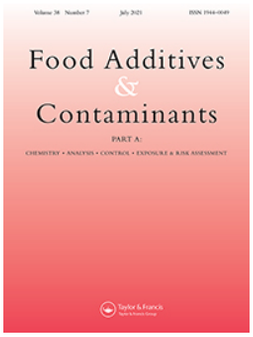

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Biosensing approaches to detect potential milk contaminants: a comprehensive review

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ABSTRACT

Accidentally present contaminants or intentionally added adulterants in milk lead potentially to delivering not only unhealthy but seriously hazardous products. Thorough, fast and sensitive analytical tools are essential for monitoring of milk quality, and for screening of any objectionable contaminants. Biosensors represent an innovative, time-efficient and on-site solution to assess milk quality in addition to their specificity towards target analytes alongside high accuracy within such complex matrices. Most biosensors use antibodies, aptamers or enzymes as the bio-receptor and rely on optical, electrochemical or thermometric transduction to generate a signal. The simplest biosensors appear to be those based on a colorimetric assay, being simple and having a signal that can be detected visually. Electrochemical sensors are more specific and sensitive, though with more complicated designs, whereas thermometric sensors have not been thoroughly explored concerning biosensing contaminants in milk. This review discusses recent advances in the field of biosensors and analyzes the various methods of bio-recognition and transduction with regard to their advantages, limitations, and application to milk products. Additionally, challenges facing further development of these strategies to fulfil the increasing demand for fast and on-line milk quality control are also presented.

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Biosensor; milk contaminant; antibiotics; hormones; pesticides; mycotoxins; melamine and urea

Introduction

Food products are constantly assessed for their quality, often using sophisticated methods that require experienced personnel, laborious steps, and expensive equipment. Milk is one of the food products used daily worldwide together with several other dairy products. Milk and milk products are an essential staple in all households owing to their richness in nutrients, i.e. protein, fat, sugars, vitamins and minerals (Stokes et al. 2000). Therefore, milk and dairy products are not only commonly included in nutritive foods but also recommended as important elements in a healthy and balanced diet (Pereira 2014). Consequently, it is crucial to ensure that milk is safe and free of contaminants that surpass the imposed regulation limits especially for being consumed daily. Conventional methods used to detect certain milk contaminants, i.e. antibiotics include microbial inhibition assays and enzyme-linked immunosorbent assay (ELISA) which is the most useful and specific one, albeit considered a cost prohibitive approach, requiring a laboratory with special equipment, experienced personnel, and ample time which might not meet the need for quick analysis of milk quality in factories (Li et al. 2015). Other analytical methods rely on chromatographic separation such as high-performance liquid chromatography (HPLC) coupled to different detectors as UV-vis spectrophotometry, tandem mass spectrometry (MS/MS), with the latter being more universal, sensitive allowing for the simultaneous detection of contaminants such as antibiotics from different classes. Chromatographic methods are extremely useful, but not suited for on-site analysis as they require analyte extraction and purification aside from the complicated and expensive device setup and the consumption of organic solvents that could be toxic to the environment if not correctly disposed of. (Zacco et al. 2007; Jayalakshmi et al. 2017; Nameghi et al. 2019).

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Potential contaminants and residues found in milk are mostly antibiotics, hormones, pesticides, and mycotoxins, which will be the focus of this review. With the recognition that most conventional quality control methods are rather tedious and inefficient, biosensors present a rapid, simple and affordable solution to assess contaminant levels in food (Meshram et al. 2018). This review capitalises on the different biosensors available to detect, identify and quantify antibiotics, hormones, pesticides, mycotoxins, beta-lactamases and melamine in milk, highlighting the advantages and disadvantages of each transduction strategy, while critically discussing different bio-recognition elements and transduction methods of each biosensor. Method validation is also discussed when available as it allows for comparison among the different methods especially with regard to each biosensor sensitivity level, specificity and accuracy (M-Heel et al. 2007).

What are biosensors?

Biosensors emerged in the 1960s after work by the pioneers Clark and Lyons and, ever since, they have been applied in many fields including food and medicine (Mehrotra 2016). A biosensor is a device that employs a biochemical reaction to detect the presence of chemicals. According to the International Union of Pure and Applied Chemistry definition, "a biosensor is a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (receptor) which is in direct spatial contact with a transducer element" (Thévenot et al. 2001). It is composed of a bio-element which is a receptor that recognises the target analyte and a transducer that converts the recognition into a signal, Figure 1. The receptor could be an enzyme, antibody, aptamer, cell, tissue, protein, DNA or other biomolecule. Aptamers are synthetic single-stranded nucleic acid ligands, which upon binding to their target, can form tertiary structures that are very selective and of high affinity. Aptamers are chemically stable, cheap and user friendly compared to other conventional analytical methods and traditional anti-body biosensors (Nameghi et al. 2019). The bio-receptor should cause a physical or chemical change when close to the transducer. As such, the bio-receptor is often immobilised on the transducer *via* adsorption, covalent binding, cross-linking or entrapment (Thapar et al. 2018). Transduction mechanism could be electrochemical, optical, thermometric, or piezoelectric.

In electrochemical biosensors, a chemical signal is generated due to interaction of immobilised bio-recognition element on electrode surface with the analyte of interest then converted into an electrical signal by the transducer. Electrochemical biosensors usually require three electrodes, a reference electrode, a counter electrode, and a working electrode. The intensity of the signal produced reflects the concentration of analyte, with its main advantage being highly sensitive though less selective. Depending on the transduction method, electrochemical biosensors can

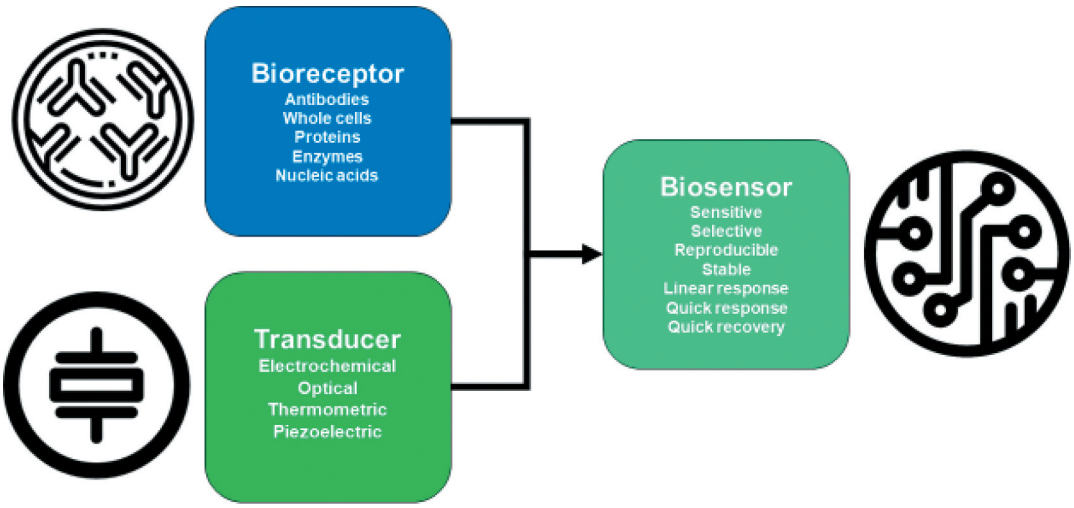

Figure 1. Diagrammatic sketch of biosensor composition and characteristics of each part.

either be voltammetric, amperometric, or potentiometric. In voltammetry, the current is measured as a function of the applied reducing potentials. Voltammetric biosensors give both qualitative and quantitative information with excellent precisions (<1%), sensitivity and wide linear dynamic range. Amperometric biosensors measures the current produced when a certain potential is applied between the working and the reference electrodes. Potentiometric biosensors are based on the potential differences between working and reference electrodes, with the analyte detected level being proportional to biosensor's response (Meshram et al. 2018; Pereira et al. 2019).

In contrast, optical transducers measure absorbance, polarisation, fluorescence, phosphorescence, chemiluminescence, bioluminescence or surface plasmon resonance produced as a result of the analyte binding to the receptor (Meshram et al. 2018). Generally, optical biosensors are divided into two main categories, label-free, and label-based. The detected signal in label-free approach is produced directly from the interaction of the analyte with the transducer. In contrast, label-based biosensors use a label, with the optical signal produced by a colorimetric, fluorescent or luminescent method (Damborsky et al. 2016).

Less commonly used, thermometric transduction measures the heat produced or consumed; they measure the change in temperature, absorption or evolution of heat that takes place due to biological reactions (Ramanathan and Danielsson 2001).

Lastly, piezoelectric transduction is used for mass-sensitive processes (Thapar et al. 2018). Piezoelectric surface like quartz crystal is functionalised with bio-recognition molecules, and an alternating potential can produce a standing wave in the crystal at a resonance frequency. The interaction between target analyte and the biomarker coated on the crystal will generate a change in the resonance frequency. This mechanical vibration change can be translated into a readable signal corresponding to the target analyte (Pohanka 2018).

A successful biosensor should be low cost, be easy to operate and require simple preparation methods (Abedalwafa et al. 2019). It should be highly selective for the target analyte and be stable to be functional over a long time. It should also be sensitive enough to measure the analytes at trace levels and to display acceptable linearity covering a wide dynamic range. A good biosensor should be accurate and reproducible, meaning it provides the same results for the same samples over time. It should also exhibit a quick response and recovery times for field applications. Rapid real-time monitoring and short recovery times are both prerequisites for functionality and reusability of stable biosensors (Meshram et al. 2018).

Potential residues and contaminants in milk

Antibiotics

Antibiotics are chemical substances derived from natural or synthetic sources that exhibit antimicrobial effects against bacteria and certain protozoa. They can be classified based on their chemical structure into β-lactams, tetracyclines, fluoroquinolones, aminoglycosides, macrolides, sulphonamides and phenicols (Abedalwafa et al. 2019). Figure 2 displays the various antibiotic classes and the most common within each class, with their corresponding structures and their minimum residue limit (MRL) in milk (European Commission 1990).

