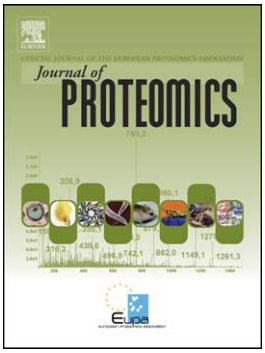

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Foodborne pathogens and their toxins

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Abstract

Foodborne pathogens, mostly bacteria and fungi, but also some viruses, prions and protozoa, contaminate food during production and processing, but also during storage and transport before consuming. During their growth these microorganisms can secrete different components, including toxins, into the extracellular environment. Other harmful substances can be also liberated and can contaminate food after disintegration of food pathogens. Some bacterial and fungal toxins can be resistant to inactivation, and can survive harsh treatment during food processing. Many of these molecules are involved in cellular processes and can indicate different mechanisms of pathogenesis of foodborne organisms. More knowledge about food contaminants can also help understand their inactivation. In the present review the use of proteomics, peptidomics and metabolomics, in addition to other foodomic methods for detection of foodborne pathogenic fungi and bacteria, is overviewed. Furthermore, it is discussed how these techniques can be used for discovering biomarkers for pathogenicity of foodborne pathogens, determining the mechanisms by which they act, and studying their resistance upon inactivation in food of animal and plant origin.

Key words:

Food borne pathogens, bacteria and bacterial toxins, fungi and mycotoxins, proteomics, foodomics

1. Introduction

Foodborne diseases are caused by consumption of food spoiled by pathogens or their toxins. These diseases are easily spread, and consequently, they are a worldwide public health problem. In 2013, there was a total of 5,196 food borne outbreaks reported in the European Union, resulting in 43,183 infected humans, 5,946 hospitalizations and 11 deaths [1]. In the United States, an estimated 9.4 million episodes of foodborne illness occur each year, along with 55,961 hospitalizations and 1,351 deaths [2]. There is a significant increase in the occurrence of foodborne illnesses due to new nutritional trends that support consuming raw and fresh food, dry products, and exotic ingredients [3]. Next to these trends, globalization of the food market is additionally affecting foodborne disease outbreaks, making food safety a universal issue [4]. This is evidenced by outbreaks of food poisoning caused by a food borne Shiga toxin producing *Escherichia coli* O104:H4 in recent years in Germany and France [5,6]. A total of 3,816 cases including 54 deaths were reported in the 2011 outbreak in Germany [6]. Therefore, detection of pathogens in food and protection against food spoilage is a task of great social, economic and public health importance.

Bacteria, viruses, fungi, and parasites that contaminate food in different stages of production and delivery and cause foodborne diseases are referred to as foodborne pathogens. Additionally, some bacteria and fungi can produce toxins, and in such cases identification of the pathogen itself is not a sufficient preventive measure for food safety. Many of these pathogens and their toxins are thermostable, and since they cannot be destroyed by typical food preparation methods (cooking, frying, freezing, etc.), food safety control becomes an even more complex issue [7].

Foodomic analytical techniques, especially proteomics, peptidomics and metabolomics, are indispensable for the monitoring of food, whether during its production, storage, or transportation, and a successful identification of foodborne pathogens and their toxins in food samples can prevent a disease outbreak. In this paper, most important bacteria and fungi falling under foodborne pathogens, as well as bacterial and fungal toxins, will be reviewed, with a particular focus on recent advances in proteomic and other foodomic methods for their detection.

2. Bacteria as food pathogens and their toxins

2.1 MALDI TOF mass spectrometry for bacterial characterization

Phenotypic tests such as colony characteristics, growth on selective agar plates, biochemical pattern characterization, and Gram staining are the methods currently used for identification of bacteria. However, these are time consuming and less practical when a fast analysis is needed. Known for its sensitivity, accuracy, and reproducibility, MALDI TOF MS is a method most commonly used for bacterial identification [8]. This approach is very rapid since it does not involve a sample preparation step, but rather relies upon the introduction of a bacterial colony onto a MALDI plate. The result is a unique intact, or trypsin digested ribosomal or intracellular protein and peptide profile of whole bacterial cells, bacterial "fingerprint", which allows an accurate identification of contaminating bacteria. Acquired bacterial MALDI TOF MS fingerprints are matched against spectral libraries previously collected under identical MALDI conditions without further identification. Consequently, the success of identification remains highly dependent on the number of well characterized food pathogen biomarker sequences in reference databases that are in the majority of cases still not publicly available [9].

An example of a successful use of MALDI TOF MS for the discrimination between bacterial subtypes is the detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP). MAP is a pathogenic bacterium that affects ruminants, such as cattle, and causes paratuberculosis (PTB). This microorganism is heat resistant, and its presence in milk is a major cause of MAP transmission to humans [10]. The most common diagnostic method used to detect MAP in cattle is Enzyme Linked Immunosorbent Assay (ELISA). However, the use of this technique is limited, and it cannot be used for detection of early infections. The reason is relatively low sensitivity. Furthermore, cross reaction with environmental mycobacteria is known to occur [11]. Thus, more sensitive proteomic approaches have been developed for MAP detection, such as 2D gel electrophoresis coupled with MS [12]. Lin *et al.* [13] have shown that MALDI TOF MS efficiently discriminates *M. avium* from other *Mycobacterium* species. These results demonstrate a substantial potential of this

method for clinical application and early detection of MAP. Furthermore, this method could be applied for fast and reliable detection of toxin producing bacteria or bacterial strains.

The major bottleneck for further implementation of MALDI TOF MS in food monitoring systems remains isolation of microorganisms, their culturing by conventional methods, and/or enrichment of anaerobic, demanding or slow growing bacterial strains [14]. Therefore, sample preparation protocols still have to be developed for viable but non culturable (VBNC) or difficult to culture food poisoning bacterial strains such as *Vibrio cholerae,* enterohemorrhagic *E. coli, Shigella flexneri,* and *Salmonella enterica* [15]. It is important to note that not all bacteria pose a health threat themselves, but proteins that they secrete can be severely toxic to humans. However, a disadvantage of MALDI TOF MS is that this technique can only detect the presence or absence of food contaminating bacteria, and it does not give any data about the expression of toxin encoding genes or toxin amounts in food [14].

