Animals are fed by grazing them and completing their basic dietary requirements with supplemented straws, and when available a minimum of feed concentrates. Whenever there is a possibility to capitalize on milk production, then it is possible to assist in some sort of improved feeding and management practices involving a greater use of green fodder and to some extent feed concentrates. Finally, semi-commercial and commercial milk production setups can be seen around urban centers where milk plants are available. This more intensive system of milk production is a response to an ever increasing demand for milk in urban markets, involving also the promotion of forage production in order to sustain the increased energy requirements to the animals for higher milk production. Of course, as parallel development, animal wastes are also increased, posing in many cases possible pollution problems, due mainly to the inadequacy of waste management practices (Cruz, 2007).

Buffalo as a source of meat, has never been a primary productive goal anywhere in the world. Only at the end of their productive career, either as farming power or milk producers, buffaloes are sent to the slaughterhouse whenever they cannot be utilized anymore for their original purpose. This is true both at the village level as well as in an intensive farming system. Males, other than being essential in the buffalo farming system for reproductive purposes and for draft power, are considered more of a burden by the owner and are therefore culled even at young age, not reaching thus the full potentiality as meat producers. If an effort has to be envisaged by the owner, this will be capitalized rather than into males, into young females which later will be able to give birth and milk. An increasing trend is though observed in countries like India, where, due to socio-religious constraints and contrary to cattle, buffaloes can be used as meat producers. India is the country with the largest export of buffalo meat together with Pakistan toward the Middle East, to Australia and Hong Kong (Uriyapongson, 2013). A major effort is made in the most relevant countries in Asia in order to save unwanted males and capitalize them into meat production. In India, both private companies and governmental institutions are aiming at different strategies to rescue newborn males, which would otherwise be immediately culled, and including them into growing protocols up to 200 kg of live body weight before slaughtering. They are definitely winning strategies, as both farmers and companies have their share of return income (Dhanda, 2013). In addition, buffalo meat is the cheapest when compared to other meat producing species, and therefore a valuable source of protein for the "weaker part of society." It has to be underlined though that in Asia there is still a large room for improvement, considering that in many other countries such strategies adopted to invest in buffalo meat production are either not in place, or not commercially sustainable (Ranjhan, 2013). Other countries, like Italy where, despite the low number of heads, the degree of genetic selection on the riverine subspecies has reached possibly the highest potential, are trying to invest deeply on the exploitation of buffalo meat relying and focusing on the higher quality of the meat product when compared to the cattle counterpart. The significantly reduced fat content (≈3%), which is deposited outside the muscle tissues, and a higher prevalence of unsaturated fatty acids as opposed to saturated fatty acid (differently of what is reported in cattle), makes the buffalo meat highly advisable for people who have difficulty in maintaining the correct blood cholesterol level. Finally, buffalo meat has a better water retention, making it softer and tasty when compared to other ruminant species, due to a reduced content of hydroxyproline, which is a component of collagen. Furthermore, recent scientific evidence reports a reduced risk of heart and circulatory problems in elderly people fed either buffalo or cattle meat (Infascelli et al., 2003). In any country, be it characterized by high or low level of wealth, such health issues could be instrumental for the development of a different approach toward the use of buffalo meat, and therefore the full exploitation of this species.

IMPROVING THE BUFFALO

In South and Southeast Asia, where most of the world buffalo population reside, the possibility to improve buffalo production as a whole, is linked to the exploitation and implementation of scientific advances and related technologies in some fundamental fields. Of course, there are countries in that part of the world, that will lead such path, due to their financial strength and input, and to their cultural approach to the improvement of living conditions. This general tendency in buffalo production traits will have to contend with the local and general sustainability of the entire process. The improvement in production of any trait (milk, meat, reproductive performance, etc.), is inevitably related to a number of genes and to environmental conditions. In the last decades, a tremendous improvement has been witnessed in the genome configuration of many animal species, following the publication of the human genome sequence in 2001, which represents a milestone in the understanding of similarity and differences among individuals in any animal population. Likewise cattle, a number of buffalo breeds have been sequenced, highlighting the identification of roughly 90,000 variants and single nucleotide polymorphism (SNP) in the buffalo genome. Such identified polymorphism in the buffalo population can be used to study the genetic "backbone" of the buffalo species, and to identify specific genetic variations which may have a significant impact on any production traits (Iamartino et al., 2013). Following SNP chips, epigenetic studies and microRNAs expression profiling in buffaloes, are helping in understanding the impact of gene diversity on economically significant traits and breeding strategies (Babar et al., 2013). Another area of strong interest for the improvement of buffalo productions, is nutrition. Of course there will always be areas in the Asian continent, where feeding buffaloes will be subjected and limited