β-Lactam antibiotics, like penicillin, are administered to cattle to treat bacterial infections by inhibiting bacterial cell wall formation but their overuse led to the appearance of antibiotic-resistant bacteria. Tetracyclines are an antibiotic family that works by inhibiting bacterial protein synthesis. Their persistence in animal products and consumption by humans may lead to liver damage, allergic reactions, and nephrotoxicity. Fluoroquinolones treat urinary tract, skin, and respiratory infections by inhibiting bacterial DNA formation. Aminoglycosides inhibit the synthesis of bacterial protein; however, their uncontrolled use predictably has led to bacterial resistance. Macrolide antibiotics prevent the protein synthesis of the bacterial ribosomes. Macrolide residues, like erythromycin, in humans, cause liver damage, intestinal flora disorder, and abnormal heart activity. Sulphonamides, i.e. sulfamethoxazole, are synthetic antibiotics that starve the bacteria of folate. Lastly, phenicols prevent bacteria from producing vital proteins. However, the overconsumption of

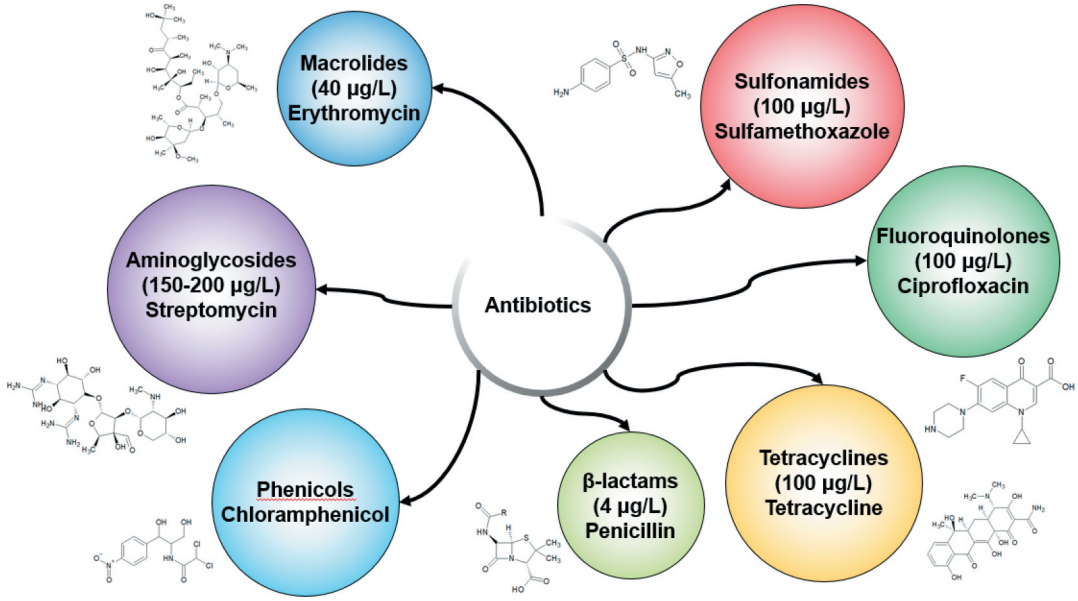

Figure 2. Classes of antibiotics with structure of each and MRL for certain compounds according to the European Council (Union 1990).

these antibiotics led to bacterial resistance, bone marrow suppression, nausea, and diarrhoea (Abedalwafa et al. 2019).

More than 60,000 tons of antibiotics were globally administered annually to livestock at the beginning of the last decade (Van Boeckel et al. 2015) as supplements or to treat bacterial infections. Their residues in milk, especially at levels higher than the MRLs, could lead to the multitude of drawbacks aforementioned. This warrants for regulating the MRL for the sum of certain antibiotics in milk (Zacco et al. 2007; Van Boeckel et al. 2015).

Moreover, new biosensor strategies should be implemented to detect the degradation products or metabolites of these antibiotics, which may be harmful active products. Few conventional methods simultaneously investigate antibiotics with their degradation products in milk (Xiao et al. 2015; Mohsenzadeh et al. 2018). Although Yehia et al. use a potentiometric sensor for the selective determination of cefquinome in presence of its degradation products but MRL was hardly approached since no biorecognition element was utilised (Yehia et al. 2016).

Hormones

The natural occurring hormones in animals have different essential physiological roles (Malekinejad and Rezabakhsh 2015b). For example, progesterone titre in milk has been used as a diagnostic tool in confirming cattle pregnancy in veterinary medicine (Hoffmann et al. 1977). However, artificial hormones are being used to increase milk production and meat in cattle, though with some adverse effects being observed such as disruption of the physiological function of the endocrine system, cancer and premature puberty in children (Malekinejad and Rezabakhsh 2015a; Kumar et al. 2018). Several studies have explored the utilisation of biosensor technology to detect and quantify hormones in dairy products, especially, milk. Typical hormones found in milk are progesterone, testosterone, somatotropin, insulin-like growth factor-1 (IGF-1), and 17β-oestradiol (E2) displayed in Figure 3 with representative structures.

Progesterone is a natural steroidal sex hormone associated with ova implantation in the uterus and embryo growth (Kumar et al. 2018). Milk analysis confirmed progesterone presence being administered in cattle to promote growth (Mottram et al. 2002). Also administered in livestock to promote growth is testosterone which is responsible for the development and appearance of secondary male sex organs and secretory glands (Kumar et al. 2018).

Somatotropin is a growth hormone that is normally used to increase milk production in USA and other countries, though forbidden in Canada and the EU due to its side effects on animal and human health (Ozhikandathil et al. 2012).

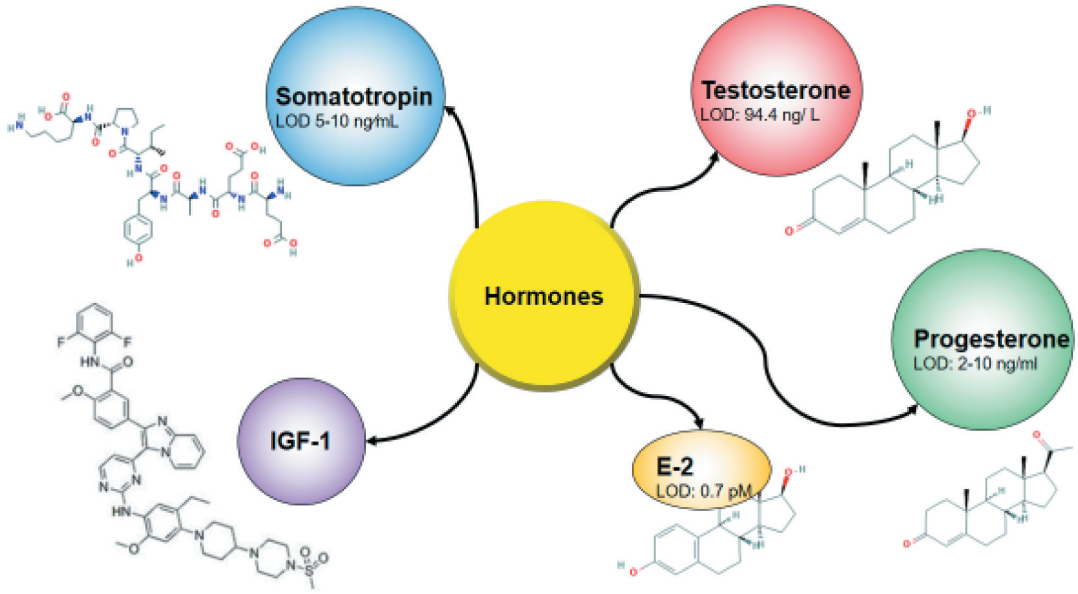

Figure 3. Hormones found in milk and their MRLs.

IGF-1 is a hormone that stimulates cell proliferation and differentiation. It is a suspected carcinogen that is not destroyed by pasteurisation and is present in milk from cows treated with recombinant bovine somatotropin (Collier et al. 1991; Guidi et al. 2001).

Lastly, E2 is the most active female sex hormone that is often administered to cattle to enhance growth and increase milk yield. However, consumption of milk contaminated with E2 even at low levels could lead to endocrine system disturbances as well as various kinds of cancers (Nameghi et al. 2019).

Pesticides

There are around 2 million tons of pesticides consumed annually, worldwide in order to obtain higher yields from agricultural fields and to counteract plant diseases, pests and weeds. However, pesticides residues in food are the cause of 1 million deaths and chronic illnesses per year (Verma and Bhardwaj 2015; Özdemir et al. 2019). Pesticides are considered not only a potential milk contaminant but also one of the major environmental pollutants due to their inevitable spread in water, soil, atmosphere, and hence passage into agricultural products (Ambrus and Yang 2016). Pesticide residues undergo degradation in soil but due to their high stability and low water solubility, they persist in the ecosystem and to exhibit long shelf life, i.e. organophosphates and organochlorines can be detected 15–20 years after their use (Campanella et al. 2011).

Figure 4 displays the various pesticide classes and the most common pesticide of each class with their corresponding structures. Organochlorides, which are used as insecticides, may cause neurodevelopment disorders, genotoxic effects and behavioural disorders (Chen, Pacheco, et al. 2016). Carbamates and organophosphorus compounds both act as acetylcholinesterase (AChE) enzyme inhibitors leading to acetylcholine accumulation at different cholinergic receptors with subsequent hyperstimulation of nicotinic and muscarinic receptors, and to overstimulate nerves with disrupted neurotransmission (Colovic et al. 2013). Due to all of the aforementioned adverse effects caused by indirect pesticide consumption in the food chain, pesticide levels in milk must be monitored on-site. As most of the adverse effects of pesticides are related to its specific binding to AChE, most biosensors used for its detection employ AChE as a recognition moiety (Chen, Pacheco, et al. 2016) as discussed later.