2.2 Application of proteomics for the detection of bacterial toxins

Bacterial toxins can be divided into endotoxins and exotoxins. Endotoxins are lipopolysaccharides (LPS), located in the outer membrane of Gram negative bacteria. These agents are seldom secreted during bacterial growth, but can be released after lysis of bacteria resulting from either autolysis or external lysis*, e.g.* as a result of the effects of antibiotics or phagocytic digestion by the host's immune system. Thus, endotoxins most often act close to bacterial growth. They are moderately toxic and heat stable [3]. Exotoxins, on the other hand, are proteins secreted by both Gram positive and Gram negative bacteria. Compared to endotoxins, they are more potent and more specific because they act enzymatically. Since they are mostly secreted, they act at a site that can be distant from the original place of bacterial growth. Some exotoxins are released only upon bacterial lysis [7]. About 16% of all foodborne outbreaks in 2013 in the EU were caused by bacterial exotoxins produced mainly by *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens,* and *Staphylococcus aureus* [2].

2.2.1 Gel free proteomic methods for bacterial toxin detection

Staphylococcal enterotoxins (SE) are gastrointestinal protease tolerant exotoxins produced by *S. aureus*. Although there are 23 defined serotypes, staphylococcal enterotoxin A (SEA) causes 80%, while staphylococcal enterotoxin B (SEB) is responsible for an additional 10% of staphylococcal foodborne illnesses [16]. The main reason for SE contamination in food is poor hygiene during food production [17]. Staphylococcal enterotoxins have a remarkable ability to resist heat, acidity, and pH changes, which makes them persist in food substrates during processing. For the detection of SE, MALDI TOF MS is used [18,19].

Although MALDI TOF is a powerful mass spectrometric technique, most analyses of staphylococcal enterotoxins are still based on immunoaffinity assays [20,21,22]. However, these assays require a specific antibody against a certain enterotoxin, and so far there are only a few available: staphylococcal enterotoxin A to E, G, H and staphylococcal enterotoxin like Q [23]. Development of antibodies against enterotoxins is difficult and expensive, and unspecific cross reactivity between the antibody and molecules with similar properties as enterotoxins is quite common. As a result, antibody free methods are becoming more popular. A label free bottom up proteomic approach for the detection of SEA and SEB in milk and shrimp was reported. After tryptic digestion, ^{13}C-labeled internal standard peptides were used for isotope dilution LC ESI MS/MS using Multiple Reaction Monitoring (MRM) on a triple quadrupole mass spectrometer [23]. The toxins were identified with detection levels of 2.5 and 5 ng/g levels of SEA and SEB, respectively. Sospedra *et al.* [24] looked for the same toxins in milk and fruit juices using single instead of tandem MS on a triple quadrupole, and achieved one order of magnitude lower detection levels. Such differences in detection levels could be explained by variable recoveries due to analyte loss during sample preparation. To overcome this problem, Protein Standard for Absolute Quantification (PSAQ) strategy was recently proposed, which uses an isotope labeled whole protein analogue of the protein target. The labeled protein is added to the matrix, resulting in the same handling of the target and the standard protein, which leads to robust quantification. By use of such assay developed for the quantification of SEA in serum, the toxin was detected by a hybrid quadrupole/linear ion trap (LTQ) mass spectrometer with a detection limit of 352 pg/mL [25]. Dupré *et al.* [26] used the same method on a LTQ Orbitrap to quantify SEB, ricin, and ETX in complex human biofluids and food matrices with lower

limits of detection close to 1 ng·mL^{-1}. Finally, levels of *B. cereus* toxin cereulide in rice and pasta were determined by LC MS/MS MRM with $^{13}C_6$ cereulide toxin as internal standard, making this a superior method to previous ones that used the antibiotic valinomycin as a standard [27].

While most heat stable exoproteins in food cannot be inactivated by thermal processing, heat labile deadly botulinum neurotoxin (BoNT) produced by some strains of *C. botulinum*, a pathogenic and sporogenic Gram positive bacteria, is easily heat denatured [28]. This extremely potent toxin causes botulism, a severe illness with a very low survival rate. Since BoNT has proteolytic activity, toxicity of *C. botulinum* is dependent upon the existence of secreted toxins, and not the bacteria itself [28]. The quantitative, well established and widely accepted assay for BoNT detection is the *in vivo* mouse bioassay, in which mice are injected with toxin dilutions and toxicity levels are observed [29]. Although this bioassay has been the gold standard for BoNT detection for years, the large experimental error, high costs and ethical concerns have pushed researchers into developing alternative *in vitro* methods for BoNT identification. Immunological detection methods such as ELISA, although more sensitive than the mouse bioassay, have the limitation of false positives due to cross reactions as well as detection of both active and inactive toxins [8]. Endopeptidase activity assays based on BoNT's intrinsic enzymatic function have been established. Mass spectrometer was coupled to the reaction chamber in order to enable rapid detection of substrate cleavage location. Kalb *et al.* [30] used this approach for differentiation of the serotype of this deadly toxin. Furthermore, MALDI TOF MS and HPLC ESI MS/MS have been directly compared for an endopeptidase activity assay based quantification of anthrax lethal factor, a *Bacillus anthracis* toxin with intrinsic enzymatic activity [31]. The quantitative data resulting from both platforms were very similar, but isotope dilution MALDI TOF MS turned out to be a faster and more robust and precise quantitative MS technique. Wang *et al.* [32] developed novel peptide substrates for an Endopep MS approach to detect all seven BoNT serotypes, opting for MALDI TOF MS because of the short sample analysis times and high throughput capability. The same group also demonstrated the use of this method for quantitative detection of four BoNT serotypes [33].

Bacillus subtilis is a sporogenic, Gram positive bacterium that contaminates flour, bread and yeast, and survives baking temperatures. Germinated spores can cause ropy bread spoilage.

