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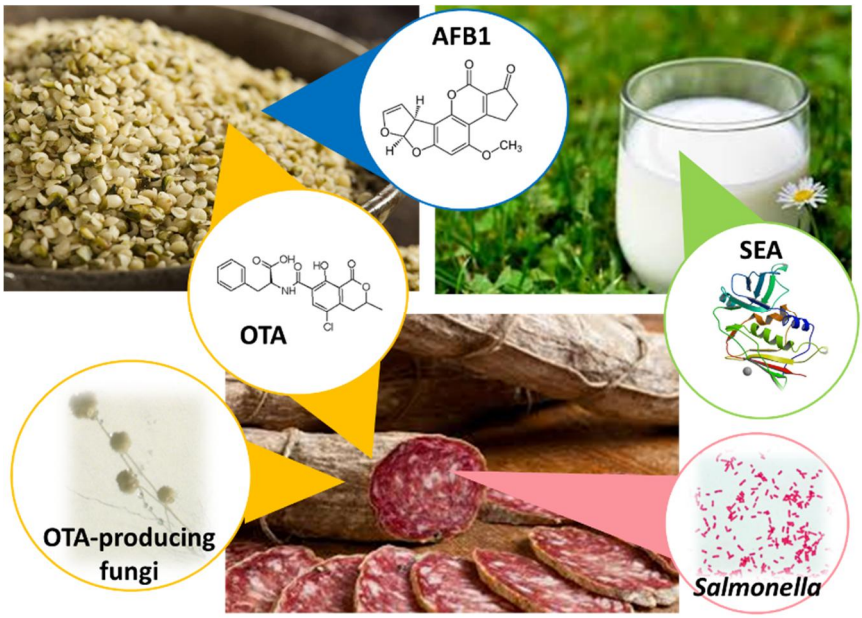
PCR-based methods for the detection of toxigenic bacteria and filamentous fungi and alternative methods for toxins detection in foods



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Supervised by Prof. Marco Arlorio

PhD program co-ordinator Prof. Domenico Osella

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Chapter 1. Introduction

1. Food safety

Food quality and safety are key topics regarding human nutrition and human wellbeing. If the last two centuries were fundamentally dedicated to the improvement of the availability of food and nutrients, starting from the last two decades of the past century a new concern about food quality and food nutritional profiles became to open new scenarios in food science area. The attention towards the presence of food contaminants in foods (both from natural and anthropic origin) is higher today than it was before, and the development of new robust, time saving and rapid methods of analysis represents a priority to guarantee food safety.

1.1. Food safety concept in Europe

The demand of sufficient, safe and nutritious food is now constantly increasing. Incidents regarding pesticide residues in fruit and vegetables, the accumulation of process contaminants like acrylamide, the overall environmental pollution and food-borne illness outbreaks have intensified concerns about healthy nutrition. The changing life styles, including more adventurous diets (e.g. “novel” foods like *microalgae* and insects) and the spread of ready-to-eat foods, the emergence of newly recognized microbial pathogens and the increasing number of immune-compromised patients has contributed to rise the attention regarding this topic. Additionally, the demand for the production and the processing of environmental and animal friendly food, which also conforms to social and labour standards, has increased *(1)*. As a result, safety issues, organic productions, animal welfare and the use of genetically modified organisms (GMOs) have been deeply discussed by the European Commission, resulting into the drawing of an EU *white paper on*

food safety (2) and the foundation of the European Food Safety Agency (EFSA) *(3)*. In the White Paper, the entire food production chain (including animal feed) is held responsible for the safety of food. The Governments of the Member States verify that the producers adequately meet this responsibility to protect the health and well-being of consumers. The document contains 84 action points that have to be processed into community laws to strengthen the food safety systems of the Member States. Within this framework, the EU introduced the General Food Law in 2002, defining general food safety principles and food safety procedures *(4)*.

Consequently, European Commission has also set the maximum level of pathogens, microbial metabolites and chemical substances admitted in each kind of food. Regulation 1169/2011 reports the new requirements for food labelling, as well as the rules for the ingredients declarations (also considering allergens presence) *(5)*, while EU Regulation 1881/2006 reports more properly the maximum tolerated level of contaminants (from biotic and abiotic origin) in foods *(6)*. For the most common toxins, like mycotoxins and marine biotoxins, EU set the sampling methods and the analysis required in the official controls *(6 - 8)*.

1.2 Microbial pathogens in foods

Approximately 30% of all globally emerging infections over the past 60 years include pathogens commonly transmitted through food *(9)*. A pathogen is an organism able to cause cellular damage by establishing in tissues, which results in clinical symptoms *(9)*. Some pathogens regularly caused diseases, while others, named “opportunistic pathogens”, infect primarily immune-compromised individuals.

The presence of pathogenic microorganisms in foods can cause different types of symptoms, principally depending on the viable microorganism or its metabolites ingestions. Infections occur when harmful microorganisms are ingested via food

and they can replicate in the intestinal tract causing illness. Some pathogens, like *Shigella* spp., require the human host as part of their life cycle, while many others have primary reservoirs in other animals or in the environment and the infection of humans is accidental. There are many reasons why foodborne diseases are a global public health challenge. Food represents an important vehicle for pathogens causing acute gastroenteritis, and, as some food-borne infections have been controlled through the application of good manufacturing practices, others emerge as new threats. With the population getting older, the number of immune-compromised or susceptible to severe outcomes from foodborne diseases has increased in many countries. Globalization has promoted the international distribution of foods and the inadvertent pathogens diffusion into new geographical areas, as well as the exposition of travellers and immigrants to unfamiliar foodborne hazards in new environments. Moreover, the diffuse habit of consuming food prepared outside the home, grows the number of people potentially exposed to the risks of poor hygiene in commercial food service settings *(10)*.

All of these new situations led to the evolution of new pathogens, the increasing isolation of highly opportunistic pathogens, the development of antibiotic resistances, and changes in virulence of known pathogens. Rapid, sensitive and reliable methods must be set for the detection of pathogens in food, to improve the management of microbiological risk. Especially in developing countries, outbreaks of foodborne diseases often go unrecognized or unreported. Inexpensive and rapid methods for outbreaks investigation and control need to be improved too. The development of robust screening and confirmation methods is a key requirement today, also considering the developing of "multi-array" based methods of analysis. Significant technical advancements were carried out during the last decades, particularly concerning the development of sensor-like devices, even if the classical microbiological methods as well as the classical approach for toxins determination and quantification are currently largely exploited.

1.3. Toxins in foods

Industrial food processing is responsible for the contamination of food with different chemical agents (e.g. metals, plasticisers, plastic monomers, neo-formed toxic compounds like furan and acrylamide and more), but also some living organisms can naturally produce toxic "chemicals". These toxins from biotic origin are not harmful to the organisms that have produced them, but they may be toxic to other organisms, including humans, when eaten.

A wide variety of microorganisms can grow and develop in food, some of them synthesizing toxins. Toxins from marine origin (often produced by dynoflagellates) are a typical example. These toxins, able to trigger different health problems, ranging from diarrhea to amnesic or paralytic effects, can easily accumulates in fish or shells consumed by humans *(11)*. Moreover, toxins from bacteria and fungi are probably the more common toxins in human diet *(12, 13)*.

The biological mechanisms of toxicity are very different for microbial toxins, ranging from cytotoxicity to teratogenesis and cancerogenesis *(14, 15)*.

Some toxins can be harmful to human health even if ingested in low amounts, while others are only toxic whenaccumulated by the organism *(16)*.

Next to the effect on health, the economic impact of food contaminations is significant when spoiled foods have to be removed from the market *(17)*. The costs of a contamination can affect both the economy and the environment, so the best approach is to avoid these contaminants to enter the food chain. This can be obtained through regulation and monitoring of the possible sources of contamination in the environment. Therefore, rapid and inexpensive screening methods are required to detect even low amounts of contaminants. For their part, the regulators can limit the presence of potentially toxic substances in food to protect the consumer from reasonably foreseeable problems.

Different methods aimed at removing toxins from foods and feeds have been suggested, but this is generally difficult to implement; raw cereals or flours with

high a content of toxins (e.g. mycotoxins) are commonly mixed and diluted with other with low content, in order to decrease the concentration of toxins to respect EU rules *(18, 19)*. Prevention of contamination with toxigenic microorganisms remains the main strategy to contain toxin levels in foods.

Because the removal of some food toxins from foods is unfeasible and other toxic compounds may be created during processing or cooking, the chronic consumption of small quantities of food toxins is unavoidable. Therefore, it is necessary to develop analytical methods for rapid and sensitive detection of the toxins (and primarily of microorganisms able to produce them) as a form of prevention of the risk given by the consumption of the toxins.

1.3.1 Bacterial toxins

Bacterial growth on food is responsible for several transformation processes, affecting both quality (sensorial, texture) and safety aspects. Many GRAS (Generally Recognized As Safe) non-toxigenic bacterial species (mainly belonging to the group of lactic bacteria, like *Lactobacillus* spp.) are useful to pilot food fermentations, particularly lactic acid fermentation *(20)*.

Besides microbial metabolites useful to some processes (e.g. volatile/non volatile molecules produced during fermentation by starter cultures), bacteria can also produce toxins. The ingestion of food containing toxins causes intoxication, while the ingestion of harmful bacteria that produce toxins in the intestinal tract is responsible for a toxin-mediated infection *(21)*. The foodborne bacteria that can cause most common toxin-mediated infections are *Shigella* spp. and Shiga toxin-producing strains belonging to *Escherichia coli* species and *Campilobacter jejuni*, while intoxications are caused mainly by *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum* and *Clostridium perfringens* (Table 1) *(22)*.

Bacterial toxins are classified into two categories: i) exotoxins and ii) endotoxins:

i) exotoxins are proteins primarily secreted by gram-positive bacteria. Temperatures over 60 °C, animal digesting enzymes and stomach acidity denature this type of toxins, leading to the decrease/elimination of their toxicity. Being proteins, they are highly antigenic, consequently they strongly stimulate the immune response. These features make them good candidates for vaccines. Based on the target, there are three types of exotoxins: cytotoxins, neurotoxins, and enterotoxins, classified depending on their mechanism of activity. Cytotoxins kill the host cell or disrupt its normal functions, like erythrotoxins produced by *Streptococcus pyogenes*, which are able to damage red blood cells *(23)*. Neurotoxins, produced mainly by *Clostridium botulinum* and *Vibrio cholerae*, cause paralysis by binding to nerve cells and preventing the release of the acetylcholine at the neuromuscular junction. Enterotoxins, like the cholera toxin or Staphylococcal enterotoxins, bind to the plasma membranes of epithelial cells lining the small intestine and induce the cells to discharge large amounts of fluids and electrolytes. This mechanism generally results in severe diarrhea and vomiting *(24)*.

ii) Endotoxins constitute part of the outer membrane of gram-negative bacteria, released into the bloodstream after the death of bacteria attacked by the immune system. Endotoxins are quite stable, even when heated (after cooking or after industrial process with temperature). The lipid portion inserts into cell membranes of white blood cells and vascular endothelial tissue, leading to fever, chills, weakness, aches, blood clots and shock. The pyrogenic response is primarily attributed to the induction of interleukin-1, which causes the hypothalamus to increase the body temperature. Septic shock occurs when endotoxins induce the secretion of Tumor Necrosis Factor (TNF), which increases blood capillaries permeability. This results in a drop of blood pressure, which causes shock and damages the kidneys, lung, gastrointestinal tract and the blood-brain barrier from which bacteria can enter the bloodstream. Among the bacteria that produce

endotoxins there are *Salmonella enterica* serovar Typhimurium, *Proteus* spp. and *Neisseria meningitidis (25).*

Table 1. Main foodborne illnesses caused by bacteria and their metabolites *(22)*.