Miscellaneous

Mycotoxins

Mycotoxins such as aflatoxin (AF) are toxic secondary metabolites biosynthesised by certain

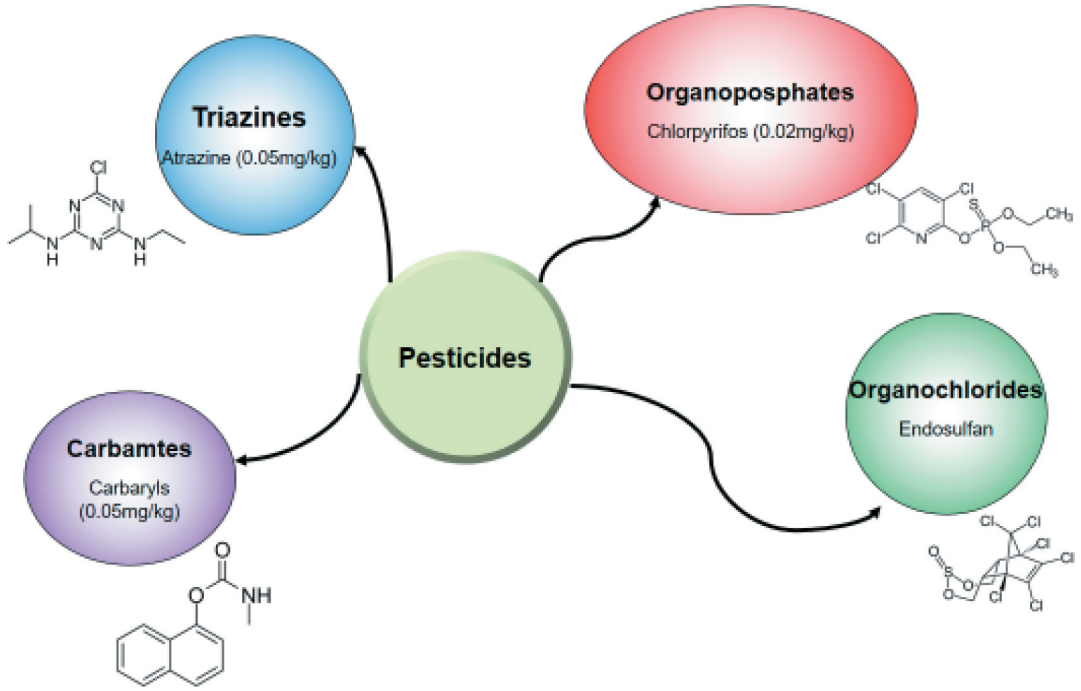

Figure 4. Classes of common pesticides and their MRLs.

parasitic fungi (*Aspergillus, Fusarium, Penicillium, Claviceps* and *Alternaria*). AF is found in the milk and urine of mammals after they have fed on contaminated feed (Grout et al. 2020). In humans, AF consumption may cause liver cancer and other mycotoxins inhibit ribosomal protein synthesis (Gurban et al. 2017; Matabaro et al. 2017).

Aflatoxins B_1, B_2, G_1, and G_2 may contaminate a large range of food products like nuts and cereals, whereas aflatoxin M_1 (AFM1) is more likely to contaminate milk and dairy products (Gurban et al. 2017). The MRL of AFM1 in milk is 0.025 μg/kg (ppb) as specified by the EU (European Commission 2010).

β-Lactamases

β-Lactamases are enzymes produced by bacteria as a defence mechanism against β-lactam antibiotics that are often added illegally to disguise β-lactam antibiotic levels in milk *via* catalysing β-lactams hydrolytic degradation (Chen, Xianyu, et al. 2016).

Melamine and urea

Both are common milk adulterants added intentionally to milk as nitrogenous compounds in order to increase non-protein nitrogen content (Sharma and Rajput 2012) and protein content (Liu et al. 2012), respectively. Milk is often diluted with water to increase its volume, whereas these substances are added to give a false high-protein content. EU MRL for melamine in dairy products is 2.5 mg/kg, whereas according to the FDA, the melamine MRL in milk is 0.25 mg/kg (Filazi et al. 2012). The MRL for urea in milk is 700 mg/L (Azad and Ahmed 2016). An increase in their amounts beyond the specified values could lead to kidney problems, especially in children. Classical quality control methods for total protein determination employ the Kjeldahl method (Moore et al. 2010) which measures only nitrogen content as an indicator of the protein content and hence cannot determine whether the nitrogen originated from protein or non-protein sources. Accordingly, spectroscopy and chromatography are employed for their detection though requiring complex setup and expensive devices.

Biosensors to detect antibiotics in milk

Table 1 lists all the biosensors used to detect antibiotics explained in the present review, with biorecognition element, transduction method,

advantages, disadvantages, and limit of detection (LOD), if available, for the reader to compare among them for any needed application.

Electrochemical biosensors

Zacco et al. developed an electrochemical magneto immunosensor to detect sulphonamide antibiotic in milk by covalently immobilising sulphonamide antibodies onto magnetic beads. A direct immunological competitive assay using sulphonamide antibiotic and horseradish peroxidase (HRP) enzyme as a label is performed on the antibody-modified magnetic bead. The magnetic beads are then captured by a magneto sensor (magneto graphite–epoxy composite) which is used as a working electrode. The electrochemical signal is detected when a substrate binds to HRP. The biosensor achieved an LOD of 1.44 µg/L which is below the MRL of sulphonamides. This method is cost-effective and could be used for on-site analysis. However, it was only tested on spiked full cream milk samples (Zacco et al. 2007).

An inkjet-printed label-free electrochemical biosensor was fabricated by Rosati et al. for the detection of ampicillin in milk. Rosati et al. (2019) printed microelectrodes using inkjet printers with and ink composed of silver nanoparticles with thiols bound to their surfaces. The particles were functionalised with ampicillin aptamers to detect ampicillin in spiked milk samples. This low cost aptamer-based biosensor displays a wide dynamic linear range 0.1–10 mg/L but low sensitivity with an LOD of 10 µg/mL, which is relatively high for MRL detection (Rosati et al. 2019).

Another electrochemical sensor that utilises aptamers was developed by Li et al. (2018) to detect multiple antibiotics simultaneously. Metal ions, i.e. Cd^{2+} and Pb^{2+}, which are attached to the aptamer of a certain antibiotic, act as signal tracers by generating differential pulse voltammetry (DPV) peaks. When the analyte is present, the interaction between the antibiotics and aptamers leads to the dissociation of dsDNA and more change in metal ions corresponding to peak current. The antibiotics tested were kanamycin and streptomycin with LODs of 74.50 pM and 36.45 pM, respectively, with a linear range from 0.1 to 100 nM, offering higher sensitivity levels at the picomolar level. This biosensor is rapid and sensitive and can detect any antibiotic simply by changing the aptamer. However, it was only tested on local milk samples from a supermarket which were subjected to centrifugation and dilution (Li et al. 2018).

Lai et al. (2012) developed an antibody displacement assay composed of gold-coated magnetic nanoparticles modified with antibodies and interdigitated electrodes. Chemiresistors measure the change in resistance across a nanoparticle film when a small organic molecule is present in milk, i.e. enrofloxacin, a fluoroquinolone antibiotic. The method is versatile and can detect different antibiotics by changing the antibodies bound to the nanoparticles with an LOD of 0.28 in aqueous PBS buffer and 2.8 pM in spiked milk samples that were not purified or centrifuged. Limitations lie in being partially selective towards certain antibiotic type and that the analyte needs to penetrate first the nanoparticle film (Lai et al. 2012)

Conzuelo et al. (2012) developed a disposable amperometric magneto-immunosensor to measure tetracyclines in milk. Its technique relies on the competitive binding on an immobilised antibody between the antibiotic and a tracer labelled with horseradish peroxidase. Hydrogen peroxide was added as an enzyme substrate in the presence of hydroquinone to mediate the redox reaction. The amperometric response was detected using screen-printed carbon electrodes and translated to indicate the amount of tetracyclines present, displaying high sensitivity level, with LODs of 8.9, 1.2, 66.8, 0.7 ng/mL for tetracycline, oxytetracycline, chlortetracycline, and doxycycline, respectively. This electrochemical immunosensor exhibits good dynamic linear range 17.8–189.6, 4.0–-242.3, 144.2–2001.9, 2.6–234.9 ng/mL for tetracycline, oxytetracycline, chlortetracycline, and doxycycline, respectively. However, it requires about 30 min for the analysis and was applied to spiked milk samples (Conzuelo et al. 2012).

Li et al. (2015) implemented an immunosensor for β-lactams by immobilising specific antibodies. Antibiotic binding to the antibody was measured by electrochemical impedance spectroscopy. The LOD of the biosensor was well below the MRL at 2.7×10^{-7} ng/mL, indicating high sensitivity. Additionally, when tested in the presence of aminoglycosides, the biosensor exhibited good specificity and selectivity towards the β-lactams present (Li et al. 2015).

Table 1. Biosensors to detect antibiotics in milk.