A proteomic investigation of this microorganism and the change of its proteome under inhibition of growth was recently performed by quantitative label free LC MS/MS and comprehensive bioinformatic analysis [34]. Using this approach, several differently expressed proteins were detected not only in *B. subtilis* but also in a couple of other investigated bacteria. Majority of those proteins were enzymes and co factors involved in protein synthesis and energy metabolism as well as chaperones including heat shock proteins [34]. Furthermore, multipronged quantitative proteomics approach was used to investigate changes in *B. subtilis* after treatment with antimicrobial agent totarol. A total of 139 proteins were found to be differently expressed using 2 DE, DIGE, and iTRAQ analyses. The study revealed significant down regulation in expression of several central metabolic primary dehydrogenases (*e.g.* glyceraldehyde 3 phosphate dehydrogenase and succinate dehydrogenase). Among up regulated proteins were those involved in anaerobic respiration (*e.g.* nitrate reductase and lactate dehydrogenase), heme biosynthesis, and cell homeostasis [35].

Clostridium difficile, a Gram positive spore forming bacterium, infects the human colon causing diarrheal infections [36]. In order to identify novel extracellular factors of *C. difficile*, proteomic techniques were used. The secretomes of the three *C. difficile* strains CDR20291, CD196, and CD630 were examined and compared [37]. LC MS analysis yielded in identification of 158 different proteins in the supernatant, most of which originate in the cytoplasm. Ternan *et al.* [36] used LC/MS based on exponentially modified Protein Abundance Andex (emPAI) spectral counting method to determine proteomic changes in response to heat stress. Sixty five proteins (37%) were modulated by 1.5 fold or more when this bacterium was grown at 41°C compared to the growth at 37°C. A 2.7 fold decrease in the flagellar filament protein (FliC) implied reduced motility at 41°C. Moreover, two groups independently reported on the identification of two novel zinc metalloproteases implicated in *C. difficile* toxicity by LC MS/MS [38,39]. Secretome of another highly pathogenic *Clostridium* species, *C. perfringens*, has been largely studied [40].

Bacteria from genus *Salmonella*, comprising two species *S. enteritica* and *S. bongori*, are a major cause of foodborne illness throughout the world [41]. Serotypes of *S. enteritica* are facultative intracellular pathogens, replicates within host cells in a membrane bound

compartment, the Salmonella containing vacuoles (SCVs). Typhoid toxin is secreted by *S. typhi* and other non typhoidal serovars (*e.g.* Javiana). This cytolethal distending toxin (CDT) in *Salmonella* consists of CdtB (catalytic DNase I like) subunit and two subunits PltA (poses ADP ribosylating activity) and PltB, which are necessary for excretion of CdtB [42]. The finding that typhoid toxin binds to and is toxic toward cells expressing glycans terminated in N acetylneuraminic acid provides insight into the molecular base for *S. typhi* host specificity [43]. *Salmonella spp.* uses two distinct type III secretion systems (T3SS), a syringe like macromolecular assembly that functions to inject effector proteins (exotoxins) into the host cell. SPI1 delivers effectors required for internalization by intestinal epithelial cells while SPI2 T3SS promotes intracellular replication and survival, eventually leading to a systemic infection [42]. Rogers *et al.* [44] presented phosphoproteomic study of phosphoinositide phosphatase (SopB), one of SPI1 effectors, on phosphorylation events important in the initial stages of infection. Tahoun *et al.* [45] demonstrated that SopB induces transformation of enterocytes promoting host colonization and invasion. Impact of SPI2 effectors onto host phosphoproteome and proteome was analyzed by Imami *et al.* [46], who showed that SPI2 effectors differentially modulate the host phosphoproteome and cellular processes (*e.g.* protein trafficking, cytoskeletal regulation, and immune signaling) in a host cell dependent manner. Host targets of SPI2 T3SS secreted SteC Ser/Thr protein kinases were identified and a unique way of actin network rearrangement enhancing *Salmonella* replication in SCV was demonstrated. Phosphoproteomic analysis were performed with phosphopeptide enrichment on a TiO_2 column and LC fractionation followed by nano HPLC LTQ Orbitrap MS. Additionally, *Salmonella* interactome was reviewed by Schleker *et al.* [47].

Some *Escherichia coli* strains, especially the Shiga toxin producing *E. coli* O104:H4, can cause severe food poisoning with sometime fatal ends [6]. On the other hand, other *E. coli* strains have been frequently used as industrial and model microorganisms, and their proteomes have been subjects of numerous investigations. Some of them are the analyses of protein changes of both bacteria under different growth conditions and for studying of the effect of different anti microbial agents [5,6,35,68]. Detection of the enteropathogen *E. coli* O104:H4 and characterization of the Shiga-toxin by use of genomic, proteomic and immunochemical methods were extensively studied [5,6]. Characterization of *E. coli* heat labile enterotoxin

was achieved by the use of two different mass spectrometric methods, namely LC/ESI MS and MALDI TOF MS, as shown in Figure 1.

The Gram positive bacteria *Listeria monocytogenes* and the Gram negative one *Yersinia enterocolitica* came in relatively late into focus as food pathogens [48,49]. *Listeria monocytogenes* can survive a broad range of temperatures, and contamination with this microorganism is of primary concern in processed food products. *Listeria* exotoxin Hemolysin Listeriolysin O was the first focus of investigation in connection to growth and virulence of this food pathogen [48,50], but analyses of proteome and its changes under the influence of biologically active peptides and synthetic inhibitors of bacterial growth by use of LC MS/MS [51] and quantitative label free LC MS/MS were recently published [35].

Gram negative enterobacterium *Y. enterocolitica* is frequently involved in human enterocolitis. Outbreaks of *Yersinia* infections are mostly associated with consumption of raw fruits and vegetables since this bacterium and its enterotoxin Yst are capable of surviving under refrigeration temperatures [49,52]. For investigation of this bacterium, its growth and toxin production, genomic and proteomic investigations were performed [53]. Different strains of *Campylobacter spp.* are also pathogen, the most prominent food pathogenic strain being C. *jejuni*, a typical bacterial cause of acute infective diarrhea in humans in many developed countries causing more food borne illnesses than Salmonella spp. [54]. *Campylobacter jejuni* is unique among bacterial species in that it possesses both N-linked glycosylation and O-linked glycosylation systems [55], while only one of these pathways can be present in some other bacterial species [56]. Glycoproteins of *C. jejuni* may contain bacteria specific monosaccharides (e.g. pseudaminic acid and diacetamidobacillosamine) [57]. These facts are important for specific detection of this bacterium in food. Current proteomic and glycomic studies are aimed at understanding the mechanisms of *C. jejuni* biogenesis and pathogenicity. Elmi *et al.* [58] analyzed the role of outer membrane vesicles (OMV) secreted by these bacteria in interaction with host cells and delivery of virulence factors. Using nano HPLC ESI 3D IT MS, more than 150 proteins were identified in OMV [58].