Etiology	Signs and symptoms	Associated foods
Bacillus cereus (enterotoxin)	Severe nausea and vomiting.	Improperly refrigerated cooked or fried rice and meat.
Bacillus cereus (diarrheal toxin)	Adbominal cramps, watery diarrhea, nausea.	Meat and stews.
Campilobacter jejuni	Bloody diarrhea, vomiting, fever and cramps.	Undercooked poultry, unpasteurized milk and contaminated water.
Clostridium botulinum (toxin)	Vomiting, diarrhea, blurred vision, dysphagia and descending muscle weakness.	Canned foods with a low acid contents, fermented foods, foods held warm for extended time.
Clostridium perfringens (toxin)	Watery diarrhea, nausea, abdominal cramps.	Meats, poultry, dried or precooked foods.
E. coli EHEC and STEC	Severe diarrhea (often bloody), nausea, abdominal pain and vomiting. Little or no fever.	Undercooked beef, unpasteurized milk, raw fruits and vegetables, salami and contaminated water.
E. coli ETEC	Watery diarrhea, abdominal cramps, some vomiting.	Water or food contaminated by human feces.
Listeria monocyogenes	Fever, muscle aches, nausea and diarrhea. Meningitis in immunocompromised patients.	Fresh sotf cheeses, unpasteurized milk, inadequately pasteurized milk, ready-to-eat foods containing meat.
Salmonella spp.	Diarrhea, fever, abdominal cramps, vomiting. *S.* Typhi and *S.* Paratyphi cause fever, headache, chills and myalgia.	Contaminated eggs, poultry unpasteurized milk or juice, cheese, contaminated raw fruits, vegetables and water supplies.
Shigella spp	Abdominal cramps, fever and diarrhea.	Food and water contaminated with fecal materials.
Staphylococcus aureus (enterotoxin)	Severe nausea and vomiting, abdominal cramps. Diarrhea and fever may be present.	Improperly refrigerated meats, potatoes, eggs, cream pastries.

1.3.2 Mycotoxins

In considering chronic health risks from raw food sources, mycotoxins are recognized as the primary concern. It is widely accepted that filamentous fungi can produce over 300 toxic secondary metabolites characterized by different degree and mechanism of toxicity. Nevertheless, scientific attention is focused mainly on those agriculturally important and that have proven (or suspected) to have effects on human health, like aflatoxins, fumonisins, ochratoxin A, deoxynivalenol and zearalenone.

Mycotoxins are chemical compounds toxic to vertebrates and other animal groups in low concentrations. These metabolites constitute toxigenically and chemically a heterogeneous group, and they are clustered together only because they can cause diseases and death in human beings and other vertebrates. In fact, fungi can also produce other low-molecular-weight toxic metabolites, antibiotics and phytotoxins, but since they are toxic to bacteria and plants respectively, they are not considered mycotoxins. Moreover, also mushrooms (phylum: Basidiomycota) poisons, that can cause diseases and even death in humans and in several animals, are arbitrarily excluded from the mycotoxin group, due to the size of the producing fungus and the exposure through intentional consumption *(26)*.

Human exposure to mycotoxins may result from consumption of contaminated vegetal foods or animal products: it is estimated that one quarter of the world's crops are contaminated to some extent with mycotoxins *(13, 27- 29)*.

Mycotoxins can be synthesized at various stages in the food chain: cereals and other crops can be contaminated both pre-harvest and post-harvest, and in animal-derived food the presence of mycotoxins can originate from the carryover into the tissue of exposed animals *(26, 30)*.

Mycotoxin-producing mold species are extremely common in nature, and they can grow on a wide range of substrates under a wide range of environmental conditions. Mycotoxin synthesis occurs when fungi mainly belonging to genera

Aspergillus, *Fusarium* and *Penicillium* find certain conditions of temperature, nutrients and water activity: these parameters strictly affect the production of the mycotoxins, as secondary metabolites.

High levels of mycotoxins (especially aflatoxins, ochratoxins and fumonisins) in food and feed commodities may have adverse effects on human and animal health, including mycotoxicosis and carcinogenic effects. The most dangerous mycotoxin is aflatoxin B_1 (AFB_1), which was proved to be hepatocarcinogenic and genotoxic. In dairy cattle, aflatoxin M_1 and M_2 (AFM_1 and AFM_2) can be found in milk and milk products obtained from livestock that have ingested feed contaminated by AFB_1 and AFB_2 *(31)*. Aflatoxins M_1 and M_2 represent the oxidrilated form of native toxins, depending on the animal metabolism.

Ochratoxin A (OTA), synthesized by several species of *Aspergillus* and *Penicillium* fungi in a huge variety of foods, including cereals, coffee, cocoa, wine (namely passito-like wines, produced with dried grapes), dried fruits, as well as in animal-derived foods, has nephrotoxic and nephrocarcinogenic effects.

Among the toxins produced by *Fusarium* spp., zearalenone, fumonisins, trichothecenes T-2/HT-2 toxin and deoxynivalenol are the most toxic and prevalent in foods and food ingredients. Zearalenone is a field contaminant implicated in numerous mycotoxicoses of farm animals, especially pigs, having estrogenic activity. Recently, zearalenone has been suspected to stimulate the growth of human breast cancer cells *(32)*. Fumonisins are also cancer-promoting metabolites, of which Fumonisin B1 is the most important. Fumonisin B1, often recovered in corn and other grains, is hepatotoxic and nephrotoxic. Trichothecenes, a family of 200-300 related cyclic sesquiterpenoids, are recognized as gastrointestinal toxins, dermatotoxins, hematotoxins and immunotoxins. T-2 and HT-2 toxin are the most toxic mycotoxins among the trichothecene group. Deoxynivalenol, although less toxic, is important because it frequently occurs at levels high enough to cause adverse effects.

2. *Salmonella* as a major pathogen in food

Major outbreaks involving *E. coli* and *Salmonella* have highlighted problems with food safety and increased public anxiety that modern farming systems, food processing and marketing may not provide adequate safeguards for public health. The ecology of food poisoning organisms and the environment in which they may grow and survive has been extensively studied, but the ability to control the diffusion of some of these organisms still needs to be improved. This may be due in part to modified production practices, lack of control of hazards at the farm level, industry difficulties in controlling hazards during production, the trend towards minimal processing of foodstuffs and longer shelf-life for many foodstuffs.

In Europe, the most commonly reported foodborne pathogens are *Salmonella* spp., *Listeria monocytogenes* and *Campylobacter jejuni (33). Salmonella* is the second cause of food poisoning and its incidence has slightly increased in the last seven years, with 88,715 confirmed cases in 2014. Among the *Salmonella* serovars, the two most commonly reported in 2014 were *Salmonella* ser. Enteritidis and *Salmonella* ser. Typhimurium, representing 44.4% and 17.4%, respectively, of all reported serovars in confirmed human cases *(33).* In particular, the DT104 phago-type *Salmonella* ser. Typhimurium is widely distributed in cattle herds and it carries several chromosomally located genes conferring the ACSSuT resistance type (resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline). The incidence of multidrug resistant strains is increasing, and more than one third of people infected by this organism requires hospitalization *(34).* Antimicrobial resistance increases the morbidity, mortality, length of hospitalization and healthcare costs. The unfeasibility of reversing antimicrobial resistance back towards susceptibility and the critical need to treat bacterial infections in modern medicine have forced

researchers and pharmaceutical companies to develop new effective antimicrobials against difficult-to-treat multidrug-resistant pathogens *(35)*. Last available data about drug-resistant bacteria in EU showed isolates of *Salmonella* and *E. coli*, especially from poultry, resistant to ampicillin, (fluoro)quinolones, tetracyclines and sulphonamides, whereas resistance to third-generation cephalosporins and colistin was uncommon *(36)*. High proportions of human *Salmonella* isolates were resistant to tetracyclines (30.3%), sulfonamides (28.6%) and ampicillin (28.2%). Multi-drug resistance (MDR) was high overall (26.0%) in the EU, and some of the investigated serovars exhibited very high MDR, such as *S. enterica* serovar Kentucky (74.6%), monophasic variant of *S. enterica* serovar Typhimurium 1,4,[5],12:i:- (69.4%) and *S. enterica* serovar Infantis (61.9%) *(36)*. However, more than half (54.8%) of all isolates from humans were susceptible to the complete range of antimicrobial classes tested. The proportions of *Salmonella* isolates resistant to the clinically important antimicrobials ciprofloxacin and cefotaxime was overall relatively low (8.8% resistant to ciprofloxacin and 1.1% to cefotaxime). Other multidrug resistant bacteria, like Methicillin-Resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Mycobacterium tuberculosis* and extended-spectrum beta-lactamase (ESBLs)-producing bacteria have become a major global healthcare problem in the 21st century.

2.1 Biology of the genus *Salmonella*

Bacteria belonging the genus *Salmonella* are Gram-negative, non-spore forming and predominantly motile bacilli belonging to the family *Enterobacteriaceae*. *Salmonella* are facultative anaerobic microorganisms, negative to oxidase test and positive to catalase test. The majority of *Salmonella* species do not ferment lactose but they can grow on citrate as a sole carbon source, decarboxylate lysine, hydrolyse urea and produce hydrogen sulphite (Fig. 1) *(37, 38)*.

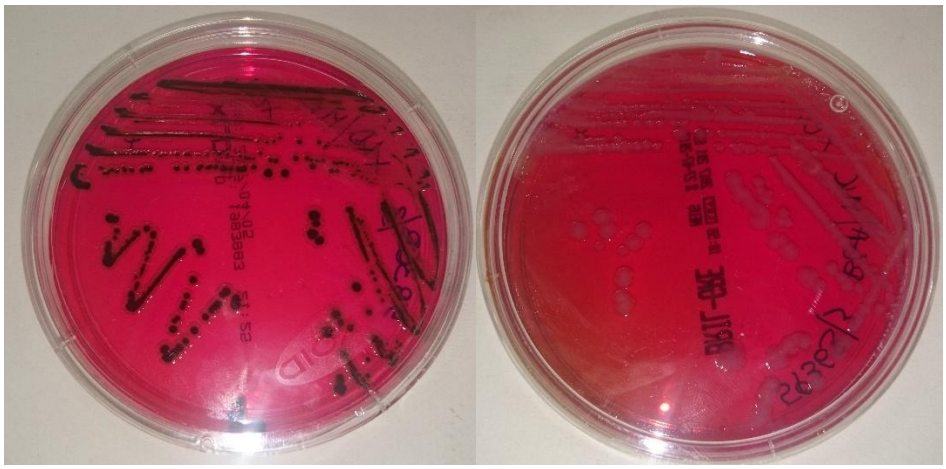

Figure 1. On the left: *Salmonella* grown on Xylose Lysine Deoxycholate agar at 37 °C for 24 hours. Typical colonies are red with black center, due to the fermentation of xylose and the decarboxylation of lysine, which cause the alkalinization of the medium allowing the formation of the black center by hydrogen sulfide forming organisms. On the right: *Salmonella* grown on Brilliant Green agar at 37 °C for 24 hours. Typical colonies are pink and opaque surrounded by brilliant red zones in the agar.

Salmonella are facultative intracellular bacteria, and their ability to survive in different host cells is crucial to exert his pathogenic action. Thanks to the adaptive acid tolerance response (ATR), which is characterized by the induction of several proteins upon exposure to mildly acidic conditions, *Salmonella* can survive to the acid environment of the stomach. In the gut, *Salmonella* adheres to enterocytes, M cells and dendritic cells of intestinal epithelium through fimbriae, and starts the invasion of host cells. The invasion activates different metabolic pathways in the host cells, which lead to the release of arachidonic acid, prostaglandins and leukotrienes, the induction of phospholipase A2 and the increase of intracellular Ca^{2+}. These modifications influence electrolytes transport and cause diarrhea. When the bacteria arrive to lymphatic vessels, they can be phagocytized by resident macrophages, disseminate through the blood stream and cause bacteremia. Microorganisms included in the phagosomes of the macrophages can

survive thanks to virulence factors encoded by SPI-2, that allow resisting to activated lymphocytes T and spread the bacteremia to several organs, included the cholecyst and, finally, in the spleen *(39)*.