Method	Bioreceptor	Residue	LOD	Advantages	Disadvantages	Refs.
Electrochemical biosensors						
Graphite-epoxy magneto immunosensor (Amperometry)	Antibodies-HRP enzyme	Sulphonamide	1.44 µg/L	Quantitative, low cost, on-site analysis	Matrix interference/need dilution pretreatment	(Zacco et al. 2007)
Inkjet-printed Ag NPs (Impedance Spectroscopy)	Aptamers	Ampicillin	10 mg/L	Eco-friendly, wide dynamic linear range, reproducible	Calibration needed, low sensitivity	(Rosati et al. 2019)
AuNP biochem resistor (Conductimetry)	Antibodies	Enrofloxacin	2.8 pM	Versatile, no sample preparation	Partially selective, analyte has to penetrate NP film	(Lai et al. 2012)
Multiplexed graphitised multi-walled carbon nanotubes/ carbon nanofibers-gold nanoparticles aptasensor (voltammetry)	Aptamers	Kanamycin, Streptomycin	74.50 pM 36.45 pM	Sensitive, reproducible, could be used for multiple antibiotics	May need signal amplification	(Li et al. 2018)
Carbon SPE magnetosensor (amperometric)	Antibodies	Tetracyclines	8.9 µg/L	Disposable, sensitive, specific	Label needed	(Conzuelo et al. 2012)
Carbon SPE (amperometric)	Antibodies	β-lactams	$2x10^{-7}$ µg/L	Specific, sensitive	Pretreatment step is needed	(Li et al. 2015)
Single-graphene nanosheets (amperometric)	Penicillinase enzyme	Penicillin	10^{-13} M	Sensitive, quantitative, reproducible	Pretreatment step is needed	(Wu et al. 2014)
Carbon SPE (amperometric)	Antibodies	Chloramphenicol	4.7 ng/L	Label-free, sensitive, specific, wide linear range 0.01–10 ng/mL	Need dilution pretreatment (2-times diluted with PBS)	(El-Moghazy et al. 2018)
Optical biosensors						
AuNP colorimetric sensor	Aptamer	Streptomycin	47.3 nM	Sensitive	Pretreatment to remove salts	(Soheili et al. 2016)
Delvotest SP-NT colorimetric sensor	*Bacillus stearothermophilus*	Sulphonamides, penicillin, cloxacillin	N/A	Simple	Lack of specificity	(M-Heel et al. 2007)
AuNP lipid capsule colorimetric sensor	Phosphatidylcholine liposome	Gentamicin	7.5 nM	Simple, sensitive, specific	Qualitative only	(Liu et al. 2016)
Surface Plasmon resonance (SPR) immunosensor	Antibodies	Sulphamethazine	1 µg/L	Label-free, quantitative	Pretreatment step (incubation 30 min)	(Sternesjo et al. 1995)
SensiQ chip (SPR) immunoassay	Antibodies	Penicillin G	8 pM	Portable, simple	Qualitative only	(Pennacchio et al. 2015)
Lab-on-a-chip multiplexed immunoassay with wavelength interrogated optical sensor (WIOS)	Antibodies	Sulphonamides, fluoroquinolones, tetracyclines	100 µg/L	Simultaneous detection of 8 antibiotics, cheap, fast, simple	Not sensitive	(Suarez et al. 2009)
SPR based sensor chip	Penicillin-binding protein 2x	β-lactams	4 µg/kg	A screening assay for routine use	Not specific	(Cacciatore et al. 2004)
WIOS sensor chip	Antigens	Sulphonamides, tetracyclines, fluoroquinolones, β-lactams	Below MRL for all antibiotics	Label-free, multi-analyte, possible chip regeneration	Not ready for field or on-site analysis	(Adrian et al. 2009)
Au-NP (fluorescence)	Aptamers	Kanamycin A	0.3 nM	Linear response, sensitive	Dye-labelled	(Li et al. 2019)
parallel affinity sensor array PASA (chemiluminescence)	Antibodies	10 Antibiotics	Below MRL except for Penicillin G	Simultaneous analysis of multiple analytes	Many calibration tests, not reproducible	(Knecht et al. 2004)
Whole-cell biosensor	Bacterial strains	Ciprofloxacin	8 µg/L	Low cost, simple, portable	Living strains short life-time	(Cheng 2018)
Gold chip SPR sensor	Hapten protein	Fluoroquinolones	2 µg/L	Low cost, portable, field analysis	Pre-treatment needed	(Fernandez et al. 2011)
Quantum dot "traffic light" assay	Antibodies	Ofloxacin, Chloramphenicol, streptomycin	0.3 µg/L 0.12 µg/L 0.2 µg/L	Quantitative, simple, fast (10 min)	Milk- matrix effect, need dilution with PBS	(Taranova et al. 2015)

Wu et al. (2014) introduced a biosensor in which haematein, ionic liquids and penicillinase are adsorbed on a film containing single-graphene nanosheets. Penicillin is hydrolysed by the action of penicillinase yielding penicilloic acid and H^+; the freed proton is measured amperometrically with haematein as a pH indicator. The biosensor has a wide range 1.25×10^{-13} to 7.5×10^{-3} M, and a low detection limit of 10^{-13} M, but it was only assessed on spiked milk samples that were free of fat and protein (Wu et al. 2014).

Lastly, El-Moghazy et al. (2018) designed a screen-printed carbon electrode coated with a layer of poly (vinyl alcohol-co-ethylene) nanofibrous membrane that is covalently bonded to a chloramphenicol antibody in order to detect chloramphenicol. The binding of the antibiotic to the antibody generates an amperometric signal. The amperometric immunosensor was only applied on diluted spiked milk samples. It displays a wide linear range 0.01–10 ng/mL and exhibits a high sensitivity which might be attributed to the high porosity of the nanofibrous membrane. The LOD of the biosensor was at 4.7 pg/mL which is below the MRL and it exhibited specificity in the presence of antibiotics from other classes (El-Moghazy et al. 2018).

Optical biosensors

Delvotest SP-NT and Copan Milk Test are two commercially available colorimetric growth inhibition-based assays used to detect antimicrobial substances in milk. Their ampoules or microplates contain spores of *Bacillus stearothermophilus*, which upon milk addition germinate producing carbonic acid that leads to a colour change of bromocresol purple. Upon the presence of antimicrobials, bacterial growth would be inhibited and consequently, no colour change is detected. Le Breton et al. attempted to validate the test kits on local milk samples that were spiked. They found that the kits are not antibiotic specific as they were able to detect several antibiotics, i.e. penicillin, cloxacillin, sulphamethazine, sulphadiazine, cephalexin and gentamicin, albeit not oxytetracycline, dihydrostreptomycin or trimethoprim. They were easy to use, rugged, sensitive and relatively stable (M-Heel et al. 2007)

A highly sensitive colorimetric-based biosensor was developed by Soheili et al. (2016) for streptomycin detection in raw milk. The biosensor is comprised of functionalised gold nanoparticles with streptomycin aptamers. The biosensor was tested on spiked milk samples of concentration range 180–1000 nM and displayed an LOD of 47.2 nM which is below the MRL. Its advantages also include a short analysis time of 80 mins. The complexity of the setup only lies in sample pretreatment to remove salts that could lead to nanoparticle aggregation (Soheili et al. 2016).

Phosphatidylcholine (PTC) is a therapeutic agent that was used by Liu et al. (2016) to design a colorimetric sensor to detect gentamicin (GMC), an aminoglycoside, in milk. The method is based on preparing a red-coloured gold lipid capsule by modifying gold nanoparticles with PTC liposomes. The hydrogen bond between GMC and PTC causes the gold lipid capsule to collapse and with the wine-red colour shifts to blue. The linear range of aminoglycosides detection was up to 0.2 mM and LOD of 7.5 nM in pretreated spiked milk samples. The biosensor also showed no cross reactivity when it was tested against other antibiotics (Liu et al. 2016). This method is simple, sensitive, specific and suitable for both qualitative and quantitative purposes.

Another type of optical biosensor was designed by Sternesjo et al. (1995) to detect sulphamethazine. This immunosensor utilises sulphamethazine antibodies based on surface plasmon resonance transduction which detects the change in the refractive index of an analyte once it binds to its recognition moiety. Sulphamethazine was immobilised to a carboxymethyldextran-modified gold film *via* covalent bonding. Sulphamethazine polyclonal antibodies were incubated with milk samples, for 30 min, to bind sulphamethazine and the free antibodies were detected. This sensor is label-free exhibiting good sensitivity with an LOD of about 1 ppb in raw and skim milk which is lower than the MRL for sulphonamides. However, this biosensor exhibited slight cross-reactivity towards other sulphonamides as sulfamerazine and sulphadiazine. Moreover, the constructed calibration curve in buffer displayed significant difference with its counterpart in milk mostly attributed to a milk matrix effect (Sternesjo et al. 1995).

Another biosensor that employs SPR technology is the modified SensiQ chip. Pennacchio et al. (2015) immobilised penicillin G antibodies on the gold surface of the commercially available chip in order to detect penicillin. The method had an LOD of 8 pM, much lower than 12 nM specified by the MRL of the EU. This method is easy to use and can be adopted outside the laboratory for on-site analysis; however, it displays a narrow dynamic range with an upper quantification limit of penicillin G at 100 pM (Pennacchio et al. 2015).

Wavelength interrogated optical sensor (WIOS) method directs a collimated light beam into the sample and if a resonance condition is achieved, light is transmitted to a photodetector with an output signal to represent resonance peaks whose position indicates the quantity of the molecule adsorbed on the chip surface. Suarez et al. employed WIOS technology to develop a lab-on-a-chip multiplexed immunoassay sensor to detect sulphonamides, fluoroquinolones, and tetracyclines in milk samples. The chip allows simultaneous monitoring of different antibiotics in the same sample. The recognition moieties used are antibodies that bind to their specific analytes, and with the remaining free antibodies being detected. Advantages of such a biosensor include being inexpensive, fast and of simple device setup due to its "one-step fabrication process" and automation. The LOD of the chip was also within the MRL of sulphonamides which is 100 μg/L. However, the incidence of a false negative was noticed although the overall test accuracy attained 95% for multiplexed measurements on blind milk samples (Suarez et al. 2009).