2.2.2 Gel based proteomic methods for bacterial toxin detection

Prior to sample analysis, separation of proteins is usually done by liquid chromatography, but gel based techniques are also used. After protein separation by SDS PAGE, subsequent band excision, and protein extraction followed by tryptic digestion, SEA was determined. When the SEA extracted from milk was analyzed, thirteen peptides were revealed with 58% sequence coverage, while nineteen peptides were identified as products of trypsin cleavage of the SEA standard with 73% coverage of the protein sequence [59].

Bacterial secretome analysis allows for the clarification of the role of secreted, membrane, and cell wall proteins in pathogenicity. Different strategies are employed to prefractionate the whole proteome in order to enrich the low abundance proteins, which are often not detected during the initial analysis [60]. In one such study, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and strong cation exchange fractionation, both combined with tandem mass spectrometry, were used to analyze the secretomes of a methicillin resistant *Staphylococcus aureus* strain (MRSA). A total of 174 distinct proteins were identified, with the expression of some proteins such as enterotoxins U and B being identified for the first time in this strain [61]. The same group combined SDS PAGE with LC MS/MS to examine the proteomes of MRSA and methicillin susceptible *S. aureus* (MSSA). They identified 261 extracellular proteins in MRSA and 168 in MSSA. Out of 144 proteins unique to MRSA, some are suggested to be the most probable virulence factors of this strain [62]. Quiblier *et al.* [63] provided new insights on the relevance of SecDF, an accessory protein in the Sec secretion pathway, in *S. aureus* pathogenicity. Using LC-MS/MS, these authors showed that deletion of *secDF* leads to an altered exoproteome, resulting in reduced adhesion, invasion, and cytotoxicity of this food borne pathogen.

The same protein, SecDF, was studied with respect to the secretion of *Bacillus cereus* toxins. A *secDF* knock out mutant showed slower growth rate, reduced virulence, and motility. It was demonstrated by use of label free mass spectrometry that three most extensively studied diarrheal enterotoxins nonhemolytic enterotoxin (Nhe), hemolysin BL (Hbl), and cytotoxin K (CytK), were less abundant in the secretome of the Δ*secDF* mutant than in the wild type bacteria [64]. Secretome of *B. cereus* was further analyzed using SDS PAGE prefractionation combined with LTQ Orbitrap XL mass spectrometer [61]. In a natural

environment, *B. cereus* is growing in the small intestine where oxygen supply is limited. In the following experiments it was cultured under three redox conditions (low oxireduction potenial (ORP) anoxic, high (ORP) anoxic, and fully oxic), after which the secretomes were compared. A total of 57 secreted virulence-related proteins were detected, out of which 31 were found for the first time in the secretome of this bacteria [65]. Further analysis of the identified proteins suggested that the redox dependent regulatory pathway may modulate the expression of several virulence factors. The same instrument, LTQ Orbitrap XL, was used for the evaluation of the role of EntD, a *B. cereus* exoprotein, in production of numerous virulence factors. Proteomics were used for in depth characterization of an *entD* knockout mutant and for investigation of the profiles and functions of cellular and extracellular proteins controlled by EntD [66]. Furthermore, exoproteome analysis of a novel strain of *B. cereus* using a 2DE MS approach demonstrated that this strain is implicated in a disease similar to cutaneous anthrax [67].

Comparative proteomic analysis of extracellular proteins of *C. perfringens* type A and type C strains using a 2 D gel electrophoresis coupled with MALDI TOF TOF revealed proteins that have not been reported in the exoproteome of any other Gram positive bacterium [68]. SagA protein, DnaK type molecular chaperone hsp70, and endo beta *N* acetylglucosaminidase were among the most abundant proteins secreted by *C. perfringens* ATCC 13124. These proteins can be used as markers for *C. perfringens* detection in food samples, since they are conserved among *C. perfringens* strains and they share low homology (<50%) with the nearest similar proteins.

Differences in proteome of *Y. enterocolitica* were investigated by 2D electrophoresis followed by MALDI TOF identification of separated proteins and by quantitative LC MS/MS of different bacterial lysates. Use of this method resulted in identification of more than 1000 bacterial proteins and detection of up and down regulated proteins during bacterial growth in the presence of inhibitors [34].

3. Fungi as food pathogens and mycotoxins

Among the eukaryotic food pathogens with the highest toxicity, next to toxin producing microalgae, are mycotoxin producing fungi. Although toxins produced by algae may be stronger in their effects, food poisoning caused by these agents is relatively rare [69]. Together with bacteria, food pathogenic fungi are the most frequent cause of food spoiling [3]. Mycotoxin producing fungi mostly belonging to *Penicillium, Aspergillus, Fusarium,* and some other strains of filamentous molds [70] tend to infect edible plants and animal feed, meaning that humans can get exposed to these toxic compounds not only through the ingestion of spoiled food, but also through the consumption of dairy products, eggs, etc. Although contamination with mycotoxins is more prevalent in developing countries, where there is a lack of suitable cultivation and processing technologies, food storage is handled poorly, and malnutrition is prevalent, there are noticeable mycotoxin occurrences in the developed countries as well [71]. It should be noted that some mycotoxins can be exploited for their beneficial properties. For example, ergot alkaloids produced by *Claviceps* mold are important substances for the pharmacological industry, where they are used for the production of anti migraine drugs, prolactin inhibitors, and anti Parkinson disease agents [72].