There are different routes that *Salmonella* can use to penetrate in host cells. *Salmonella* can be recognized and phagocytized by macrophages. Phagocytosis of Gram-negative bacteria involves multiple receptors, which recognize microbial molecules including lipopolysaccharide and flagellin and the binding to ligand, can affect the phagosome maturation (*40)*. *Salmonella* can also actively enter into host cells using a type III secretion system (T3SS1), a system of almost 20 proteins organized in a needle-shaped structure that release effector proteins in host cells and facilitate the process of invasion. The T3SS1-mediated invasion is regulated by at least 15 effector proteins, including SipA, SipC, SopB/SigD, SopD, SopE2 and SptP, which induce rearrangement of the actin cytoskeleton, promote the formation of membrane ruffles and the rapid internalization of the bacteria and modulation of the inflammatory response. Several other T3SS1 and T3SS2 effector proteins interact with ubiquitin pathways of the host cell *(41)*. In addition, *Salmonella* can adhere through fimbriae and non-fimbrial adhesins on the surfaces and can be internalized via a T3SS1-independent process *(42)*. Once in cells, *Salmonella* survives and replicates within a modified phagosome, termed the *Salmonella*-containing vacuole (SCV), which initially expresses on its surface early endosome markers (Fig. 2). These markers are degraded within 60-90 min post invasion and replaced by lysosomal glycoproteins, typical markers of late endosomes and lysosomes *(43)*. Interactions with the host cell endocytic pathway mediated by a variety of T3SS1 and T3SS2 effector proteins regulate the movement of the SCV from the cell periphery to the microtubule-organizing centre, in the perinuclear area, where the SCV-enclosed bacteria replicate *(44, 45)*. Then, when intracellular replication has started, *Salmonella* induces the formation of filaments from the SCV's surface *(46)*. Subsequently, the T3SS2 and

other factors involved in nutrient acquisition and limitation of antibacterial mechanisms are induced.

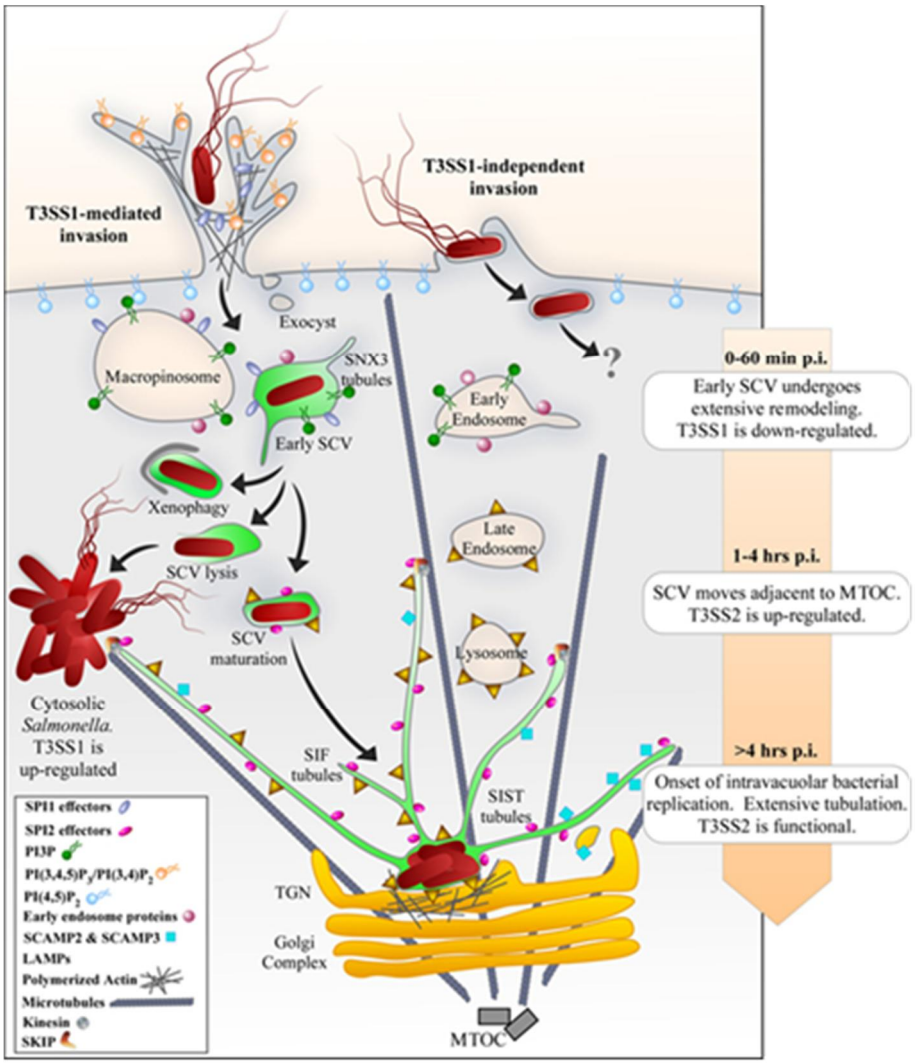

Figure 2. Invasion of a cell of the gut endothelium by *Salmonella* through adhesion or through receptor mediated endocytosis. In the last case, several effectors drive actin-mediated ruffling and internalization of the bacteria into a modified phagosome or SCV, which relocate to a juxtanuclear location where intravacuolar replication happens *(43)*.

2.2 Virulence factors of *Salmonella*

Infection of human host by *Salmonella* is a complex process involving components of both the pathogen and the host. To colonize a host, the pathogen must survive to the resident microbiota present on host surfaces, as well as to the host innate immune system. Several factors, mainly proteins, facilitate the process of invasion and are necessary for the colonization of host cells. These specific virulence factors are still poorly understood *(47)*. Amongst these factors, *Salmonella* have two T3SSs necessary for active internalization in host cells, a type I secretion system and other factors such as fimbriae, flagella and ion transporters that have important roles in establishing and maintaining the intracellular niche. The Salmonella Pathogenicity Islands (SPI) on the chromosome encodes many virulence factors. *Salmonella* has two SPI: the first encoding genes involved in the surviving in the intestinal environment and the colonization of host cells, the latter involved in the intracellular replication and in the diffusion of the bacteria in the bloodstream. Invasion and early post-invasion processes are regulated by T3SS1, flagella, fimbriae and non-fimbrial adhesins. Flagellar-based motility increases the invasiveness *(48)*, even if flagellin is a potent inducer of innate immunity, since it is translocated into the cytosol by T3SS1, resulting in activation of the inflammasome and caspase-1-mediated cell death *(49 - 51)*.

The survival of *Salmonella* in the eukaryotic host cells is permitted by the expression or the down-regulation of several factors. Once in host cells, flagella are usually down-regulated, although inside macrophages they may be induced with T3SS1 and used for escape *(52)*. To survive the reactive oxygen species produced by the phagosome NADPH oxidase of host cells in response to the bacterial invasion, *Salmonella* uses a superoxide dismutase *(53)*. Moreover, in the host, iron availability is limited due to the activity of iron-binding proteins such as transferrin and Nramp1 (Natural resistance-associated macrophage protein

one), a divalent metal-proton symporter found in macrophages, neutrophils and dendritic cells *(54)*. *Salmonella*, which needs iron for growth, produces two siderophores, enterobactin and salmochelin *(55)*. Salmochelin is a glucosylated derivative of enterobactin resistant to lipocalin-2, which prevents bacterial iron acquisition in the inflamed intestinal *(56)*. Other metal ions, Mg^{2+}, K^{+} and Zn^{2+}, are required for intracellular survival. *Salmonella* has three distinct systems for uptake of Mg^{2+}: CorA, MgtA and MgtB, each essential for virulence *(57)*, the ZnuABC high-affinity Zn2+ uptake system, and the Trk system, a multiunit protein complex that functions as a low-affinity K+ transporter *(58)*.

Several serotypes of *Salmonella*, including *Salmonella* ser. Typhimurium, are known to harbour a virulence plasmid of variable sizes, although not all isolates belonging to these serotypes carry the virulence plasmid *(59, 60)*. All plasmids contain the 7.8 kb salmonella plasmid virulence (spv) locus, which harbours five genes designated *spv RABCD*. Two genes, *spvB* and *spvC*, encode the principal factors for plasmid-mediated virulence of serovar Typhimurium *(61)*. Both are translocated via the T3SS2 into host cells *(62, 63)*. SpvB ADP-ribosylates actin, destabilizes the cytoskeleton, and is associated with host cell cytotoxicity *(62)*. SpvC has phosphothreonine lyase activity and can inactivate the mitogen-activated protein kinases Erk1/2, JNK and p38 in mammalian cells *(63)*.

Salmonella ser. Enteriditis and *Salmonella* ser. Typhymurium produce exotoxins including a heat-stable cytotoxin, which act by inhibiting the protein synthesis, leading to the host cell lysis and to the dissemination of the bacteria. The cell lysis can also be due to chelating properties of the toxin, observed in Caco-2 and Hep-2 cells, respectively from heterogeneous human epithelial colorectal adenocarcinoma and human epithelial type 2 of human laryngeal carcinoma *(64)*.

In addition to the cytotoxin, *Salmonella* ser. Typhimurium produces a heat-labile enterotoxin (Stn) that causes the increase of intracellular cAMP, the consequent

increase of sodium and chlorine ions and the efflux of water and electrolytes into the intestinal lumen *(65)*. This important virulence factor is encoded by the gene *stn*, located in an 800-bp *ClaI-EcoRI* genomic DNA fragment and highly conserved in *S. enterica* serotypes, while *S. bongori* lacks the *stn* gene, that is therefore exploited to discriminate the two species *(66)*. The Stn enterotoxin (29 kDa), is structurally and immunologically similar to both cholera toxin and the heat-labile enterotoxin (LT-I) of *E. coli,* being constituted by two subunits A (Active) and B (Binding), in a 1:5 ratio. The B subunit of the toxin binds to a component of the cell membrane known as glycolipid globotriaosylceramide, causing the induction of tubular membrane invaginations for the bacterial uptake into the cell. When the protein is inside the cell, the A subunit interacts with the ribosomes to inactivate them and stop the protein synthesis, leading to the death of the cell.

2.3 Occurrence of *Salmonella*

In industrialised countries, the main reservoir of *Salmonella* is the intestinal tract of farm animals. The microorganisms, excreted in faeces, may contaminate soil, bedding, feedstuffs and water, and continue the cycle of contamination *(67, 68, 69)*. Live poultry at all stages of the farming process can be carriers of *Salmonella*. *Salmonella* can colonize eggs both through transovarial transmission in infected poultry and through broken or weak eggshell membranes, and dairy products can get contaminated through poor handling practices at the farming level (such as milking of animals) *(70, 71)*. Despite other sources, like contact with infected or carrier animals, environment or person-to-person, foodborne salmonellosis is the most relevant one with a high global impact in human health. Hence, there are many ways that allow *Salmonella* to enter into the food chain: during the processing of raw meat, bacteria of the digestive tract can colonize surfaces or meat, while fresh fruit and vegetables can be contaminated through contaminated

freshwater and soil or through fertilization with contaminated animal manure. Human usually are infected through the consumption of contaminated food and water. It is estimated that 95% of *Salmonella* infections are due to the consumption of contaminated foodstuffs and low numbers of organisms, as low as 10 cells, are able to cause infections especially in infants and elderly *(72, 73)*. Contamination often occurs when organisms are introduced into preparation areas and are allowed to replicate in food, due to inadequate storage temperatures and/or cooking or cross-contamination of ready-to-eat food *(74)*.

The major symptom caused by *Salmonella* is gastroenteritis, however, a few serotypes belonging to typhoidal serotypes, like *Salmonella* ser. Typhi, *Salmonella* ser. Paratyphi A, *Salmonella* ser. Paratyphi B and *Salmonella* ser. Paratyphi C, can cause enteric fever. Usually salmonellosis is self-limiting, resolving in few weeks, but occasionally the infection becomes systemic (5-10% of infected persons), causing other extra-intestinal infections especially in the risk groups (infants, young children, older people and the immune-compromised patients) *(75)*. It was estimated that non-typhoidal *Salmonella* causes around 93.8 million of illnesses and 155.000 deaths each year worldwide *(76)*.