Cacciatore et al. employed digoxigenin-labelled ampicillin to bind penicillin-binding protein to detect β-lactam penicillin residues in milk. β-Lactams in positive samples would bind to the penicillin-binding protein, whereas the non-complexed protein would form a complex with the ampicillin and be detected using SPR transduction. The assay was tested on defatted raw milk samples and was declared non-specific due to the influence of other antibiotics in the milk matrix. Additionally, the authors recommend pretreatment and centrifugation to achieve better sensitivity (Cacciatore et al. 2004).

A label-free sensor chip to detect antibiotics from four different antibiotic families, i.e. sulphonamides, fluoroquinolones, β-lactams, and tetracyclines was fabricated by Adrian et al. Bioreceptors, mainly antigens, are immobilised on a waveguide grating, with WIOS being used to detect the change in refractive index caused upon analytes binding. When subjected to blind milk samples, the chip detected and identified the different antibiotic families at levels below their MRLs in a short time of 40 min. With further modifications, the author expects to improve the bench-top biosensor to make it suitable for field analysis. They also aim to improve reproducibility, which required signal amplification by reagents (Adrian et al. 2009).

Li et al. (2019) reported the development of an aptamer-based biosensor to detect the aminoglycoside kanamycin A. The method relies on the fluorescence output signal that develops when kanamycin A forms a complex with a dye-labelled aptamer that is modified on gold nanoparticles. In the absence of the antibiotic, the signal is quenched. The LOD of this method was at 0.3 nM with a linear range of 0.8–350 nM (Li et al. 2019).

A similar chemiluminescence based signal output is a parallel affinity sensor array (PASA) which has a chip-based ELISA. This biosensor, developed by Knecht et al. (2004) was used to detect contaminants in drinking water and was extended to detect antibiotics in milk. Hapten proteins are immobilised on the chip instead of antibodies, making it an indirect competitive immunoassay. This assay allows for the screening of a spectrum of several antibiotics from different families though requiring several calibration tests for each antibiotic. All antibiotics were detected at levels below their MRL except for penicillin G which was detected at its MRL range. The assay was performed on spiked pasteurised whole milk without further preparation. Additionally, the assay time was around 5 min. Limitations lie in being not robust enough and requiring several calibration tests, in addition to possible cross-reactivity (Knecht et al. 2004).

Cheng (2018) designed a whole-cell biosensor to detect the fluoroquinolone ciprofloxacin. They immobilised live bacterial strains onto a commercially available charge-coupled device called LumiSense. The strains emitted luminescence when ciprofloxacin was detected, with an LOD of 8 ng/mL, which is below the MRL. This

method is portable and of low cost with short response time 20–80 min, but not specific aside from its short operational life due to the use of live bacterial strains (Cheng 2018).

Fernandez et al. (2011) modified the SPReeta Evaluation Kit SPR3 created by Texas Instruments Inc. to create a biosensor that can determine fluoroquinolones in milk. The kit has three-channel gold chips that were activated with mixed self-assembled monolayers and immobilised with fluoroquinolone haptenized protein. As the fluoroquinolone binds to the antibody, SPR signal decreases due to a change in the refractive index. Minimal treatment of the spiked local milk samples decreased matrix interferences and three samples were measured in less than 30 min. The biosensor is inexpensive, reusable and portable being suited for on-site analysis, and with an LOD of 2 μg/L for enrofloxacin (Fernandez et al. 2011).

Taranova et al. (2015) developed a "traffic light" immunochromatographic assay to simultaneously identify multiple antibiotics in milk. The biosensor is a test strip consisting of three lines of different colours. Antibodies are labelled with quantum dots as fluorescent markers when excited by a UV source. The visibility of the line colours indicates qualitative analysis while the fluorescence intensity of the lines indicates quantitative analysis. When tested against spiked milk samples, the biosensor displayed a wide dynamic range and a high sensitivity level for ofloxacin, chloramphenicol, and streptomycin with LODs at 0.3, 0.12, and 0.2 μg/L, respectively, which are 80–200 times lower than those attainable with the corresponding ELISA assay using the same monoclonal antibodies. Its advantages are the short analysis time of 10 min, quantitative multi-contaminant analysis, analyte recovery, reproducibility and minimal sample preparation (Taranova et al. 2015). However, quantitative analysis based merely on test line signal is unreliable as it is vulnerable to changes by factors such as temperature (Chen et al. 2019).

Thermal biosensors

Zhou et al. (2013) designed the enzyme thermistor (ET) which is a colorimetric biosensor that can detect heat released during enzyme-catalysed hydrolytic degradation. ET detects consumed antibiotic penicillin G which in turn determines the amount of β-lactamase enzymes present. This is important because β-lactamase enzymes are used, illegally, to lower antibiotic levels in milk *via* catalysing the hydrolytic degradation of β-lactams. ET has an enzyme column on which β-lactamases are immobilised and as penicillin G flows through the column it gets converted to penicilloic acid with the release of heat that is directly proportional to the consumption of the antibiotic. This biosensor was tested on spiked local milk samples and exhibited an LOD of 1.1 U/mL and a linear range of 1.1–20 U/ml. It remained stable for 60 days and provided accurate quantitative information in 5 min per injection. The results agreed with "established culture-based assays". However, it should be noted that some interference was observed due to the complexity of milk samples composed of fats, proteins, sugars, casein micelles that could additionally clog the column (Zhou et al. 2013).

Piezoelectric biosensors

To detect sulphadiazine in milk, Bhand and Mishra (2017) developed an electrochemical quartz crystal nanobalance that detects small mass changes caused by the chemical binding of the antibiotic to anti-sulphadiazine antibody that was immobilised on gold surface of the piezoelectric transducer gold quartz crystal. This method was described as being highly sensitive when it assessed centrifuged and diluted milk samples with an LOD of 10 ng/ml. Antibiotic recovery was 98.6–100.5%. Additionally, it can be automated using flow injection analysis. Its disadvantages are the narrow dynamic range 50–200 ng/ml and the cross-reactivity with structurally similar antibiotics (Bhand and Mishra 2017).

Biosensors to detect hormones in milk

Table 2 lists all the biosensors used to detect hormones explained in the present review, with bioreecognition element, transduction method, advantages, disadvantages, and limit of detection (LOD), if available, for the reader to compare among them for any needed application.

Electrochemical biosensors

Mottram et al. (2002) developed a disposable screen-printed carbon electrode (SPCE) biosensor system with monoclonal antibodies attached on the surface of the SPCE. Progesterone is labelled with alkaline phosphatase and the product of the electrochemical oxidation is detected and considered inversely proportional to the unlabelled progesterone present in milk. The biosensor was calibrated and tested on fresh milk samples and displayed a narrow linear range of 3–20 ng/ml. A major limitation of the biosensor is being irreproducible, especially, when milk is at risk of microbial infection resulting in a change of the detected electrochemical properties (Mottram et al. 2002).

An electrochemical immunosensor was developed by Dong et al. (2017) for progesterone detection in milk. This biosensor is based on the immobilisation of thionine-graphene oxide composites on a glassy carbon electrode in addition to progesterone coating antigen. The free progesterone competes with progesterone coating antigen for binding to a biotin-progesterone antibody to yield an electrical signal for the quantitative determination of progesterone. The biosensor displayed good specificity, reproducibility, stability, and is considered environmentally friendly as thionine-graphene oxide is non-toxic. It has a low detection limit of 0.063 ng/L in spiked milk samples and the results agreed with LC-MS/MS analysis with a correlation coefficient of 0.998. However, its limitations are the required sample dilution by 100 times and its long analysis time of 3 h per run limiting its usage for screening large number of milk samples (Dong et al. 2017).

An aptasensor was designed by Nameghi et al. (2019) based on the immobilisation of split DNA strands on a gold electrode surface to detect the presence of 17β-oestradiol (E2). When E2 is present on the surface of the sensor where the split aptamers are bound, split1-E2-split2 complex is formed on the surface of the electrode. This technique offers a fast analysis time (30 min) and displays high selectivity in the presence of other compounds alongside a very high sensitivity level with an LOD of 0.7 pM and linear ranges of 3–300 pM and 300 pM-9 nM in spiked milk samples (Nameghi et al. 2019).

Optical biosensors

Tschmelak et al. (2005) developed a total internal reflection fluorescence-based immunoassay that utilises a commercially available monoclonal anti-progesterone antibody linked to a fluorescent dye to detect progesterone in milk. The dyed anti-body is mixed with the analyte and with the mixture transported over the sensor surface where the free binding sites on the antibody can only bind the immobilised progesterone derivative on the transducer surface. The fluorescence signal is inversely proportional to progesterone level. The biosensor was tested on spiked UHT, raw and fresh milk samples and exhibited LODs of 45.5–56.1 pg/ml, which is below the MRL. The biosensor is automated and only required milk dilution (Tschmelak et al. 2005).

Similarly, an enzyme immunoassay (EIA) biosensor was developed by Claycomb et al. (1998) to measure progesterone levels in bovine milk. To achieve the desired sensitivity, EIA used covalent carbohydrate-binding microtiter wells, monoclonal antibodies, horseradish peroxidase as the enzyme label, and 3,3′,5,5′- tetramethylbenzidine-dihydrochloride (TMB) to serve as the substrate (Claycomb et al. 1998). The assay time was 15 min, making it suitable for real-time analysis. It was tested on diluted and undiluted fresh milk and dilution was proven necessary to improve assay resolution. The EIA biosensor displayed a dynamic range of 0.2–20 ng/ml. Further building on the EIA, the same group of authors improved the specificity of the biosensor to measure progesterone levels but at the follicular phase. The slight difference lies in coating the anti-progesterone on small disks of nitrocellulose membrane. Progesterone labelled with horseradish peroxidase was used as the conjugate and TMB as the chromogenic substrate for HRP in presence of hydrogen peroxide. Free progesterone in the sample and enzyme-labelled progesterone compete for antibody-binding sites on the nitrocellulose membrane; followed by a change in the absorbance due to oxidation of the chromogenic substrate. The sample handling was all automated with the developed biosensor sensitive enough for progesterone detection in milk at follicular phase (below 1.0 ng mL^{-1}) in real-time. However, it suffers from

Table 2. Biosensors to detect hormones in milk.