Fungal genome sequencing was a significant boost in proteomic analysis and most of the fungal species broadly recognized as food pathogens have been sequenced. In fungal proteomics, gel based techniques have been used for prefractionation of protein extracts to study hydrophobic proteins, such as membrane proteins [73]. Protein separation on SDS PAGE, followed by LC MS/MS analysis on an Orbitrap MS, was used for the identification of *Aspergillus niger* secretome associated with growth, confirming the previously done *in silico* predictions [74]. Using the same approach, Ferreira de Oliveira *et al.* [75] inspected protein secretion in *A. niger* microsomes upon D xylose induction. Secretome of *Fusarium graminearum* was analyzed using gel electrophoresis followed by Q TOF detection. Out of 87 identified proteins, 63 were predicted to be secretory [76]. A variation of SDS PAGE, termed Blue Native PAGE (BN PAGE), has been used for the identification of *Trichoderma harzianum* secretome [77]. Furthermore, 2D electrophoresis in combination with MALDI TOF proved to be an excellent method for the detection of 121 different *Penicillium chrysogenum* extracellular proteins and their isoforms [78]. The fungal secretome can also

be quantified using isotope assisted quantification methods like Isobaric Tags for Relative and Absolute Quantitation (iTRAQ), as was done in the case of *Aspergillus fumigatus* [79], *Phanerochaete chrysosporium* [80], and *Trichoderma reesei* [81]. An alternative approach to chemical labeling is metabolic labeling, which introduces stable isotopes into the growth medium, with stable isotope labeling by amino acids in cell culture (SILAC) being the most popular strategy [73]. In fungal proteomics, SILAC has been broadly used for studying *Saccharomyces cerevisiae* proteome [82]. However, despite its potential, it is rarely used for examining filamentous fungi [73]. An alternative to chemical and metabolic labeling, label free MS methods, are slowly being introduced in fungal proteome research. Quantitative proteomics, based on emPAI spectral counting method, were used for examining the secretome of *Thermobifida fusca* on different lignocellulosic biomass [83]. Normalized Spectra Abundance Factor (NSAF), another spectral counting method, was used to determine relative protein quantification in *Uromyces appendiculatus* [84].

Prevention of fungal growth is the only certain approach in battling mycotoxin ingestion. It should be noted that the visible absence of mold does not necessarily correlate with the lack of mycotoxin presence, since mycotoxins are resistant to food processing and they can persist in the food long time after their biosynthesis [69]. Thus, research should be focused on the detection of mycotoxins themselves, rather than the fungi producing them. Recent advances in analytical methods have ameliorated mycotoxin detection and quantification in various food matrices (see Table 2.)

The exact functions of mycotoxins are not yet elucidated, but most authors agree that their most plausible function is elimination of competitive microorganisms from their surroundings. Already in low concentrations, these secondary metabolites are toxic to vertebrates and other animal groups. Belonging mainly to *Aspergillus, Penicillium,* and *Fusarium* genera, mycotoxins grow in a wide range of food commodities during production, storage or transportation [71]. Contamination of food products with mycotoxins can cause severe health problems and many of these substances have been classified as cytotoxic, carcinogenic or mutagenic [3]. Despite temperature treatments such as cooking and freezing, mycotoxins enter the food chain since most of them are heat stable. These characteristics make the control of their formation difficult but necessary, since such contaminants pose a serious risk to public health. Although more than 400 different

mycotoxins have been identified, only some of them frequently occur in food products and can cause adverse effects in different organs. Most investigated groups of these low molecular weight food contaminants are aflatoxins, ochratoxins, patulin, and *Fusarium* mycotoxins (fumonisins, zearalenone, trichothecenes, deoxynivalenol) [85].

Because of the harmful effects of mycotoxins, next to the prevention of fungal growth, food decontamination and detoxification strategies have been developed. Degradation or enzymatic transformation of mycotoxins to less toxic compounds by bacterial transformation in the intestinal tract of animals prior to resorption is a useful method for biological control of several mycotoxins [86]. Additionally, decreasing the bioavailability by use of mycotoxin binding agents in the gastrointestinal tract is a worthy technique for reducing the uptake as well as exposure to these toxic substances [86].

3.1 Sample preparation methods for mycotoxin detection

Since food is a complex matrix, optimization of sampling and sample preparation protocols used to detect mycotoxins in food continues to be a high priority among regulatory agencies and commodity industries worldwide [87]. Sample preparation can be a bias of an analytical method since the experimental data's accuracy, reproducibility and certainty depend on the quality of the cleanup technique. This becomes even more critical for analysis of food matrices containing very low concentrations of target substances, such as mycotoxins. The simplest sample preparation method used in mycotoxin detection is the “dilute and shoot” approach, a direct injection of diluted sample into the LC without further cleanup. Many groups have shown that this approach, when followed by LC MS/MS detection, provides a robust method for quantification [88,89]. The most widely used sample pre concentration approach in mycotoxin analysis is solid phase extraction (SPE). As shown in Figure 2, Campone *et al.* used pressurized liquid extraction and online SPE extraction coupled with UHPLC MS/MS for fully automated detection of aflatoxins and Ochratoxin A (OTA) in dried fruits [90]. *Alternaria* toxins alternariol, AME, altenuene, tentoxin, and tenuazonic acid (TeA) in wheat, tomato juice and sunflower seeds were detected using SPE on polymeric based columns, and toxins were separated and quantified by LC MS/MS using matrix matched standard calibration [91]. The recovery limits for target substance ranged between 71 and 113%, with quantification limits reaching between 1 and 10 µg/kg. The drawback of this

method is that it is time consuming, requires large amounts of organic solvents, and it is expensive [92]. Furthermore, absorption loss is often noted and the sample solid phase interaction can be affected by *e.g.* pH and solvent type. Solid phase microextraction (SPME) is a solvent minimized SPE technique in which only a few milligrams of sorbent are applied. This technique has been used for the determination of OTA in wheat and maize grains [93]. Saito *et al.* used the same sample preparation method coupled online with LC atmospheric pressure (AP) ESI MS to determine OTA and Ochratoxin B (OTB) in nuts and grain samples [94].