2.4 Taxonomy of the genus *Salmonella*

The genus *Salmonella* is composed of two species, *S. enterica* and *S. bongori*, with *S. enterica* divided into the subspecies *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* based on biochemical and genomic modifications (Table 2) *(77)*. Each subspecies is further divided into serotypes based on the presence of specific surface molecules, namely O antigen, H antigen and to a lesser extent the Vi antigen *(78)*. The O antigen, or somatic antigen, is the carbohydrate component attached to the core oligosaccharide of the lipopolysaccharides molecule. Each O antigen has been designated a number for identification and strains that do not express O antigens are referred to as rough

in the antigenic structure details, having outer membrane more penetrable by hydrophobic antimicrobial *(79)*. All O antigens are listed sequentially following the White-Kauffmann-Le Minor scheme *(80)*. The H antigen is the filamentous portion of the flagellar component of the bacterium and the antigenic differentiation is related to differences in the middle portion of the flagellin protein. Most *Salmonella* cells can express two different H antigens (diphasic). The phase 1 antigen is encoded by the *fliC* gene and the phase 2 antigen is encoded by the *fliB* gene. Most cells only express 1 antigen at a single time. Cells that can only express 1 antigen are referred to as monophasic, they can occur naturally in some serovars or through loss of either the *fliC* gene or *fliB* genes in serovars that are usually diphasic, such as *Salmonella* ser. Typhimurium. The lack of a phase 1 or phase 2 antigen is denoted by a "-" in the antigenic structure, as described in some *Salmonella* ser. Typhimurium. Non-motile strains do not express phase 1 or phase 2 antigens and are denoted with two "-" in the antigenic formula. The Vi antigen, or capsular antigen, is present only in few serotypes, like *S*. Typhi, *S*. Paratyphi C and *S*. Dublin. Serotyping is achieved by agglutination test on glass slides with the corresponding anti-sera. To date, there are over 2,500 serovars identified within *S. enterica*, but they do not all frequently cause diseases in humans and domestic animals *(80)*.

Strains of *Salmonella* spp. resistant to antimicrobial drugs are now widespread in both developed and developing countries, due to the use of growth promoters in livestocks and the incorrect use of antimicrobial agents for treatment in humans and animals *(81 - 83)*. Therefore, the occurrence of *Salmonella* strains resistant to quinolones, fluoroquinones, and third generation cephalosporins has increased in food animal sources *(84, 85)*. Since 1990s, the isolation of the multi-resistant strain of *Salmonella* ser. Typhimurium definitive phage type (DT) 104 has increased. This strain displays resistance to six commonly used antimicrobials: ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines (R-type ACSSuT) *(86)*.

Table 2. Classification of *Salmonella* species *(77)*.

Species	Subspecies	Subgenus	Number of serotypes
enterica	*enterica*	I	1454
	salamae	II	489
	arizonae	IIIa	94
	diarizonae	IIIb	324
	houtenae	IV	70
	indica	VI	12
bongori	-	V	20

2.5 Methods for *Salmonella* detection and subtyping

With the arising of multi-drug resistant strains, the simple identification of the *Salmonella* species is no longer sufficient to establish the risk to which consumers are exposed, and genotyping is required. Current testing of food and environmental samples for the presence of *Salmonella* is performed by detecting the pathogen, identifying the isolate as a specific *Salmonella* serovar and subtyping the isolate for the possible association with any clinical cases of salmonellosis. Detection methods are based on traditional bacterial culture procedures through serial enrichments of increasing selectivity, which leads to the isolation of *Salmonella* on selective-differential agar media, as established by ISO6579:2002/Cor1:2004 *(87)*. The isolation and identification of *Salmonella* from foods through culture-dependent traditional methods requires at least 5 days to obtain a result, which must be confirmed through biochemical testing. Also, the confirmation test takes days, if not performed trough automated technologies that allow simultaneous testing of multiple analytes. Following a non-selective pre-enrichment for 16-20 hours in buffered peptone water, two selective enrichments in Tetrathionate broth (Müller Kaufmann) and Rappaport-Vassiliadis Soy peptone (RVS) broth are carried out for 18-24 hours. An aliquot of each enrichment is then plated on two selective media, Brilliant Green Agar (BGA)

and Xylose-Lysine-Desoxycholate (XLD) agar, and incubated for 18-24 hours. After incubation, five suspected colonies of *Salmonella* from each medium (grey-reddish-pink and slightly convex colonies on BGA, or slightly transparent red colonies with a black centre and a pink-red zone around them in XLD) are finally transferred onto non-selective media, like Nutrient agar plates for biochemical confirmation of *Salmonella* and serotyping.

Salmonella presence is confirmed when the bacterium results oxidase negative, negative to Voges–Proskauer test for the digestion of glucose to acetylmethylcarbinol, do not produce urease, indole negative, non-lactose fermenter or O-Nitrophenyl-β-D-galactopyranoside (ONPG) negative, Lysine Decarboxylase positive (Fig. 3). All these tests can be performed singularly, by using panels, like API 20E test, or by using automatized instruments.

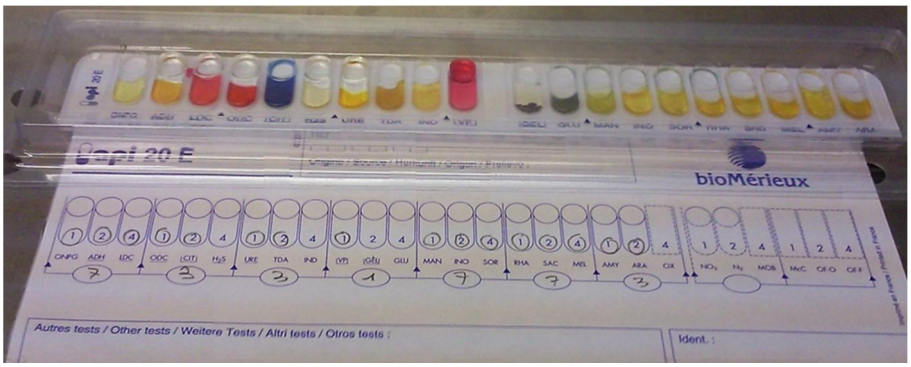

Figure 3. API 20E strip for the identification of Enterobacteriaceae. The metabolization by *Salmonella* of the dehydrated substrate contained in each well cause the turning of indicator. The positivity to the reactions is reported in the reading table, obtaining a numerical profile. Each profile identify a certain species.

A faster alternative to the microbiological identification is the fluorescent quantitative real-time PCR, which is the most sensitive method for detection, monitoring and measurement of pathogen levels. There are several commercial

kits that apply genetic methods for the detection of *Salmonella.* Most of them exploit *16S*, *hilA, Prot6e*, *gapA*, *recA*, *rpoB* genes.

To detect and track foodborne disease outbreaks and to track sources of bacterial contamination throughout the food system, methods able to allow the identification at strain level are required. The use of subtyping methods furthermore provides an opportunity to improve the knowledge about the population genetics, epidemiology, and ecology of different foodborne pathogens. Several methods can be used for subtype *Salmonella* strains. Traditional typing methods based on phenotypic traits, antibiotic susceptibility profiles, serotyping, and phage typing, provide insufficient information for epidemiological purposes. More informative molecular subtyping methods have been developed based on three main mechanisms of discrimination: i) restriction analysis of bacterial DNA, ii) Polymerase Chain Reaction (PCR) amplification of specific genetic targets and iii) the identification of DNA sequence polymorphism at specific loci in the genome.

Serotyping is performed to determine to which of the 2500 *Salmonella* serovars a specific isolate belongs. This is necessary for epidemiological purposes, and it is the first approach used for looking for evidence of links between cases. Serotypes of *Salmonella* are defined based on the antigenic structure of both somatic or cell wall (O) antigens and flagellar (H) antigens. These antigens are detected using slide agglutination with commercially produced antisera, using a resuspended colony from an agar plate in case of the O antigens, while H antigens are identified using a suspension of broth culture, as described in ISO 6579-3:2014. The serotype is deduced from the specific pattern of agglutination reactions using the Kauffmann-White classification scheme.

A further discrimination of *Salmonella* isolates is achieved by subjecting the isolates to *in vitro* antibiotic susceptibility testing against antibiotics of different classes. Disk diffusion method of Kirby and Bauer is commonly used following the guidelines of Clinical and Laboratory Standards Institute *(88).* Antibiotics

commonly used for *Salmonella* serovar Thyphimurium are ampicillin, cefotaxime, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfonamides, tetracycline and trimethoprim–sulfamethoxazole. The data about the antibiotic susceptibility profile is also useful to choose the most effective therapy to give to patients.

Subtyping through phage typing exploits the selective ability of bacteriophages to recognise particular receptors on the surface of the bacterial wall and to infect and consequently lyse certain strains of *Salmonella*. The profile obtained by the susceptibility of each isolate to the lysis by a panel of different bacteriophages allows the identification of the phage-type. Phage typing resulted useful in the description of pandemic clones of *Salmonella*, such as *Salmonella* ser. Typhimurium DT104, but a limited number of phages are avalaible, thus excluding the discrimination among different untypeable strains *(89)*. A further drawback of this technique is the need of adequate maintenance of phages, to assure accurate and reproducible results. Consequently, only suitable well-trained laboratories like National Reference Centers are able to use this technique.

Among DNA based techniques, the most frequently used is Pulsed-Field Gel Electrophoresis (PFGE), which is considered the gold standard *(90)*. Other techniques commonly used for *Salmonella* genotyping are plasmid typing, ribo-typing, Amplified Fragment Length Polymorphisms (AFLP) and recently Multi-Locus Variable number tandem repeat Analysis (MLVA).

PFGE profiles provided a DNA ‘‘fingerprint’’ that reflects the DNA sequence of the entire bacterial genome. To perform PFGE, an optimal number of cells are embedded in an agarose plug and treated with detergents and enzymes, such as sarcosine and proteinase K, to lyse the embedded cells and release the genomic DNA (Fig. 4). The plug is then washed and treated with a rare cutting restriction enzyme, such as *XbaI*, *BlnI*, or *SpeI (91, 92)*. The plugs are then inserted into the wells of an agarose gel, and the DNA separated in alternated electric fields. Following electrophoresis, the pattern of DNA separation is visualized by staining

the gel with a fluorescent dye. The gel-banding pattern from one isolate can be compared with those of other isolates, and information about the relatedness of the strains can be resolved. PFGE is characterized by a high degree of reproducibility, both within and between laboratories, and the recent introduction of computerized gel-based data collection and analysis systems allows better standardization between laboratories, thus creating the ability to rapidly compare restriction fragment patterns from isolates analysed from remote locations. Large databanks that house PFGE patterns from isolates around the world will greatly enhance *Salmonella* outbreaks detection. For *Salmonella*, a PFGE pattern database, the PulseNet system, has been developed by CDC (Center for Deseases Control and prevention) and it is commonly used worldwide to examine the diversity of different pulsotypes *(93)*.