to local availability by relying mostly on crop residues. It's only within semi-intensive and intensive management systems, that feeding practices have relevance on their effect on productivity and environment. As for the latter effect, it is a well-known condition that livestock significantly contribute to greenhouse gas emission with regard to carbon dioxide, methane, and nitrous oxide (Steinfeld et al., 2006). Likewise cattle, in buffaloes too, strategies can be implemented in order to reduce methane emission and rumen methanogen bacteria by altering level of intake, frequency of feeding, type of feedstuff, ratio of forage to concentrate, type of carbohydrates, etc. (Boadi et al., 2004; Hook et al., 2010). Even natural compounds like tannins and saponins may help in tackling such task, by including them as feed additives thanks to their intrinsic anti-methanogenic activity (Beauchemin et al., 2008). As a result, converging from the above mentioned actions in buffalo management, "...manipulation of dietary fermentation and rumen enhancement would result in improved rumen fermentation end-products and reduced methane emission, thus enhancing productivity" (Wanapat and Kang, 2013). Lastly, a third approach to ameliorate buffalo productivity within its specific and diversified geographical domain and environmental constraints, is characterized by a global effort in enhancing reproductive efficiency through the application of newly developed reproductive technologies. It is well-known that buffaloes tend to be affected by a series of reproductive inefficiencies, as part of their physiologic condition, ranging from being tendentially seasonal as they move away from the equator, to delayed puberty and to long anestrus period, but on the other side being significantly longer-lived when compared to the cattle counterpart. Historically, in the early 80's buffaloes were addresses for the very first time to check on their responsiveness following hormonal administration for follicle development and *in vivo* embryo development after AI (Drost et al., 1983). Unfortunately, to date, not much progress has been made using the same multiple ovulation and embryo transfer (MOET) approach (Presicce, 2007). Lately, buffaloes have increasingly been the subject of interest on which to apply the latest available technologies like *in vitro* embryo production, with a variable degree of success, but definitely making the buffalo a species of interest for that particular type of genetic exploitation (Gasparrini, 2002). As previously anticipated, the use of sexed semen has been tested in buffaloes, its feasibility has been proved both via AI and *in vivo* embryo production, as well as via *in vitro* embryo production (Liang et al., 2008; Lu et al., 2010), and the trend is now to have buffalo sexed semen commercially available worldwide. In Asian countries, where the swamp subspecies is predominant, there is a tendency to use semen from river buffaloes to crossbreed with swamp females, in order to have F1, F2, and back-crossing. Such crossbreeding offspring is characterized by a larger body size and higher milk and meat production ability when compared to the original swamp lines, together with a fertility not at all compromised. This is an additional strategy aiming at optimizing productive features typical of the river sub-species, while insisting in an environment with swamp predominance.

BUFFALO DISEASES

Buffaloes, like other domestic animals are exposed to parasitic infestations, microbial infections, toxic agents, and even dietary deficiencies (Hartung, 1994). Buffaloes are more or less susceptible to the same most common diseases and parasitic infestations observed in domestic cattle (Thomas, 2008). Clinical symptoms of most common buffalo diseases are very similar to cattle, and in general, buffaloes are more resistant to most of the diseases than domestic cattle. This feature favors the buffalo to survive in hot humid regions, which are usually conducive to diseases to a higher frequency. Therefore, in the same ecosystem, the effect of disease on buffalo and it's productivity is often less deleterious than on cattle. Variations in temperature, weather, rainfall, and sunshine in combination with seasonal shortages of feed and water could also influence the status of health and disease. Generally, animals with poor sanitary nutrition and health conditions are prone to be affected with diseases. Likewise other domestic farm animal species, newborn and young buffalo calves are less resistant to diseases than adult buffaloes. Therefore, calf mortality is the major cause of losses in the buffalo species. Calf pneumonia or diarrhea resulting from management, environmental, nutritional, and physiological variations, and various infectious and parasitic agents (Snodgrass et al., 1986), are the most important causes of buffalo calf mortality (Subasinghe, 1986; El-Ghari et al., 1994; Galiero et al., 1994; Islam et al., 2013). Second, most frequently observed calf disease is related to naval ill or joint affections. Naval pathologies occur frequently in calves born in unhygienic environment, with no disinfection treatment of the naval and at the same time receiving little colostrum (Radostits et al., 1994). Haemorrhagic septicaemia (HS) which is commonly known as pasteurellosis is the major threat to adult water buffaloes (Islam et al., 2013). This is caused by the bacterium *Pasteurella multocida*. Buffaloes are more susceptible to HS and die in larger number than cattle. Buffaloes though, are comparatively more resistant to many diseases including contagious bovine pleuro-pneumonia, foot root, foot and mouth disease (FMD), anthrax, black quarter, and mastitis, than cattle (Thomas, 2008). Incidence of mastitis is high in countries where high yielding buffaloes are kept for milk production. On the other end, buffaloes are comparatively less resistant to tuberculosis than cattle (Lall et al., 1969). Buffaloes are affected with tuberculosis when they are kept under unsanitary conditions. Sporadic outbreak of cowpox, rabies, tetanus, actino bacillosis, and ringworm in buffalo is also reported in many Asian countries, and regional variations are observed in the incidence of these buffalo diseases. For example, FMD incidence is rare in buffaloes in Egypt but is high in Myanmar and some islands of Indonesia. In India and Sri Lanka, the incidence of FMD is also low compared to cattle. In a recent study in Bangladesh, it was found that about 64.2% buffaloes rising at farmer's condition are infected with gastro-intestinal parasites. Parasitic load is also higher in young animals compared to adult buffaloes. It is comforting that tests, diagnostic procedures and treatment measures developed for domestic cattle can also be used efficiently in buffaloes (Thomas, 2008). A number of

vaccines are available for the most common buffalo diseases. In most cases, buffalo diseases can effectively be controlled through proper vaccination and deworming at regular intervals.

CONCLUSION

Despite the significantly lower number of buffalo heads around the world, in comparison to cattle, they are going to still significantly impact more on Asian countries and their zoo-economies, against the continuous mechanization and introduction of dairy cattle heads. We are witnessing improved living conditions and health standards, leading to increased life expectancy, together with a gradual increase in Asian and world human population. Such inevitable facts urge us to ensure that efforts are properly addressed in various fields of scientific enquiry, in order to enhance buffalo production in a sustainable and holistic manner.

AUTHOR CONTRIBUTIONS

GD and GP designed the concept of this manuscript and subsequently drafted and finalized it for submission. TN and PD contributed in this manuscript through providing research information and revising it critically.

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