Recently, two alternative extraction methods have been developed: dispersive liquid liquid micro extraction (DLLME) and QuEChERS (quick, easy, cheap, effective, rugged, and safe), both being known for their applicability in a multi mycotoxin analysis. DLLME was developed as an upgrade to liquid liquid extraction (LLE) (95). A big disadvantage of LLE is that this method is time consuming and that it requires relatively large volumes of organic solvents. The optimized DLLME method proved to be simple, quick and efficient, and the amounts of solvents necessary for extraction are much lower. Emidio *et al.* [96] used DLLMe coupled to LC MS/MS to determine six estrogenic mycotoxins in water samples using bromosolvents for extraction, instead of the typically used chloroform, chlorobenzene and other highly toxic and environmentally unfriendly agents. Wang *et al.* [97] used a similar approach for the detection of *Fusarium* mycotoxin zearalenone (ZEN) in maize products. Furthermore, a DLLME procedure for the determination of Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), and OTA in rice was proposed [98]. The other newly introduced extraction procedure, QuEChERS, is a simple sample preparation process consisting of three steps: extraction of the sample by adding a solvent, partitioning of water with salts, and cleanup using dispersive SPE. Although initially introduced for cleaning up extracts in pesticide analysis [99], it has been used successfully for simultaneous detection of multiple mycotoxins [100,101,102,103]. Coupled with an LC MS/MS detection method, the use of QuEChERS extraction resulted in detection of ZEN, deoxynivalenol (DON), AFB1 and their metabolites in eggs [104], while Pizzutti *et al.* [105] identified 36 mycotoxins in wines using Waters Quattro Premier XE tandem mass spectrometer in Multiple Reaction Monitoring (MRM) mode.

3.2 Immunochemical methods for mycotoxin detection

There are two still most frequently used types of analytical methods for the detection of mycotoxins: immunochemical methods and chromatographic methods. The most commonly used immunological methods are Surface Plasmon Resonance (SPR) and ELISA. They both depend on the specificity of the antibodies directed against mycotoxins. Before an SPR biosensor can be used for analyte detection, a capturing agent needs to be immobilized on the surface. Mycotoxin DON was detected with high selectivity by applying the molecular imprinting of a polymer onto a SPR chip [106]. The same method was used for determination of citrinin in red yeast rice [107]. Next to imprinting polymers, aptamers, single stranded DNA or RNA oligonucleotides, can be bound to the surface of the SPR chip [108]. Zhu *et al.* [109] used an OTA targeting aptamer, cross linked via biotin to streptavidin that was immobilized on the sensor chip surface. This highly reproducible method with low detection limits was used for OTA quantification in wine and peanut oil [109]. Presented papers demonstrate that SPR is a method that can be further developed for fast, high throughput routine determination of mycotoxins in different classes of food.

Several groups generated monoclonal antibodies and performed an indirect competitive ELISA (IC ELISA). This method was used for the screening of maize silage for paxilline [110], detection of ustilotoxin B in rice false smut balls and rice grains [111], or for quantitative determination of OTA in corn and feed samples [112]. Equally as HPLC MS, IC ELISA can be employed as a rapid and accurate screening of mycotoxins in food samples. Quite a few new immunochemical detection methods have been proposed recently, such as immunoaffinity columns coupled with a fluorometer for mycotoxin determination in meat products [113], and rapid screening of aflatoxin B1 in beer by fluorescence polarization immunoassay [114]. Although immunochemical methods have been proven to be very successful for detection of a single mycotoxin, they frequently fail when concurrent analyses of multi mycotoxin samples are required. Whether monoclonal antibodies, aptamers or immunoaffinity columns are used, each of these methods depends on a single biological interaction between a mycotoxin and its specific ligand. For complex mycotoxin samples, chromatographic methods are preferred.

3.3 Chromatographic methods for mycotoxin detection

The most widely used chromatographic method for mycotoxin detection and quantification in complex matrices is LC coupled with MS or tandem MS/MS. The existence of *Fusarium* mycotoxins beauvericin (BEA), enniatins (ENNs) (A, A1, B, B1), fusaproliferin, and moniliformin was evaluated by LC ESI MS/MS in 65 rice samples [115]. Beauvericin that was found in 26 out of 65 rice samples was the most prevalent mycotoxin. Enniatin A1 was the only identified member of ENNs, while fusaproliferin and moniliformin were not detected [115]. Gas chromatography (GC) has also been applied for determination of *Fusarium* mycotoxins [116]. Escrivá *et al.* used GC MS/MS to determine seven trichothecenes in laboratory rat feed [117]. Deoxynivalenol was the most prevalent trichothecene followed by 15 acetyldeoxynivalenol, nivalenol and 3 acetyldeoxynivalenol, while neosolaniol, diacetoxyscirpenol and fusarenon X were not identified in any sample. However, LC has replaced GC since mycotoxins are polar compounds, and GC analysis involves an additional derivatization step that is not needed in LC. It should be noted that for the analysis of certain mycotoxins, an alternate method is required. Ochratoxin A, for example, is best determined by LC with fluorescence detection (LC FLD) [93]. Fluorescence detection is also widely used in the detection of ergot alkaloids. Köppen *et al.* [118] developed an HPLC FLD method coupled with SPE and a cleanup procedure based on sodium neutralized strong cation exchange for the detection of 12 ergot alkaloids in rye flour and wheat germ oil. Beaulieu *et al.* [119] used LC FLD to evaluate the diversity and distribution of ergot alkaloids in morning glory (Convolvulaceae) seeds and seedlings, as well as the variation in toxin distribution among species.