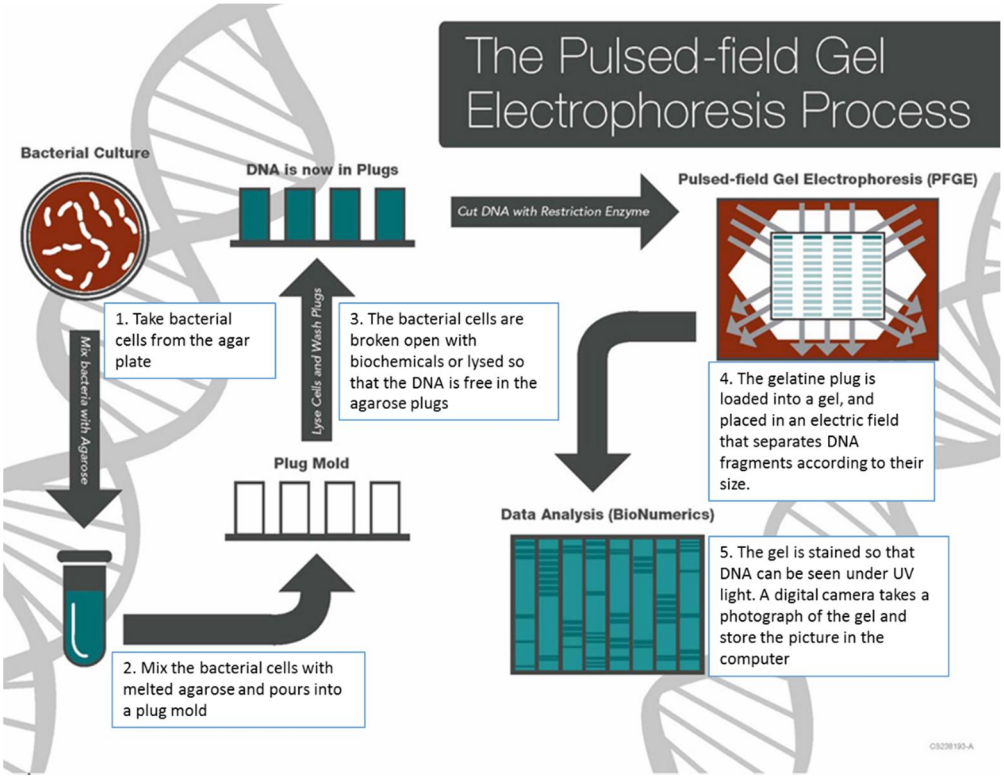

Figure 4. PFGE workflow *(modified from 93)*.

Another technique based on restriction fragment analysis is plasmid typing, in which plasmid DNA digested with enzymes such as *HindIII* is separated through gel electrophoresis. If the plasmid lacks restriction sites for a particular enzyme, the plasmid will not be cut. Variability can occur also among unrestricted plasmids, depending on the degree of supercoiling. The main drawback of this approach is that all *Salmonella* strains that do not carry plasmids show the same profile. Moreover, plasmids can be gained or lost in response of certain conditions, limiting the possibility of applying this technique to determine relatedness of isolates involved in foodborne outbreaks.

Through the digestion of chromosomal DNA by restriction enzymes, followed by gel electrophoresis, banding patterns are generated. This approach can be coupled with Southern blotting, using probes for repeated DNA elements, like ribosomal RNA gene sequences (rRNA) or IS200 sequences. For *Salmonella*, the use of IS200 sequences, which are approximately 700 bp in length and are randomly located around the genome *(94*), would not be effective in differentiating all isolates, since some strains lack IS200 sequences.

In ribotyping, frequent cutting restriction enzymes are used to cut total genomic DNA, then restriction fragments are transferred to nylon membrane and incubated with a probe homologous to the highly conserved regions of rRNA *(95).* Sequence differences in the regions flanking the rRNA gene lead to variability in the size of the junction fragments, which produce distinct patterns that can be used to discriminate between related strains. Ribotyping is highly reproducible, produce relatively few bands, it has been largely automated and other supplemental enzymes can be used if more discrimination is needed, but it may not adequately separate unrelated isolates within a particular serotype *(96).* The disadvantages of this technique relies on the limited number of rRNA genes in some serotypes and on the fact that a mutation, or other genetic changes, could alter the size of fragments containing a portion of the rRNA gene *(97).*

A combination of restriction analysis and PCR amplification is employed in AFLP method. This combination increases the sensitivity, requiring only a small amount of genomic DNA to determine the relatedness of bacterial strains, and it results in a high reproducible and accurate technique *(98)*. Following the digestion with one or more enzymes, known DNA fragments, named linkers, are ligated to the free DNA ends. These linker oligonucleotides contain sequences complementary to the restriction sites and are recognized by PCR primers that allow the selective amplification of the restriction fragments. The separation of the amplicons by gel electrophoresis leads to characteristic separation profiles, which can be compared among strains. AFLP shows though some limitations, since the reaction products are best processed with an automated DNA sequencer, and the variability among different sequencing platforms could potentially limit some inter-laboratory data comparison *(99)*.

The Multi-Locus Variable number tandem repeat Analysis (MLVA) is a recent technique, which through the identification of polymorphic DNA sequences allows the profiling and molecular subtyping of *S. enterica* subspecies. Bacterial genomes have many regions with nucleotide repeats in coding and non-coding DNA sequences. When these repeats are directly adjacent to each other and their number at the same locus varies between isolates, the respective genomic regions are called Variable-Number Tandem Repeat (VNTR) loci. The repeats at the same locus can be identical or their nucleotide sequences can differ slightly. Multilocus VNTR analysis (MLVA) allows determining the number of tandem repeat sequences at different loci in a bacterial genome. The MLVA genotyping method is based on the amplification of DNA fragments that contain variable copies of tandem repeats, followed by accurate sizing of the PCR products through agarose gel electrophoresis. Recently, the use of primers labelled with different fluorescently coloured dyes and capillary electrophoresis carried out on an automatic DNA sequencer allows MLVA amplicons to be analysed in one single run. The different fluorophore molecules incorporated in the amplicons absorb

the laser energy and release light of different wavelengths, which are then identified by the detector in the DNA sequencer. Exploiting a specific software, each locus is distinctly recognised on the electropherograms according to its colour, and, based on their amplicon sizes, the repeat number per MLVA locus is automatically calculated (Fig.5). Once the number of repeats in a set of VNTR loci (alleles) for a bacterial isolate is assessed, an ordered string of numbers corresponding to the number of repeat units at each locus is provided. The profile number can be easily compared to the VNTR loci from the complete sequence of a *Salmonella* genome that is already present within the database. This choice resulted useful especially for inter-laboratory comparison, when standardization is required. Moreover, reduced typing time, high resolution, suitability for large-scale automation are the main advantages for resolving large and complex outbreak situations. Their drawbacks include high assay-specificity and the current lack of standardisation for many bacterial species. In Europe, only the *Salmonella enterica* subspecies *enterica* serovar Typhimurium MLVA assay has achieved generally accepted standardisation *(100)*.

Different typing techniques have strengths and weaknesses that will affect their usefulness in determining the source and relatedness of *Salmonella* isolates. The use of several methods is often required for the effective discrimination at strain level, because only the identification based on different parameters can give complete information. The choice of the molecular method depends on a number of factors.

Serotyping is an essential first step in characterizing a Salmonella isolate, and can separate strains to a certain degree; however, isolates within a particular serotype will need to be further distinguished with techniques such as PFGE, MLVA or AFLP. An important feature of each of these genotypic techniques is that their discriminatory power can be somewhat suitable to the needs of a situation. A recent work has indicated that the ability of PFGE to separate among closely related isolates can be significantly enhanced when multiple reactions with

different enzymes are compared *(101)*. The same happens when multiple loci are screened and compared through MLVA *(100)*, and when the number of unknown nucleotides in primer design for AFLP are reduced *(102)*.

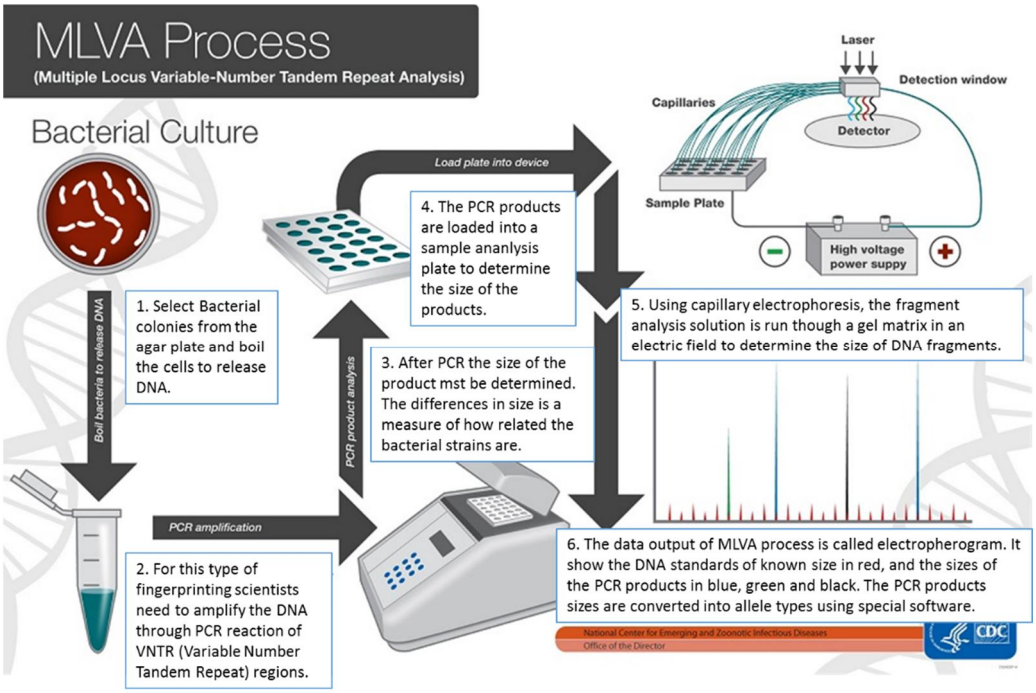

Figure 5. MLVA workflow *(modified from 93)*.

Certain typing methods do not work in particular situations; for example, many strains of *Salmonella* lack plasmids, repetitive sequences, or useful phage receptors, or separate as smear patterns in gels and thus cannot be typed. Therefore, alternative and robust typing methods will be required to distinguish between these un-typeable isolates.

Another limit of the typing method is linked to the reproducibility. The lack of reproducibility can be due to minor changes in the amplification conditions that lead to variability in the profiles generated, or to techniques that rely on visual interpretation of DNA bands.

Therefore, techniques that rely on stable, defined markers for typing provide the most reproducible typing results, however they may not provide enough discriminatory power for long-term epidemiologic studies, due to their genetic instability. Additionally, to improve reproducibility and utility of typing results, it is best to limit the number of times an isolate is transferred on culture media. In fact, repeated passing of *Salmonella* on artificial media can lead to genetic changes *(103)*, and if changes occur in a portion of the genome targeted by a typing method, such as a restriction site for PFGE, the pathogen causing foodborne disease may be incorrectly classified. Likewise, the loss of plasmid in culture would have an effect on an isolate's plasmid profile, potentially altering the results of other restriction-based methods where the plasmid DNA makes up part of the restriction profile.

Additionally, factors such as the time needed to get the results, the cost of the supplies, and the cost of the equipment to perform the typing method are important for picking a molecular method. Some techniques, even if discriminatory, require specialized and expensive equipments, and thus are not always feasible. For example, to use MLVA or AFLP as a high throughput typing method, an investigator needs access to an automated DNA sequencer. Therefore, the selection of the most appropriate molecular typing method for the characterization of *Salmonella* in the food chain will rely upon the needed level of discrimination and the resources that are available to carry out the typing.

Overall techniques such as AFLP, MLST, MLVA, and PFGE have provided the best levels of discrimination among the *Salmonella* isolates; however, none of these methods appears to be clearly superior to the others. Nonetheless, in a study carried out by our group, only MLVA has allowed to discriminate adequately among closely related isolates and to identify the source of *Salmonella* strain. Therefore, the careful selection of methods and the order of deployment will help to maximize the discriminatory power of a typing scheme to characterize foodborne *Salmonella* infections.