Method	Bioreceptor	Residue	LOD	Advantages	Disadvantages	Refs.
Electrochemical biosensors						
SPCE	Antibody	Progesterone	3–20 µg/L	Automated, sensitive	Not reproducible	(Mottram et al. 2002)
Amperometric immunoassay	Antibody	Progesterone	0.063 ng/L	Specific, reproducible, cheap, eco-friendly	Time-consuming, needs sample preparation	(Dong et al. 2017)
Aptasensor (Voltammetry)	Aptamer	E2	0.7 pM	Selective, stable, cheap, sensitive	Time consuming	(Nameghi et al. 2019)
Optical biosensors						
River Analyser – RIANA (totalinternal reflecting fluorescence)	Antibody	Progesterone	45.5 ng/L	Sensitive, automated	Expensive, dilution needed	(Tschmelak et al. 2005)
Nitrocellulose membrane assay (Absorbance)	Antibody	Progesterone	Less than 1 µg/L	On-line measurement, automated, sensitive, rapid (10 min)	Low accuracy at low concentrations (0.5 ng/ml)	(Delwiche et al. 2001)
FO-SPR	Antibody	Progesterone	0.6 µg/L	Automated, no preparation, sensitive	Not reproducible	(Daems et al. 2016)
Nanoparticle-enhanced SPR	Antibody	Progesterone	13 ng/L	Sensitive	Complicated setup, slow	(Wang et al. 2019)
Automated SPR biosensor	Antibody	Progesterone	3.56 µg/L	Automated, fast analysis	Expensive	(Gillis et al. 2002)
Fluorescence quantum dots immunoassay	Antibody	Progesterone	0.1 µg/L	Sensitive, simple,	Ten-fold water dilution to minimise matrix interferences	(Trapiella-Alfonso et al. 2011)
Enzyme Immunoassay (EIA)	Enzyme	Progesterone	N/A	Sensitive, fast	Expensive	(Claycomb et al. 1998)
SPR-based BIA technology	Antibody	IGF-1	4 µg/L	Specific, sensitive, rapid, no sample preparation, reproducible	Expensive	(Guidi et al. 2001)
Gold nanoisland lab-on-a-chip	Antibody	Somatotropin	5 µg/L	Simple, user friendly	Not sensitive, time-consuming	(Ozhikandathil et al. 2012)
Reflectometric Interference Spectroscopy (RIS)	Antibody	Testosterone	94.4 µg/L	Selective	Not sensitive	(Rau and Gauglitz 2012)
Poly (N-isopropylacrylamide) microgel-based etalons	Aptamer	E2	3.2 pM	Selective, no pretreatment needed	Not sensitive	(Jiang et al. 2018)
Graphene quantum dot nanocomposites (Voltammetry)	Aptamer	AFM1	10 fM	Quantitative, sensitive, accurate, rapid	Complicated setup, short shelf life	(Kordasht et al. 2019)
Single-walled carbon nanotubes electrode (Amperometry)	Antibodies	AFM1	0.2 µg/L	Quantitative, low cost, easy operation, rapid	Centrifugation step is required	(Abera et al. 2019)
Magnetic nanoparticles on Screen Printed Carbon Electrodes (amperometric)	Antibodies	AFM1	0.1 µg/L	Quantitative, reproducible, sensitive	Centrifugation step is required	(Paniel et al. 2010)
Sweet Sensor (Glucose sensor)	Polyclonal antibody conjugated with Enzyme	AFM1	27 ng/L	Easy to use	Complex procedure to fabricate	(Giovanni et al. 2019)

unsatisfactory accuracy notably at low concentration levels as the 95% confidence prediction interval for a 0.5 ng/mL was estimated to be 0.2–1.5 ng/mL (Delwiche et al. 2001).

Also to measure progesterone levels in milk, Daems et al. (2016) developed a biosensor based on automated fibre optic surface plasmon resonance (FO-SPR). The device consists of disposable FO-SPR sensor tips, a tungsten halogen lamp, and a UV-VIS spectrophotometer. This automated assay is simply based on sample binding the gold surface of the sensor tip, leading to a shift in the spectrum that is proportional to progesterone level. The LOD of the biosensor was 0.5 ng/ml when tested on spiked milk, and when it was tested on bovine milk samples, it agreed with the ELISA results with an ICC of 0.97. The biosensor had an assay time of 20 min and researchers aim to improve its automation (Daems et al. 2016).

Wang et al. (2019) designed a novel SPR biosensor chip also to detect progesterone in milk. It relies on the use of large magnetic nanoparticles to amplify SPR signals, along with the presence of molecular linkers between the nanoparticles and the antibodies. It was tested on cow's milk directly and exhibited sensitivity with an LOD of 0.038 ng/ml. Nevertheless, such a biosensor has a complicated setup and requires a long run time of *ca*. 50 minutes (Wang et al. 2019).

Gillis et al. (2002) designed an SPR biosensor composed of progesterone covalently immobilised to the BIACORE 2000 and CM5 sensor chip. A fixed amount of monoclonal anti-progesterone antibody is mixed with the bovine milk sample, and amount of free unreacted antibody is detected using biomolecular interaction analysis. The LOD is at 3.56 ng/ml when tested on cow's milk, with full automation displaying 5 min per run. It covers the concentration range 0.5–50 ng/ml and was found to be reusable and stable with less than 1% change over the course of 129 runs (Gillis et al. 2002).

Trapiella-Alfonso et al. (2011) devised another antibody-based immunoassay using semiconductor quantum dots (QDs) as fluorescent labels to detect and quantify progesterone levels in cow's milk. Colloidal water-soluble CdSe/ZnS QDs are conjugated to an antigen derivative and the resulting complex is then employed with unlabelled anti-progesterone monoclonal antibodies as the biological recognition elements. The detection limit was at 0.1 ng/mL in cow's milk and the biosensor covers a linear range of 0.3–14.5 ng/ml. The narrow fluorescence spectrum of QDs maximises the sensitivity of the biosensor which was also found to be reproducible with 3.5% RSD after 2 days of experiments. However, a 10-fold water dilution pretreatment step was essential to minimise matrix interferences (Trapiella-Alfonso et al. 2011).

Ozhikandathil et al. (2012) designed a biosensor based on the use of three-dimensional gold nanostructure morphology that is assembled into a microfluidic device to detect bovine somatotropin. When gold nanoparticles bind to somatotropin followed by UV light irradiation, the resultant complex causes a shift in the gold band. The biosensor has an LOD of 5 ng/ml and does not require skilful staff or complex instrumentation. However, it is time-consuming as it takes 70 minutes per run, but has a wide linear concentration ranging from 5 to 1000 ng⁄mL (Ozhikandathil et al. 2012).

Reflectometric interference spectroscopy (RIfS) was used by Rau and Gauglitz (2012) to design a label-free biosensor to detect testosterone in milk. A diode array spectrometer detects characteristic interference patterns when a halogen white light source is reflected onto an optical fibre. Anti-testosterone antibodies with free binding sites bind to the immobilised testosterone derivatives on the sensor surface as recognition elements. The LOD of this assay is 94.4 ng/L in (3.5 % fat) milk and LOQ is 229.3 ng/L. The biosensor had a recovery rate of 70–120% and a run time of 15 min (Rau and Gauglitz 2012).

Jiang et al. (2018) developed an aptasensor to detect E2. This biosensor is composed of thermoresponsive polymer poly(N-isopropyl acrylamide) (pNIPAm) microgel-based etalons that are modified by E2 binding 75-mer DNA aptamer to its outermost Au layer. The aptamer binds first to E2 with the resulting complex undergoing a conformational change to a form that can block the diffusion of salt ions into the microgel layer proportional to E2 levels in solution. The biosensor displayed LOD of 0.9 pg/ml in commercial skim milk, 8.4 pg/ml in 2% milk and 4–9.1 pg/ml in farm milk. This device does not need sample pretreatment and is reusable for 5 times with only washing required. It is selective, but exhibited low cross-reactivity with progesterone (Jiang et al. 2018).

Biosensors to detect pesticides in milk

Table 3 lists all the biosensors used to detect pesticides explained in the present review, with biorecognition element, transduction method, advantages, disadvantages, and limit of detection (LOD) if available for the reader to compare among them for any needed application.

Electrochemical biosensors

Mishra et al. (2012) integrated a screen printed electrode (SPE) into a flow cell for the detection of milk borne pesticides. Chlorpyriphos-oxon (CPO), ethyl paraoxon (EPOx) and malaxon (MOx) in milk were detected *via* an amperometric signal that is related to enzymatic activity. Acetylcholinesterase enzymes (AChE) were immobilised on electrodes by engulfment in a photo-cross-linkable polymer, with the automated flow-based biosensor to quantify the three organophosphate pesticides in milk. This method is rapid and quantified the three pesticides in less than 15 min, exhibiting a narrow linear range of 3×10^{-10} to 5×10^{-9} M with good reproducibility. The LODs were at 5×10^{-9} M, 5×10^{-12} M and 5×10^{-10} M for EPOx, CPO and MOx, respectively, in spiked milk. It also had good recovery of 95% in lower fat milk, thus making the biosensor more suitable for low-fat samples. Additionally, the electrodes are expensive and not reusable (Mishra et al. 2012).