Extremely sensitive and precise mass spectrometry ionization techniques, namely MALDI TOF and ESI MS, are mainly used in the field of food safety assessment [87]. With the advance of technology, high resolution mass spectrometers (HRMS), with a resolving power higher than 10 000 and with a mass accuracy of less than 5 ppm, seem to be preferred for mycotoxin detection in food, with Orbitrap standing out as the mass spectrometer of choice [120]. Senyuva *et al.* have written an excellent review on the use of Orbitrab HRMS in food analysis [121]. UHPLC combined with a single stage Orbitrap was successfully used for a quantitative identification of 33 compounds (a mixture of mycotoxins, pesticides and antibiotics) in milk and wheat flour [122], while Lattanzio *et al.* [123] determined T 2 and HT

2 toxins and their glycosylated derivatives in cereals. Tamura *et al.* [124] identified and quantified fumonisins in corn sample, using the same approach. When it comes to quantification, isotope dilution (ID) LC MS seems to be the method of choice for internal standardization, since it relies on signal ratios instead of on signal intensities. This method was used for the detection of OTA in various commercial and homemade fermented soybean paste products, and it yielded in reliable and reproducible results with only 2% standard deviation [125]. The ID LC MS/MS method was optimized for the identification of patulin in apple products, using $^{13}C_7$patulin as the internal standard [126]. Moreover, a stable isotope dilution assay was developed for the determination of tenuazonic acid (TeA) in human urine. The urinary excretion of TeA was monitored in two volunteers that ingested 30 µg TeA and the limits of detection and quantification of TeA in urine were 0.2 and 0.6 µg/l, respectively [127].

4. Conclusions

In addition to other commonly used techniques, the use of foodomics, especially proteomics, peptidomics and metabolomics, will result in a better, faster and more efficient monitoring of food during its production, storage, and transportation. The identification of foodborne pathogens and their toxins will help in fast identification of contaminated raw materials or food samples and prevent a disease outbreak.

Use of high throughput methods for sample preparation and further, targeted development of MALDI TOF MS will promote the use of these technologies for routine analyses of food samples.

Technological advances will also lead to further optimization of Orbitrap and Fourier transform ion cyclotron MS technology, bringing the ultra high resolution mass spectrometry (UHRMS) with a resolving power higher than 100 000 to a wider range of users, and it will further promote the use of LC MS/MS in analytics of toxins and identification of disease biomarkers.

Immunochemical and other methods that are already used for the detection of foodborne bacteria and their toxins, as well as molds and mycotoxins, still have a potential for routine

use, especially in the near future. Miniaturization and introduction of new high throughput technologies will further yield lower limits of detection, reduction of sample and solvent amount, and significantly shorter analysis time.

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Figure and table legends

Figure 1.

Characterization of heat-labile enterotoxin (LTB) of enterotoxigenic *Escherichia coli* by liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS) and MALDI TOF mass spectrometry.

Upper part– LC ESI MS analysis was used to obtain the molecular weight of the LTB protein. The deconvoluted mass spectrum (in the inset) of *E. coli* toxin eluted at retention time of 55 min provided a calculated MW of 12003.64.

Lower part – MALDI TOF spectra of intact LTB protein (black line) and of its form after reduction and carboxymethylation (gray line).

Adapted from Reference [130].

Figure 2.

Fully automated determination of fungal toxins in dried fruits by pressurized liquid extraction and online solid phase extraction cleanup coupled to ultra-high pressure liquid chromatography-tandem mass spectrometry (Adapted from Reference [92], with permission].

Table 1.

A list of most common bacterial toxins and proteomic methods most frequently used for their detection.

Table 2.

A list of most common fungal mycotoxins and proteomic methods most frequently used for their detection.

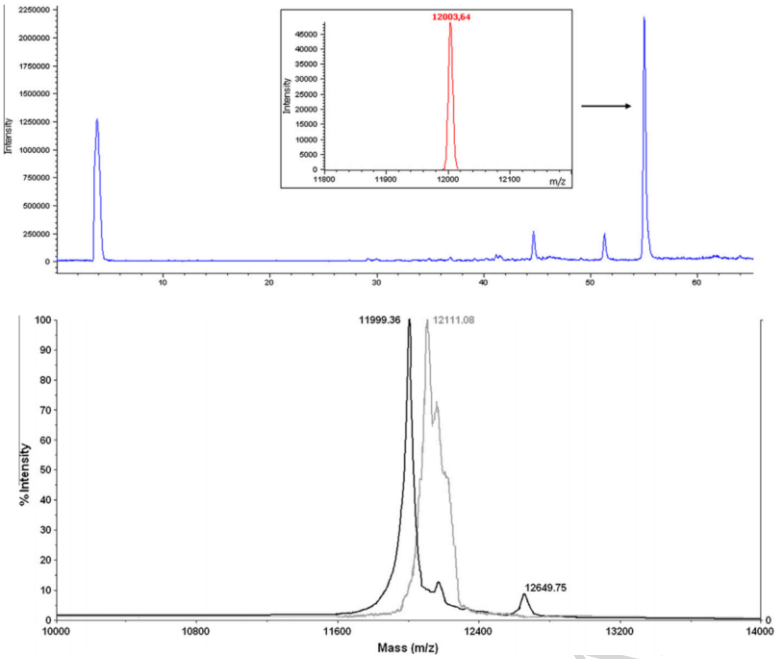


Figure 1

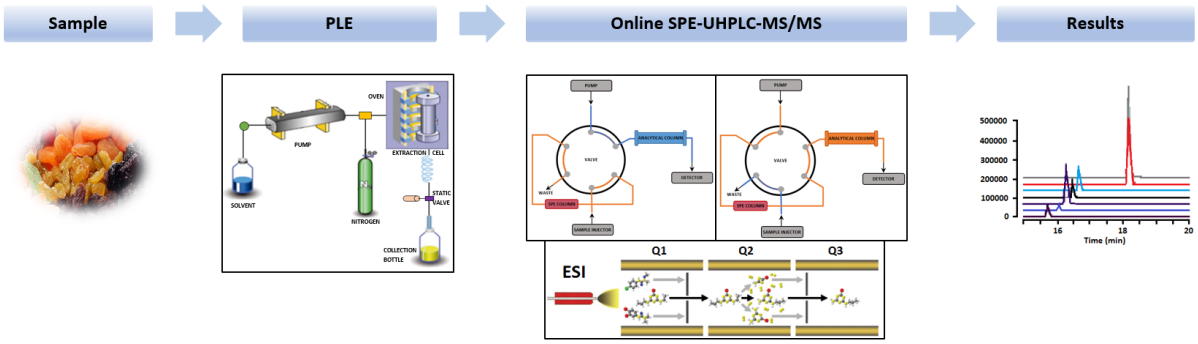


Figure 2

Table 1.