3. Staphylococcal Enterotoxins

Staphyloccal Enteroxins (SEs) are short and extracellular proteins produced mainly by *Staphylococcus aureus*, although other *Staphylococcus* species have also proved to be enterotoxigenic. SEs constitute a family of more than 20 different exotoxins, which share similarities in structure *(104)*. Their mature length is approximately 220–240 amino acids, depending on the toxin, and their molecular size is on average ~25 kD and have significant sequence variability, but, when folded, display similar three-dimensional structures *(105)*. The three dimensional structure of several SEs has been determined by crystallography *(106)*. They are elliptical in shape and have two major unequal domains composed mostly of β strands and a few α-helices. The two domains are separated by a shallow cavity. The larger of the two domains contains both the amino and carboxyl termini. Mutational analysis of both SEA and SEB implicated this cavity in the binding to T cell receptors (TcR) *(107)*. Another region on SEA identified by mutational analysis, found interacting with the TcR Vβ 7 and 8.1, is tyrosine 66, while a stretch of amino acids from 45 to 58 on SEB was found to be involved in the binding to class II major histocompatibility complex (MHC) molecules that are expressed by antigen presenting cells (APC). Several of these enterotoxins have a Zn-binding site that contributes to their interaction with class II MHC molecules *(108)*.

SEs are resistant to heat as well as to acid and to gut proteases, including pepsin, trypsin, rennin and papain *(109)*. Therefore, they may not be completely denatured by mild cooking of contaminated food. Since *S. aureus* grows over a wide range of temperatures and pH, the bacteria may grow in a wide assortment of foods, including meat and meat products, egg products, salads, bakery products and milk and dairy products. In fact, several SE-outbreaks have been caused by food contaminated with SE-producing strains left at temperatures that allow rapid

growth and replication of the bacteria. The main symptoms associated to staphylococcal enterotoxins are fever, since SEs are pyrogenic, emesis and gastroenteritis. The ingestion of less than 1 μg of toxin cause vomiting, diarrhea, prostration, severe dehydration, dizziness and abdominal pain, which are the main symptoms of food poisoning and toxic shock syndrome *(110).* In an outbreak due to Staphylococcal Enterotoxin A (SEA) contaminating chocolate milk, the amount of toxin infecting the children was reported to be only 200 ng *(111).* The disease is usually self-resolving, is rarely lethal and the elderly and young children are more susceptible.

Although there are more than 20 distinct staphylococcal enterotoxins, only the most commonly found in foods have been studied. Staphylococcal Enterotoxin A (SEA) is the most common SE encountered in food poisoning outbreaks; SEB is associated with food poisoning too, but has been studied for potential use as an inhaled bioweapon, while Staphylococcal Enterotoxin D (SED) is suggested to be the second most common staphylococcal toxin associated with food poisoning worldwide. Staphylococcal Enterotoxin E (SEE) has also been documented in some cases of food poisoning, while Staphylococcal Enterotoxin F (SEF) has been implicated in toxic shock syndrome *(110, 112).* SEA, SED, and SEE share 70–90% sequence homology, while only 40–60% with Staphylococcal Enterotoxin B (SEB) and Staphylococcal Enterotoxin C (SEC) *(113, 114).*

The economic impact of food-borne diseases is substantial. In 2014, 393 food-borne outbreaks caused by staphylococcal toxins were reported in Europe *(115).* This represents 7.5% of all outbreaks, a small increase compared with 2013, when 386 outbreaks caused by staphylococcal toxins were reported by 12 member States. As in previous years, France reported the majority (89.6%) of the outbreaks, being correlated to raw milk cheese consumption. *S. aureus* may occur in the milk of animals with clinical or sub-clinical mastitis or as the result of poor

hygienic practices during milk collection. The high incidence of staphylococcal food poisoning is often due to the insufficient pasteurization/decontamination of an originally contaminated product source or to its contamination during preparation and handling by individuals who are carriers of the organism *(116).* The ability of *S. aureus* to produce detectable amounts of enterotoxins in food depends on whether or not the strain is enterotoxigenic and whether the environmental conditions necessary for enterotoxin synthesis exist.

3.1. Biology of staphylococcal enterotoxins

S. aureus is an ubiquitous Gram-positive coccus, which usually colonizes skin and mucosal membrane of humans and domestic animals, becoming an opportunistic pathogen when the carrier's immunity system is compromised *(117, 118). S. aureus* expresses a wide array of cell-associated and secreted virulence factors, which make it a versatile pathogen capable of a wide range of infections. The secreted factors include various cytotoxins, exotoxins, exfoliative toxins and enzymes able to turn host components into nutrients that the bacteria may use for growth. Among the other secreted factors are exotoxins that include SEs and toxic shock syndrome toxin.

SEs are globular, single-chain proteins, with molecular weights ranging from 19 to 29 kDa, divided into four phylogenetic groups based on their amino acid sequences *(119).* Staphylococcal enterotoxins genes are encoded by pathogenic islands and are horizontally transmitted *(120).* SEs have an elliptical shape and contain two unequal domains, A and B, separated by a shallow cavity, which is involved in the binding to T cell receptors *(107).* Several of these enterotoxins have a Zn-binding site that contributes to their interaction with class II MHC molecules *(108).* Even though there are some differences in SE structures, these toxins share the ability to bind to the major histocompatibility complex proteins of their hosts.

SEs exert cytotoxic effect by forming pores and altering the chloride ion permeability of the membrane of mucosal cells of the intestinal wall. This mechanism promotes the death of the cells and a secretory diarrhea in few hours after the ingestion of the toxins. In contrast to the case of many other bacterial enterotoxins, specific cells and receptors in the digestive system have not been unequivocally linked to oral intoxication by a SE. It has been suggested that SEs stimulate the vagus nerve in the abdominal viscera, which then transmits the signal to the vomiting center in the brain *(121, 122).*

In addition, SEs act as super-antigens, binding as unprocessed molecules to major histocompatibility complex (MHC) class II molecules on antigen presenting cells, and subsequently activate T-cells through interaction with the variable region of the T-cell receptor a-chain (TCR-Vp) *(123).* This results in the activation of between 2 and 20% of all T-cells, ultimately leading to proliferation and the production of a variety of cytokines, which can lead to systemic shock *(124).* SEs evolved several modes of interaction with MHC class II molecules. SEA contains two MHC class II binding sites: a zinc-dependent site, located in domain A, which presumably binds MHC β chain, and a minor binding site in domain B, which is not zinc dependent *(125).* It may be that cooperation between the two binding sites is responsible for the high affinity of SEA for MHC class II molecules.

The release of inflammatory mediators causes vomiting and gastrointestinal damage *(126).* Inflammatory changes are observed in several regions of the gastrointestinal tract, but the most severe lesions appear in the stomach and the upper part of the small intestine. The diarrhea sometimes associated with SEs intoxication may be due to the inhibition of water and electrolyte reabsorption in the small intestine *(127).*

3.2. Methods for the detection of staphylococcal enterotoxins

Heat treatment that is commonly used in food processing can kill *Staphylococcus*, but cannot inactivate the staphylococcal enterotoxins *(128).* Crude SEA was reduced from 21 μg mL^{-1} to < 1 μg mL^{-1} after heating at 100 °C for 130 min, and purified SEA (0.2 mg mL^{-1}), however, was completely inactivated in buffer after heating at 80 °C for 3 min or 100 °C for 1 min *(129).* Thermal stability of SEs is influenced by the nature of the food, the pH, the presence of NaCl and the type of toxin. SEA, for instance, is relatively more stable to heat at pH 6.0 or higher than at pH 4.5–5.5, while SED is relatively more stable at pH 4.5–5.5 than at pH 6.0 or higher *(130).* Depending on these facts, it is very difficult to predict the real elimination of the toxins in thermally processed foods. The European Commission has set the maximum level of coagulase-positive staphylococci in cheeses and milk-derived products to 10^5 cfu/g in 25 g of samples, thus not avoiding the presence of the toxins *(131).* Currently, the presence of staphylococcal enterotoxins is searched in dairy products contaminated by *S. aureus* at a level higher than 10^5 cfu/g. Enterotoxins must be absent in 25 g of these foods *(131).* European legislation also stipulates the reference procedure for SE analysis in milk and dairy products, which is based on extraction, dialysis concentration and immunochemical detection using one of the two approved assays (Ridascreen® SET Total, VIDAS® SET2) *(132).* While Ridascreen® SET Total is a sandwich Enzyme-Linked Immunosorbent Assays (ELISA), VIDAS® SET2 is an Enzyme Linked Fluorescent Assay (ELFA). Both of the assays are based on the capture of the toxin in the samples by antibodies adsorbed onto a solid phase before the detection by an enzyme-labelled antibody, which generate a colorimetric signal in the first case or a fluorescent signal in the latter case. Both these methods have been considered the most practical and powerful method for the analysis of SEs in foods because of their sensitivity and reliability.

Recently, a mouse polyclonal antibody-based sandwich ELISA has been reported to detect SEA in milk and cheese at concentrations as low as 0.064 ng/mL *(133)*, whereas a monoclonal antibody-based sandwich ELISA has reached the LOD of 0.0282 ng/mL *(134)*. These findings together with the raise of rapid and high-throughput adaptable technologies could be exploited for the development of alternative methods for detection and quantification of enterotoxins. Among the most promising direct high-throughput methods, the xMAP technology *(http://www.luminexcorp.com/)* is a stable and reproducible method, which was successfully tested for detecting bacterial toxins in foods *(135)*. The xMAP technology is based on the principles of flow cytometry and uses microspheres containing a mixtures of fluorescent dyes that are used to identify a set of microspheres characterized by a spectral address. In the sandwich immunoassay format, microspheres of several sets are coupled with specific binding antibodies, which bind to the toxin when it is present (Fig.6).

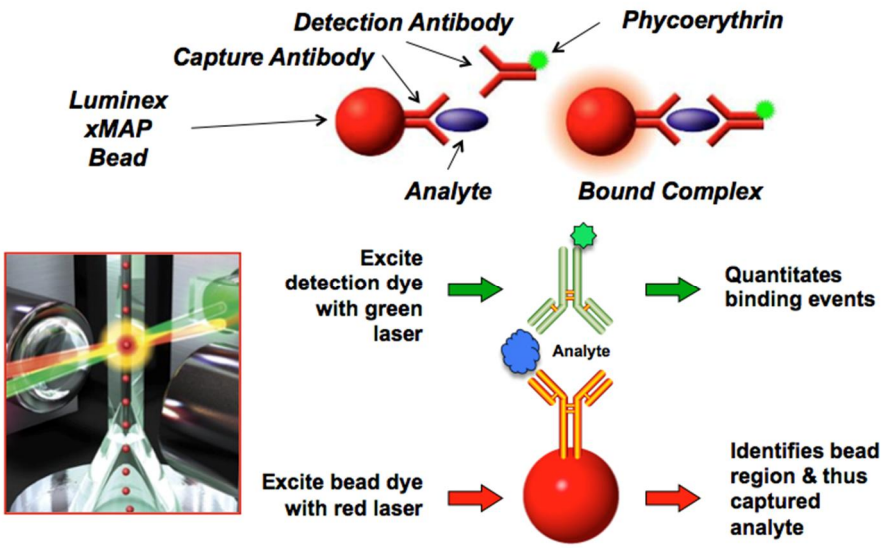

Figure 6. General overview of the Luminex xMAP immunoassay detection scheme.

Conjugated microspheres of different sets can be mixed to create a multiplex assay for several desired analytes, up to 500 various tests in each single sample. The mixtures of microspheres are incubated with the sample and then the detection of the immunocomplex toxin-antibody is performed using biotinylated detecting antibodies and streptavidin conjugated with phycoerythrin as fluorescent dye. The specialized flow cytometer detects each involved spectral address of microspheres as well as intensity of phycoerythrin fluorescent signal from each spectral address.