An AChE inhibition-based biosensor was developed by Zhang et al. (2005) to detect organophosphate and carbamate pesticides in milk. Three engineered variants of *Nippostrongylus brasiliensis* AChE enzymes were immobilised on SPEs, with enzyme activity reflected by the amount of thiocholine formed at the anode due to acetylthiocholine chloride hydrolysis to be measured amperometrically. The biosensor quantitatively detected pesticides in 2 out of 10 commercial milk samples. The LOD of carbaryl was 20 µg/L in spiked 3.5% milk. As such, this biosensor, although reproducible with recovery of 89–107%, detects all AChE inhibitors as a sum parameter and does not yield quantitative information or specify pesticide type (Zhang et al. 2005).

Tomassetti et al. (2015) developed a conventional amperometric immunosensor and a novel SPE biosensor to detect triazine herbicides in milk. The conventional amperometric sensor employed platinum electrodes along with a hydrogen peroxide electrode transducer that was modified with electrodeposited Prussian blue to enable hydrogen peroxide detection proportional to free atrazine levels in milk. In untreated spiked bovine milk samples, the conventional biosensor had an LOD of 5.0×10^{-8} M and linear range $10^{-10} - 2.8 \times 10^{-5}$ M with 1 h analysis time. The second method employing the SPEs is commercially available, displaying a wide dynamic range of 5.0×10^{-8} – 2.0×10^{-5} M with a satisfactory sensitivity (LOD of $2.3 \times 10^{-}8$ M) and 25 min analysis time. Even though the SPE biosensor was more sensitive and had a lower analysis time, both biosensors displayed major cross-reactivity with other triazines and minor with carbaryls (Campanella et al. 2011; Tomassetti et al. 2015).

Another use of horseradish peroxidase was in the fabrication of a potentiometric transducer immunosensor by Yulaev et al. (2001) to detect the triazine simazine. Anti-simazine antibodies were immobilised on different types of electrodes and with the gold planar electrode found the most efficient support for immunosensing. The biosensor displayed 14 min analysis time and was stable for 15 days and 250 tests with less than 10% signal decrease. It had an LOD of 96 ng/ml in milk spiked with 100 ng/ml simazine and LOD of 6 ng/ml in unspiked milk. This biosensor was also able to detect simazine in cucumbers (Yulaev et al. 2001).

Optical biosensors

Biochips, also known as microarrays, are arranged on small solid glass or silicon surfaces and allow for multiple simultaneous analyses. Mishra, Bhadauria, et al. (2010) utilised a biochip with a chemiluminescence enzymatic assay that was based on the inhibition of the enzyme butyryl cholinesterase to detect organophosphate pesticides methyl paraoxon, methyl parathion, and malathion. The detection ranges were 0.005–50, 0.5–1000 and 0.5–1000 µg/L, respectively, and methyl paraoxon was detected in spiked 0.5% milk with an LOD of 0.0005 µg/L and around 98.6% mean recovery. The biosensor produced results within 12 min and displayed 74% stability at the end of a 6 week period. This device setup is

Table 3. Biosensors to detect pesticides in milk.

Method	Bioreceptor	Residue	LOD	Advantages	Disadvantages	Refs.
Electrochemical biosensors						
Carbon SPE Flow cell (Amperometric)	AChE	CPO EPOx MOx	$5x10^{-12}$ M $5x10^{-9}$ M $5x10^{-10}$ M	Quantitative, sensitive	More suited for low-fat milk samples, expensive electrodes	(Mishra et al. 2012)
SPE (Amperometric)	AChE	Carbaryl	20 µg/L	Reproducible	Qualitative only	(Zhang et al. 2005)
H_2O_2 sensor (Amperometric)	Antibody – HRP conjugate	Triazine	$5x10^{-11}$ M	Sensitive, selective, very wide dynamic range	Expensive, complex	(Campanella et al. 2011)
Screen printed/H_2O_2 sensor from BVT Technology (amperometric)	Labelled Antibody	Atrazine	2.3×10^{-8} M	Selective, sensitive	Expensive, complex, long analysis time (1 h)	(Tomassetti et al. 2015)
Gold planar – electrodes (Potentiometric)	Antibody	Simazine	3 µg/L	On-site analysis, 14 min, no pretreatment,	15-days sensor life time	(Yulaev et al. 2001)
Optical biosensors						
Chemiluminescence biochip assay	Enzyme	Methyl paraoxon, methyl parathion, malathion	0.001 µg/L	On-site analysis, quantitative, 12 min. analysis	Dilution and filtration needed	(Mishra, Bhadauria, et al. 2010)
Multi-array biosensor	Enzyme	Diuron, chlorpyrifo, Catechol	1 ng/kg 0.6 µg/L 1.2 µg/kg	Quantitative, 10 min analysis	Labelled	(Silletti et al. 2015)
SPR-based sensor	Antibody	Triazine (Atrazine)	5.30×10^{-8} M	Good recovery, Easy, 25 min analysis time	Narrow dynamic linear range	(Tomassetti et al. 2015)

miniaturised and thus can be used at the field level (Mishra, Bhadauria, et al. 2010).

Silletti et al. (2015) combined an optical system integrated with an array of bio-mediators and a fluorescence activity as the transducer to detect different compounds from different classes and could be used online through fluidic systems. The multi-array biosensor was composed of six cells for contemporary measurements with three excitation light sources (red, yellow and blue). The analysis of three pesticides *i.e.* diuron (phenylurea), chlorpyrifos (organophosphate) and catechol (herbicide) gave a respective limit of detection of 1.1 ng/kg, 0.6 µg/L and 1.2 µg/kg within 10 min in spiked half-milk. They also had linear ranges 1 nM-1 µM, 1.-4–8.5 nM and 5–25 nM, respectively (Silletti et al. 2015).

An SPR immunosensor was developed for the determination of different triazine compounds. Tomassetti et al. (2015) investigated this optical biosensor in a real comparative study with two amperometric immunosensors. All the sensors showed satisfactory recovery ranges of triazine pesticides (93–103%), although SPR-based biosensor displayed a relative narrower linear range 0.1–-1.5 µM with a lower sensitivity (LOD at 53 nM) and a longer analysis time of 1 h for spiked milk (Tomassetti et al. 2015).

Piezoelectric biosensors

Liu et al. (2013) designed a biosensor constituted of molecularly imprinted polymer microspheres placed on a piezoelectric quartz crystal chip as a recognition element to detect endosulfan, an organochlorine pesticide. This device is highly sensitive with an LOD of 5.59 ng/ml in pretreated milk. It displayed a linear range of 10–40 ng/ml and was stable for 6 months and reusable for 6 times. It had a recovery of 101.8–108% and was found to be selective for endosulfan (Liu et al. 2013).

Biosensors to detect miscellaneous contaminants in milk

Table 4 lists all the biosensors used to detect mycotoxins, melamine and urea explained in the present review, with bio-recognition element, transduction method, advantages, disadvantages, and limit of detection (LOD) if available for the reader to compare among them for any needed application.

Mycotoxins

Gurban et al. (2017) reported an optical biosensor-based approach to detect aflatoxin M_1 (AFM1) formed by the hydroxylation of aflatoxin B_1 in animal feed and excreted into milk. The sensor employs the covalent immobilisation of aflatoxin with horseradish peroxidase (AFM1-HRP) conjugate on the surface of the amino-functional, SiO_2 –TiO_2 based sensor using glutaraldehyde, and with specific antibody to AFM1 added as a bio-recognition moiety. Milk samples first require either filtration, centrifugation or size exclusion centrifugation. AFM1 was detected in the dynamic range of 0.001–0.1 ng/ml which is within its MRL for milk (Gurban et al. 2017).

Kordasht et al. (2019) further improved AF detection by designing a graphene quantum dot nanocomposite modified with chitosan functionalised by amine groups and an aptamer as the recognition molecule to detect AFM1 in milk. The differential pulse voltammetry transduction method detected a change in potential when AFM1 was bound to the sensor, providing ultra-sensitive detection with a wide linear range of 0.1 µM to 1 fM in spiked local and pasteurised milk, which is well below the MRL. The biosensor is reproducible for three conducted analyses and sensitive, however, the setup is complex to fabricate and has a short shelf life of only 2 days (Kordasht et al. 2019).

A chronoamperometric biosensor was fabricated by Abera et al. (2019) to detect AFM1 in milk. It is composed of single-walled carbon nanotubes functionalised on dispense printed electrodes and then coated with antibodies. AFM1 was conjugated to horseradish peroxidase (HRP) and the AFM1-HRP conjugates oxidised the chromogenic substrate TMB buffer, changing its colour to blue leading to its detection. Inactivating HRP stops the reaction and allows for measurement with an LOD of 0.2 µg/L and a linear range of 0.01–1 µg/L in spiked milk. It is considered low cost, easily operated and of rapid response within 25 min at room temperature. Centrifugation was required to remove fat and

Table 4. Biosensors to detect mycotoxins, melamine and urea in milk.