Bacteria	Toxin	Detection method
Staphylococcus aureus	Staphylococcal enterotoxin (SE)	SDS PAGE MALDI TOF MS [59] MALDI TOF/TOF MS [18] LC MS [24] LC MS/MS [23] PSAQ MS [25] PSAQ MS/MS [26] IC ELISA [20] Immuno capture PCR ELISA [21]
Escherichia coli	Shiga toxin (Stx)	MALDI TOF/TOF MS [128]
	Heat labile enterotoxin	MALDI TOF MS [129] LC MS [129]
Clostridium botulinum	Botulinum neurotoxin (BoNT)	Mouse bioassay [29] Endopep MS [30,32,33]
Clostridium perfringens	Epsilon toxin (ETX)	PSAQ MS/MS [26]
Clostridium difficile	*Clostridium difficile* toxin A (TcdA), *Clostridium difficile* toxin B (TcdB)	SDS PAGE LC MS/MS [38,39]
Bacillus anthracis	Anthrax lethal factor	ID MALDI TOF MS [31] LC MS/MS [31]
Bacillus cereus	Cereulide	LC MS/MS [27]
Bacillus subtilis	*B. subtilis* enterotoxin	2DE MALDI TOF/TOF[35] SDS PAGE LC MS/MS [34]
Campylobacter jejuni	Cytolethal distending toxin (CDT)	SDS PAGE LC MS/MS [58]
Listeria monocytogenes	Listeriolysin O	LC MS/MS [51]
Yersinia enterocolitica	*Yersinia stable toxin* (Yst)	MALDI TOF MS [53]
Salmonella sp.	*Salmonella enterotoxin* (Stn)	LC MS/MS [46]

Table 2.

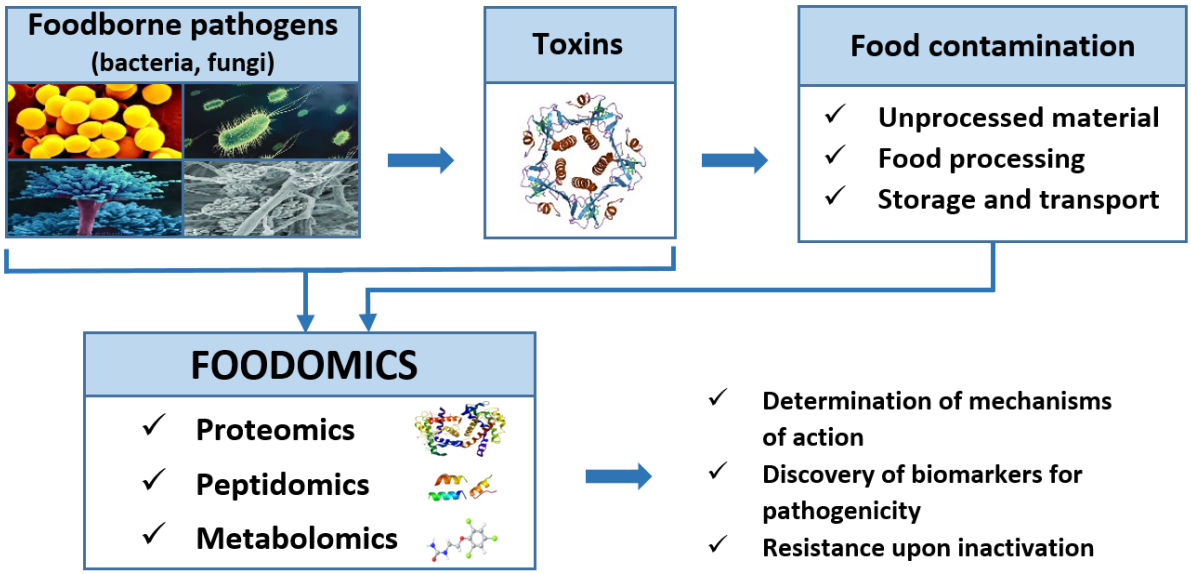
Fungi	Mycotoxin	Detection method	Sample preparation method
Aspergillus	Aflatoxin	LC MS/MS [104] Fluorescence polarization immunoassay [114]	DLLME [98] SPE [90]
	Ochratoxin	LC MS [94] LC MS/MS [90] ID LC MS [125] SPR [109] IC ELISA [112]	SPE [90] SPME [93,94] DLLME [98]
Fusarium	Deoxynivalenol	LC MS/MS [104] SPR [106]	QuEChERS [104]
	Zearalenone	LC MS/MS [104]	"Dilute and shoot" [88] DLLME [97] QuEChERS [104]
	Fumonisins	Orbitrap HRMS [124]	QuEChERS [124]
	Trichothecenes	GC MS [117] Orbitrap HRMS [123]	"Dilute and shoot" [88]
Penicillium	Patulin	ID LC MS/MS [126]	SPE [126]
	Citrinin	SPR [107] LC MS/MS [91]	SPE [91]
Claviceps	Ergot alkaloids	LC FLD [118,119]	SPE [118]

Biological significance

Comprehensive and comparative view into the genome and proteome of foodborne pathogens of bacterial or fungal origin and foodomic, mostly proteomic, peptidomic and metabolomic investigation of their toxin production and their mechanism of action is necessary in order to get further information about their virulence, pathogenicity and survival under stress conditions. Furthermore, these data pave the way for identification of biomarkers to trace sources of contamination with food-borne microorganisms and their endo- and exotoxins in order to ensure food safety and prevent the outbreak of food-borne diseases. Therefore, detection of pathogens and their toxins during production, transport and before consume of food produce, as well as protection against food spoilage is a task of great social, economic and public health importance.

Conflict of interest

The authors have declared no conflict of interest.



Graphical abstract

Highlights

- Use of foodomics techniques for better and more efficient detection of food pathogens and their toxins
- High throughput methods for sample preparation and analysis will promote use of foodomics for routine food analyses
- Technological advances will bring will bring ultra high resolution MS to wider range of users in food analytics
- Already used immunological and chromatographic methods, especially in high throughput mode still have great potential for detection of foodborne microorganisms and thei r toxins
- Identification of biomarkers will result in fast identification of contaminated food