A further innovative approach uses Surface Plasmon Resonance (SPR) for the detection of antigen-antibody in real-time in a label free environment. Surface plasmon resonance (SPR) biosensors, like Biacore system (*https://www.biacore.com/lifesciences/index.html*) exploit the change of the refraction angle of polarized light between media of different refractive index. In Biacore systems, the glass of the sensor chip and the sample solution constituted the media with different refractive index, while the conducting film is a thin layer of gold on the sensor chip surface. At a certain combination of angle of incidence and energy (wavelength), the incident light excites “plasmons” (electron charge density waves) in the gold film. As a result a drop occurs in the intensity of the reflected light. A plot of the intensity of reflection against the angle of reflection produces an SPR curve (or profile). The sensor-chip provides the physical conditions necessary to generate the SPR signal. Attachment of specific recognition elements on the gold surface (usually antibodies, but potentially also other kind of probes, like Peptide Nucleic Acids or Aptamers), and passivation of the gold surface to non-specific binding, provides a condition for monitoring for the presence of specific analytes. The interaction takes place on the gold-covered side of the sensor chip, opposite from the side where the light is reflected (Fig. 7). Sample containing analyte is supplied in a controlled fashion to the sensor surface through a microfluidic system. The sensor surface itself forms one wall of a flow

cell, which is an integral part of the microfluidic system. Protein toxins are of sufficient size to be detected directly by SPR.

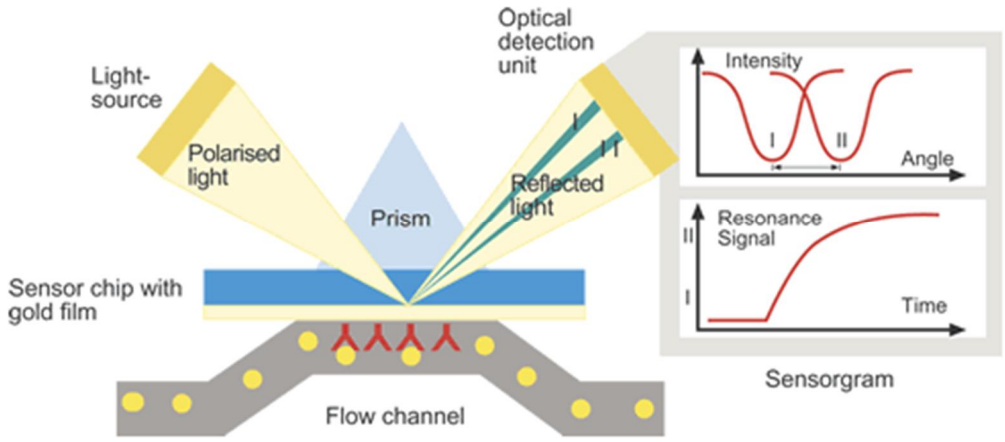

Figure 7. Principle of Surface Plasmon Resonance used in Biacore instrument.

Various other alternative methodologies, based on bioassays or molecular based assays have been developed to detect SEs, including microbiological and molecular methods to test for toxin production *(136)*. The PCR approach is known to provide information about the presence of genes encoding SEs, but it does not provide information about their expression, resulting inadequate to fulfil the requirements established by EU.

4. Aflatoxins and ochratoxins in foods

Contamination of food and agricultural commodities by toxigenic fungi is a widely neglected problem all around the world. Therefore, mycotoxins can be produced in a great variety of food commodities, when suitable condition of temperature, humidity and pH occur in food. The presence of mycotoxins in raw agricultural products poses severe health and economic concerns. The Food and Agriculture Organization (FAO) estimates that about 1000 million metric tons of foodstuffs each year are lost, globally *(137)*.

In the field, airborne spores can germinate on damaged kernels, especially when drought, flood, insect infestation and delayed harvest occur. During the processing and the storage of plant foods, conditions such as inadequate drying and warm humid environment promote mould growth and the potential synthesis of mycotoxins *(138)*. If animals consume contaminated feeds, mycotoxins can distribute and accumulate in their tissues and result in animal-derived foods *(26, 139)*. In fact, the crops contaminated above the limit of regulation for human consumption are directed to animal feed, if they respect the more permissive limits for animal feed (140). Contamination of feeds with mycotoxins is an important concern for farmers due to both acute and chronic intoxication in animals, causing the decrease of productivity and health damages *(141)*.

Aflatoxins and ochratoxins are amongst the most dangerous and frequently isolated mycotoxins. Aflatoxins are a major issue in cereals notably corn, nuts, such as peanuts, pistachio and Brazil nuts and oil seeds *(142)*. Other commodities, like wheat, oats, millet, barley, rice, soybeans, beans and sorghum, are less susceptible to aflatoxin contaminations in the field and if stored under conditions of high moisture and temperature.

Ochratoxin A is often detected in barley, oats, rye, wheat, coffee beans, and other plant products, with barley having a particularly high likelihood of contamination. There is also concern that ochratoxin may be present in certain wines, especially those from grapes contaminated with *Aspergillus carbonarius,* and in dry-cured meat. Frequently, crops can be contaminated by different fungal genera, and more than a mycotoxin can be produced. OTA and AFB1 are among the most frequently observed combinations of mycotoxins in different plant products *(143, 144).*

Considering their diffusion and the toxic effect for both humans and animals, the maximum level of aflatoxins and OTA is subjected to legal regulation both on national and international bases. Maximum levels of aflatoxins (aflatoxins B1, B2, G1, G2 and M1) and ochratoxin A are established in Commission Regulation (EC) No 1881/2006 *(6)*, while provisions for methods of sampling and analysis for the official control of mycotoxins including aflatoxins and ochratoxin A are set in Commission Regulation (EC) No 401/2006 *(7)*.

The maximum levels admitted for aflatoxins and ochratoxin A in different categories of food are showed in Table 3 and Table 4 respectively.

Table 3. Maximum levels of aflatoxins admitted in each food category by EC No 165/2010 *(145)*.

Foodstuffs	**Maximum level B1 (µg/kg)**	**Maximum level B1+B2+ G1+G2 (µg/kg)**	**Maximum level M1 (µg/kg)**
Groundnuts to be subjected to sorting or other physical treatment before human consumption or use as ingredient in foodstuffs	8.0	15.0	-
Nuts to be subjected to sorting or other physical treatment before human consumption or use as ingredient in foodstuffs	5.0	10.0	-
Groundnuts, nuts and processed products thereof, intended for direct human consumption or use as ingredient in foodstuffs	2.0	4.0	-
Dried fruits to be subjected to sorting or other physical treatment before human consumption or use as ingredient in foodstuffs	5.0	10.0	-
Dried fruits and processed products thereof, intended for direct human consumption or use as ingredient in foodstuffs	2.0	4.0	-
All cereals and all products derived from cereals including processed cereal products, with the exception of some foodstuffs (*)	2.0	4.0	-
*Maize to be subjected to sorting or other physical treatment before human consumption or use as ingredient in foodstuffs	5.0	10.0	-
Raw milk, heat-treated milk and milk for the manufacture of milk-derived products	-	-	0.05
Following spices: *Capsicum* spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika); *Piper* spp. (friuits thereof, including white and black pepper); *Myristica fragrans* (nutmeg); *Zingiber officinale* (ginger); *Curcuma longa* (turmeric)	5.0	10.0	-
*Processed cereal-based foods and baby-foods for infants and young children	0.1	-	-

Table 4. Maximum levels of ochratoxin A admitted in each food category by EC No 1881/2006 *(145)*.

Foodstuffs	**Maximum OTA levels (µg/kg)**
Unprocessed cereals	5.0
All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption with the exception of foodstuffs (*)	3.0
Dried vine fruits (currants, raisins and sultanas)	10.0
Roasted coffee beans and ground roasted coffee, excluded soluble coffee	5.0
Soluble coffee	10.0
Wine	2.0
Grape juice, grape nectar, grape must	2.0
*Processed cereal-based foods and baby foods for infants and young children	0.5
*Dietary foods for special medical purposes intended specifically for infants	0.5
Green coffee, dried fruits other than dried vine fruit, beer, cocoa and cocoa products, liqueur wines, meat products, spices and liquorice	-

4.1. Aflatoxins: chemical characteristic, toxicity and bioavailability

Aflatoxins (AF) were isolated and characterized for the first time after the death of more than 100,000 turkeys ("turkey X disease", England, 1960), which were fed with mould-contaminated peanuts *(146)*. Aflatoxin B1, B2, G1 and G2, which name were chosen from their fluorescence under UV light (B= blue, or G= green) and relative chromatographic mobility during thin-layer chromatography, are the

most important members of the aflatoxins group, which count over a dozen other aflatoxins, some coming from bio-transformations (metabolism) that occur in mammals. Aflatoxin B1 (AFB1) is considered the most potent natural teratogen, mutagen and carcinogen known for animals and humans and is usually the major aflatoxin produced by toxigenic strains *(26, 147)*. Aflatoxicoses, the diseases caused by aflatoxin consumption, can be acute resulting in death, or chronic, resulting in cancer, immune suppression, and other pathologies. The main route of exposure to AFs is via the ingestion of contaminated foods, but also the inhalation of airborne spores and the absorption through the skin can cause hepatic and gastrointestinal injuries. In 2002, 4 aflatoxins (AFB1, AFG1, AFB2, AFG2) were confirmed as a Group-1 agent by International Agency for Research on Cancer (IARC), since they significantly increased risks for hepatocellular carcinoma (HCC), especially when act synergistically with hepatitis B virus (HBV) infection, as measured by aflatoxin-specific biomarkers in cohort *(148 - 150)*.

The liver is the primary target organ in all animals including poultry, fish, rodents, and primates, with differences in species susceptibility: trout, ducklings and pigs are highly susceptible, with ruminants being less susceptible *(151)*. Moreover, within a given species, the magnitude of the response is influenced by age, sex, weight, diet and the presence of other mycotoxins and pharmacologically active substances *(152)*. Both pre-harvest and post-harvest contamination of cereals, figs, oilseeds, nuts, tobacco are common, associated in the first case to drought stress and in the latter to the moisture content of the substrate and the relative humidity of the surroundings. Presently, it is estimated that human consumption of aflatoxins ranges between 0 and 30,000 ng/kg/d, with an average intake of 10 to 200 ng/kg/d *(153)*. The maximum acceptable levels of AFB1 in cereals, peanuts, and dried fruits, either for direct human consumption or as an ingredient in foods, has been set by the European Committee Regulations (ECR) as 4 ppb for total aflatoxins (AFB1, AFG1, AFB2, and AFG2) and 2 ppb for AFB1 alone.

Aflatoxins can be found also in milk products, when cows consume aflatoxin-contaminated feeds and metabolically biotransform aflatoxin B1 into a hydroxylated form called aflatoxin M1 *(154).* Although AFM1 produced by the hepatic metabolism is less toxic as compared to the parent compound, the mutagenicity and potential carcinogenicity of AFM1 remains an issue for public health and strict maximal levels have been set in EU not only for aflatoxins in animal feeds, but also for AFM1 in milk. The European Community and the Codex Alimentarius have fixed the limit of AFM1 intake to a maximum of 50 ng/kg.

Aflatoxins are bisdihydrodifuran or tetrahydrobisfuran united to a coumarin substituted by a cyclopentanone or a lactone (Fig. 8). Aflatoxins can be classified into two groups: the first consisting of bisfuran-coumarin-cyclo pentanons, which include AFs of series B and M, AFQ, AFP, and aflatoxicol; the second group consisting of bisfuran-coumarin-lactones, which contain AFs of series G.

AFB1, AFB2, AFG1 and AFG2 are synthesized by aflatoxigenic fungi, while the other AFs (M1, M2, P1, Q1, G2a, B2a and AFL) result from microbial or animal metabolism *(155).*

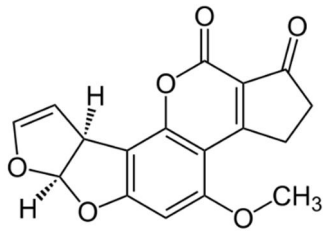

Figure 8. Chemical structure of aflatoxin B1.