Method	Bioreceptor	Contaminant	LOD	Advantages	Disadvantages	Refs.
Mycotoxins						
Pt/ZnO/AChE/Chitosan bioelectrodes (Voltammetry)	AChE	Melamine Urea	3 pM 1 pM	Sensitive, response time 20 min	Narrow linear range 1–20 nM	(Ezhilan et al. 2017)
NH_4 ion-sensitive electrodes (Potentiometry)	Urease enzyme	Urea	2.5 x 10^{-5} M	Reproducible, cost-efficient, disposable	Short shelf life	(Trivedi et al. 2009)
SiO_2–TiO_2 electrodes	Antibody	AFM1	5 ng/L	Quantitative, sensitive	Sample preparation step	(Gurban et al. 2017)
Graphene quantum dot nanocomposites (Voltammetry)	Aptamer	AFM1	10 fM	Quantitative, sensitive, accurate, rapid	Complicated setup, short shelf life	(Kordasht et al. 2019)
Single-walled carbon nanotubes electrode (Amperometry)	Antibodies	AFM1	0.2 µg/L	Quantitative, low cost, easy operation, rapid	Centrifugation step is required	(Abera et al. 2019)
Magnetic nanoparticles on Screen Printed Carbon Electrodes (amperometric)	Antibodies	AFM1	0.1 µg/L	Quantitative, reproducible, sensitive	Centrifugation step is required	(Paniel et al. 2010)
Sweet Sensor (Glucose sensor)	Polyclonal antibody conjugated with Enzyme	AFM1	27 ng/L	Easy to use	Complex procedure to fabricate	(Giovanni et al. 2019)
dELISA AuNP strip	Antibody	AFM1	0.028 µg/L	Sensitive, specific, on-site analysis	One-time use, expensive	(Wang et al. 2011)
Surface Plasmon-Enhanced Fluorescence Spectroscopy	Antibody	AFM1	0.6 ng/L	Rapid, reproducible, sensitive	Miniaturisation required and needs testing on real milk samples	(Wang et al. 2009)
Multiplexed Planar Waveguide Fluorescence Immunosensor	Antibody	AFM1, Melamine	0.045 µg/L, 13.37 µg/L	Quantitative, sensitive, rapid, simultaneous detection of aflatoxin & melamine	Labelling, pretreatment and complex equipment required	(Guo et al. 2016)

allow AFM1 detection in the skimmed layer (Abera et al. 2019).

Paniel et al. (2010) deposited magnetic nanoparticles coated with anti-AFM1 antibodies on screen printed carbon electrodes to detect AFM1 in commercial milk. The amperometric response is measured, with an LOD at 0.01 ppb which is below the MRL of AFM1 (0.05 ppb). It exhibited a linear range of 0.01 to 0.1 ppb, with the additional advantages of being sensitive, quantitative and reproducible. Likewise, the aforementioned biosensor (Abera et al. 2019), milk samples were centrifuged and only the skimmed milk layer was spiked with AFM1 for testing. Future modifications aim to further the biosensor for on-site analysis of milk specimens (Paniel et al. 2010).

Giovanni et al. (2019) designed a "sweet sensor" to detect AFM1 in whole milk. A monoclonal antibody was conjugated to the *Saccharomyces cerevisiae* enzyme invertase which converts sucrose into glucose and fructose. The produced glucose is measured using a glucometer, thus reflecting AFM1 levels. The LOD was at 27 ppt which is lower than the MRL (50 ppt). Despite the complicated design encompassing nitrocellulose and blocking proteins, this biosensor has the potential to be used for on-site analysis due to the ease of operation of the glucometer (Giovanni et al. 2019).

Wang et al. (2011) used an immunostrip to detect low molecular weight AFM1. Antibodies were conjugated to gold nanoparticles that yield a red colour when binding takes place. The 10 min assay is specific and sensitive to the interaction between the liquid test sample containing aflatoxin and the antibody-gold nanoparticles. The biosensor exhibited an LOD of 0.5 ng/ml when tested against spiked milk. It is suitable for the detection of ultra-trace amounts of AFM1 but it only provides qualitative information and is not reusable (Wang et al. 2011).

Surface plasmon-enhanced fluorescence spectroscopy was applied by Wang et al. (2009) to detect AFM1 in milk. Anti-AFM1 antibodies were labelled with fluorophores. The LOD was at 0.6 pg/mL which is less than the MRL and results were obtained within 53 min runtime. The biosensor is sensitive and reproducible, but was tested on centrifuged 30% milk powder dissolved in deionised water and spiked with AFM1. However, this biosensor has yet to be tested on real milk samples and needs to be miniaturised for field detection (Wang et al. 2009).

A multiplexed planar waveguide fluorescence immunosensor was developed by Guo et al. (2016) to simultaneously detect melamine and AFM1. Anti-melamine and anti-AFM1 antibodies were labelled with fluorophores for recognition. The sensor showed a linear range for AFM1 and melamine at 0.073–0.4 ng/mL and 26.38–270 ng/mL, respectively, and an LOD of 0.045 and 13.37 ng/mL, respectively. This method is considered quantitative, sensitive and able to detect two compounds from different contaminant families in 20 min. However, labelling and pretreatment are required along with complex device setup warranting for further improvement to be used for on-site applications (Guo et al. 2016).

Melamine and urea

A Pt/ZnO/AChE/chitosan bioelectrode was developed by Ezhilan et al. (2017) with cyclic voltammetric detection of AChE inhibition to simultaneously detect melamine and urea in milk. The biosensor detects melamine and urea with LODs of 3 pM and 1 pM, respectively, which is below the MRL for both chemicals. This biosensor was tested on cow milk that was adulterated with a binary mixture of melamine and urea and displayed good recovery (99.96–102.22%). The biosensor displayed repeatability RSDs of 0.27–0.7% and reproducibility RSDs of 1.4–1.7% (Ezhilan et al. 2017). However, it exhibited a narrow linear range 1–20 nM. The major limitations of such enzyme-based sensors lie in the immobilisation of enzymes, high cost, and limited working conditions such as pH, temperature and humidity (Sha et al. 2017).

Potentiometric detection of urea in spiked skimmed cow milk samples was achieved by Trivedi et al. (2009) using NH_4^+ ion-sensitive electrodes with immobilised urease enzymes. Urease decomposes urea into various products including NH_4^+ which is detected using electrodes and correlated to the amount of urea present in the milk. This biosensor is reproducible, cost-efficient and disposable, with an LOD of 2.5×10^{-5} mol/L. The

biosensor was found stable for around 30 days. For disadvantages, pH lower than 7 decreases enzyme activity and the maximum working temperature is 313 K. Lastly, Na^+ and K^+ present in samples interfere with the electrode and decrease sensitivity (Trivedi et al. 2009).

Renny et al. (2005) also developed an enzyme-based piezoelectric biosensor that correlates the electrical signal produced from amount of ammonia gas to the amount of urea present in milk. Although this biosensor displayed a rapid response of 3 min in spiked milk, it requires further analyses of other factors as temperature and residual pressure exerted by other gases (Renny et al. 2005).

Lastly, Mishra, Mishra, et al. (2010) designed a thermistor biosensing system with urease enzyme immobilised on controlled pore glass which packed into a column inside thermistor. The specific heat released from selective enzymatic hydrolysis was found proportional to urea concentration in the milk samples. This biosensor displays a rapid response (2 min) with a linear range 1–200 mM permitting detection of low and high urea levels in adulterated milk. Moreover, the immobilised urease column exhibited a good operational stability up to 3 months when used continuously at room temperature (Mishra, Mishra, et al. 2010).

Conclusion

Accidentally present contaminants or intentionally added adulterants in milk pose an obstacle in the milk industry, as they lead to delivering not only unhealthy but potentially seriously hazardous products. Thus, the development and implementation of more suitable assessment methods, to fulfil the regulatory authorities and industry needs has the potential to reduce hazard risk to public health. Biosensors have proven that they present an innovative, rapid, sensitive and cost-effective method to detect different types of milk contaminants *i.e.* antibiotics, hormones, pesticides and mycotoxins in milk as well as the illegally added beta-lactamases and melamine. The simplest biosensors appear to be those that include colorimetric transduction since they are easy to use and portable, albeit lacking sensitivity and accuracy due to potential background interference. Electrochemical sensors, though requiring somewhat complicated instruments, provide accurate qualitative and semi-quantitative preliminary results. The biosensors that are preferred for field or on-site use should be portable, simple and sensitive and accordingly, electrochemical sensors are not preferred for such purpose (Li et al. 2019). One of the recommended biosensors reviewed is the quantum dot "traffic light" assay which is a colorimetric test strip that detects several antibiotics in milk aside from being quantitative, rapid and does not require extensive preparation. Thus, it is suitable for on-site analysis and could be improved by adding more strips to detect more antibiotics type. Another biosensor that is practical for the on-site analysis is the fluorescence quantum dot assay that measures progesterone and high sensitivity and requires no preparation.

Most reviewed biosensors appeared to target the detection of the contaminant itself, i.e. intact metabolites or pesticides, with no emphasis on their degradation or metabolised products in milk which could be equally if not more hazardous as in case of aflatoxins.

There are several general challenges for using biosensors for milk quality control. First, milk samples are generally complex matrices and biosensors are often single-analyte systems. With regard to contaminant types that appeared to be most affected by milk matrix effect in its detection using biosensors, pesticides were the most obvious and in most biosensor types.

The second is the short shelf and operational life of the biosensors. Lastly, there is always the obstacle of translating the research material into actual field application, especially since most of the milk samples used in research have been spiked with contaminants to validate the concept. During the implementation of any new biosensor factors should be taken into consideration including the simultaneous detection of the contaminants, their metabolites and/or their degradation products notably if the latter are pharmacologically active.

Each biosensor possesses certain advantages and limitations raising the question about the effectiveness and real practical application on a large scale of this biosensor technology. Which, in turn, should exhibit high selectivity, sensitivity and repeatability. Alongside, it should offer a cost-effective and a fast analysis tool for effective routine milk analysis.

Therefore, future efforts should be made on exploiting more tolerant and environmentally bio-recognition elements alongside a best transduction method. It would be of value to combine nanotechnology with biotechnology assessment tools in a sort of bimodal detection system to develop a nanosensor that could provide a new insight due to its small size and different capabilities. Few biosensors utilise nanotechnology and as such, it is an area to be potentially pursued for in the future. Due to the relative novelty of both the biosensing technology and nanotechnology, these fields are dynamic and could be combined in a way that enables the detection and quantification of contaminants in milk. The development of biosensors for ensuring milk quality appears to be rewarding not only for the milk industries but also for several other downstream products in which milk serves as the key ingredient, such as yoghurt, cheese, cream and fermented milk and development of detection methods for these contaminants in such more complex matrices should now follow.

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Declaration of interest

The authors declare no conflicts of interest and they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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