Following ingestion with food, AFB1 is absorbed in the intestinal tract, of which the duodenum appears to be the major site of absorption. Due to its low molecular weight, AFB1 is passively diffused into cells of the intestinal epithelium and then in blood and in other organs, especially in the liver, where it is activated. The activation is due to cytochrome-P450 dependent mono-oxygenase: most of the metabolic products such as AFM1, AFQ1, and AFP1 are less toxic than the parent AFB1, but aflatoxin B1-8-9-exo-epoxide (AFBO) is toxic and highly reactive. AFBO alkylates DNA by binding with the N7 position of the guanine residues, forming the 8,9-dihydro- 8-(N7-guanyl)-9-hydroxy-AFB1 and causing irreversible damage in DNA *(156, 157)*. If this modification is not repaired before DNA replication, it may cause GC to TA transversions, DNA strand breaks and point mutations, which lead to hepatocarcinogenesis. Mutations in the *p53* tumor suppressor gene, resulting for example in the R249Sp53 protein expression, may lead to inhibition of apoptosis, inhibition of p53-mediated transcription and stimulation of liver cell growth *in vitro (158, 159)*. AFB1-related HCC was demonstrated associated to *p53* gene mutation. Studies made in China and Brazil, areas with high exposure to AF, revealed that a conversion of G to T at the codon 249 in exon 7 of of the *p53*gene in HCC patients results in R to S mutation in the p53 protein *(160, 161)*.

In presence of water AFBO is hydrolized into AFB1-8,9-dihydrodiol, and becomes available to link serum proteins like albumin. Other reactions like hydroxylation and demethylation are performed in order to limit the toxicity of AFBO. AFBO, which represent the most reliable urinary biomarker for aflatoxin recent exposure, can be also conjugated by glutathione S-transferase (GST), β-glucuronidase and sulfate transferase, in order to facilitate the excretion through bile liquid, urine and milk, in the case of lactating animals *(162)*. Aflatoxin B1 (AFB1)-glutathione (GSH) conjugation is the major pathway for the detoxification of aflatoxin metabolites.

4.2. Ochratoxins: chemical characteristic, toxicity and bioavailability

Ochratoxins (A, B, and C) are secondary metabolites produced by *Penicillium* and *Aspergillus* filamentous fungi and in particular the A form (OTA) exerts hazardous effects in animals and in humans *(163)*. OTA was isolated for the first time and chemically characterized in 1965 in South Africa, when some corn meal was intentionally inoculated with *Aspergillus ochraceus (164, 165)*. In further studies, OTA was proved to be nephrotoxic, hepatotoxic, embryotoxic, teratogenic, neurotoxic, immunotoxic, genotoxic, and carcinogenic for many animal species with differences in the effects between species and sex: in pigs the amount of absorbed toxin is about 60%, in rodents it is much lower, while humans have the highest oral bioavailability (93%) *(166 - 168)*. Considering its effects, OTA has been classified as a group 2B by the International Agency for Research on Cancer (IARC) and World Health Organization (WHO), meaning possibly carcinogenic for humans, and it has been putatively implicated in the aetiology of Balkan endemic nephropathy (BEN) and related to urinary-tract cancer *(169, 170)*. Chronic exposure to low OTA doses could be even more dangerous than acute exposure to a high dose *(171)*. Humans are normally exposed to OTA through several routes, dietary intake being the most prominent. Dermal contact or inhalation exposures are of a minor importance with respect to the general population, although occasionally these routes may also play a role. The European legislation estimated the TDI for OTA at 5.8 ng OTA kg^{-1} body weight per day. However, as there are differences in food intake (per unit of body weight) for children, adolescents and adults, for a given concentration of a contaminant, a child or an adolescent will receive different exposure than an adult *(172)*.

OTA producing-fungi were found to be a contaminant in a wide variety of foodstuffs. Ochratoxin A is a main concern in cereals, wine, coffee, spices and dried fruits, but significant levels can be found also in cocoa, malt and beer, bread

and bakery products, cheese, poultry meat and kidneys, pig kidneys and pork sausages *(173 - 178).*

Chemically, ochratoxin A is a para-chlorophenolic moiety containing a dihydroiso-coumarin group that is amide-linked to L–phenylalanine. OTA (C20H18ClNO6; IUPAC name: N-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-isochromen-7-yl]carbonyl}-L-phenylalanine) is a white, odourless, heat stable, crystalline solid agent with poor aqueous solubility *(179)* (Fig. 9). Depending on the microenvironment, OTA exists in non-ionic, monoanionic (OTA^-), and dianionic (OTA^{2-}) forms. OTA does not completely denatured during baking and resists against three hours of high pressure steam sterilization at 121°C, while at 250 °C or during coffee roasting it is only partially degraded *(180 - 183).*

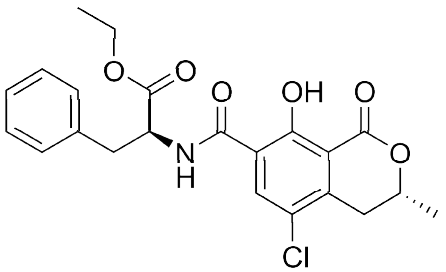

Figure 9. Structural formula of ochratoxin A

The non-ionic and monoanionic forms of OTA are absorbed from the stomach and the jejunum without known specific transport mechanisms *(184).* In the small intestine the Multidrug Resistance efflux transporter (MRP2) might slow down its absorption, since it acts transporting OTA back to the intestinal lumen. In vitro studies suggest that OTA alters the intestinal barrier and absorption functions, without causing effects on chloride secretion *(185).* In bloodstream, almost all OTA is bound by albumin. Two binding sites are recognised: the primary is

located on subdomain IIA (Sudlow's Site I) and the secondary, with much less affinity, on subdomain IIIA (Sudlow's Site II) *(186, 187).*

The tissue distribution of OTA is species-dependent and it is also largely influenced by many factors including the amount of toxin, the way of ingestion, the composition of the diet, and the health status of the body. However, the major targets of OTA toxicity are kidneys, liver, skeletal muscles and fat tissues *(188).* The toxicity exerts in the kidneys and the liver is mainly caused by two special transport mechanisms responsible for the active cellular uptake of OTA: the organic anion transporters (OATs) in the kidneys, and the organic anion-transporting polypeptides (OATPs) in the liver *(189, 190).* In the kidneys, basolateral OATs are mainly responsible for the uptake of OTA from blood into the tubule kidney cells, and the apical OAT4 transporter may be involved in the urinary reabsorption of OTA, resulting again with its accumulation in tubule cells. Furthermore, low doses of OTA can lead to the increased expression of more organic anion transporter isotypes in rat kidneys *(191).*

OTA can be bio-transformed and metabolized not only in the liver, but also in tissues, blood, and urine of animals and/or humans, exploiting other mechanisms. Both phase I and phase II enzymes transform OTA in metabolites that show low or no toxicity. Proteolytic enzymes and enzymes of the microbiota can hydrolyse part of OTA to Ochratoxin α (OTα), while in alkaline conditions the lactone ring is opened, resulting in a highly toxic compound called lactone-opened OTA (OP-OA) *(192).* The formation of further low toxic hydroxyl metabolites of OTA product of phase I reactions has been observed in different species *(193).* Some studies suggest that after dechlorination OTA is transformed to Ochratoxin B, which is less genotoxic *(194).* Among phase II reactions, sulfate, glucuronide, hexose/pentose (hex/pen-OTA) and glutathione (OT-GSH) conjugations of OTA are described as well *(195).*

The damages produced by OTA in cells are various. OTA acts as an inhibitor of phenylalanine t-RNA synthase and phenylalanine hydroxylase, resulting in the inhibition of protein synthesis. In addition to these non-specific ways of protein synthesis inhibition, OTA may influence the transcription of many proteins resulting in specific intracellular effects *(196).* OTA also has a negative effect on ATP (Adenosine Triphosphate) production. In fact, OTA interferes with the expression of phosphoenolpyruvate-carboxykinase (PEPCK), a key enzyme in gluconeogenesis, can penetrate into the mitochondria and probably binds to proteins involved in the maintenance of the membrane potential and in the oxidative phosphorylation, by interfering with phosphate transport and by inhibiting the electron transport *(197).* OTA also displays genotoxic effect, since the toxin forms electrophilic products that can covalently bind to DNA causing mutations, and subsequent formation of tumors *(198)* as well as oxidative damage for lipids and proteins *(199).* OTA can also cause both apoptotic and necrotic cell death, depending on the amount of toxin. It alters the expression of genes GADD153, GADD45, clusterin, p53, MAPK-, ERK-, p38, and JNK causing potentially apoptosis *(200).* In human kidney cells, OTA was proved to block the metaphase/anaphase transition, resulting in aberrant mitotic formations, giant cells and chromosomal instability, which lead to carcinogenesis *(201).*

4.3. Aflatoxins-producing fungi

Aflatoxins are produced mainly by four *Aspergillus* species: *Aspergillus flavus* Link ex Fr, *Aspergillus nomius* Kurtzman, Horn and Hesseltine, *Aspergillus parasiticus* Speare, and *Aspergillus tamarii (202). Aspergillus arachidicola, Aspergillus bombycis, Aspergillus ochraceoroseus, Aspergillus pseudotamari, Aspergillus toxicarius* and *Aspergillus parvisclerotigenus* are aflatoxin-producing species too, but they are less frequently found *(203 - 205).* The agronomically and economically most important aflatoxin producers are *A. flavus*, hence the name afla-toxin, and *A. parasiticus*. These species usually grow on living and decaying

plant matter, but they can colonize various commodities, especially corn, groundnut and cottonseed. *A. flavus*, the most common species involved in pre-harvest aflatoxin contamination of crops and groundnuts, is able to produce aflatoxins B1 and B2, whereas *A. parasiticus* can produce also aflatoxins G1 and G2 *(202)*.

Aspergillus are saprophytic soil-borne imperfect filamentous fungi, they can grow on wide variety of substrates when soil temperature ranges from 12 to 48 °C, with optimum of 25 to 42 ºC, and water potentials is as low as - 35 MPa. *Aspergillus* are ubiquitous in nature and have important roles in natural ecosystems and human economy because they are able to recycle starches, hemicelluloses, celluloses, pectins and other sugar polymers and degrade more refractory compounds, such as fats, oils, chitin and keratin. Both *A. flavus* and *A. parasiticus* reproduce only by asexual means, but can undergo genetic recombination through a parasexual cycle. There are great qualitative and quantitative differences in the toxigenic abilities displayed by different strains within each aflatoxigenic species: about half of *A. flavus* strains produce aflatoxins *(206)*. The incidence of non-toxigenic strains of *A. flavus* has shown to be variable depending on the geographical origin *(207, 208)* and on the substrate *(209)*.

As for the most micro fungi, the species identification belonging the *Aspergillus* genus currently is performed through the observation of microscopic morphological structures, like the conidiophores, cleistothecia, Hülle cells and sclerotia *(26)*. Morphological identification of Aspergillus mostly follows the protocols of Raper & Fennell *(210)*, Pitt & Hocking *(211)* and Samson and collaborators *(212)*. Molecular tools, especially phylogenetic species recognition, are increasingly being used with the internal transcribed spacers of the non-ribosomal DNA (ITS) now accepted as the official DNA barcode for fungi *(213)*. However, this locus is insufficient for correctly identifying all 180 species belonging the *Aspergillus* genus, and thus a secondary identification marker is

needed. The colonies of genus *Aspergillus* grow rapidly and consist of a dense felt of erect conidiophores, which vary from the shade of white, pink and yellow to brown, green and black. The conidiophores, which bring the reproductive structure, do not usually present any ramification and end with a vesicle. The apical vesicle forms the aspergillar head together with phialides, conidia and *metulae*, when present. Phialides can originate directly from the vesicle, in the case of uni-seriate heads, or can originate from *metulae* in the case of bi-seriate heads (Fig. 10). Conidia, the asexual spores, can be smooth or rough-walled, hyaline or pigmented, and are produced in long dry chains, which may be divergent (radiate) or aggregated in compact columns (columnar). Some species may produce Hülle cells, single cell or chain of cells with thick smooth wall, or sclerotia, irregular or round compact masses of iphae. Both Hülle cells and sclerozia are structure resistant to environmental stresses *(212)*.

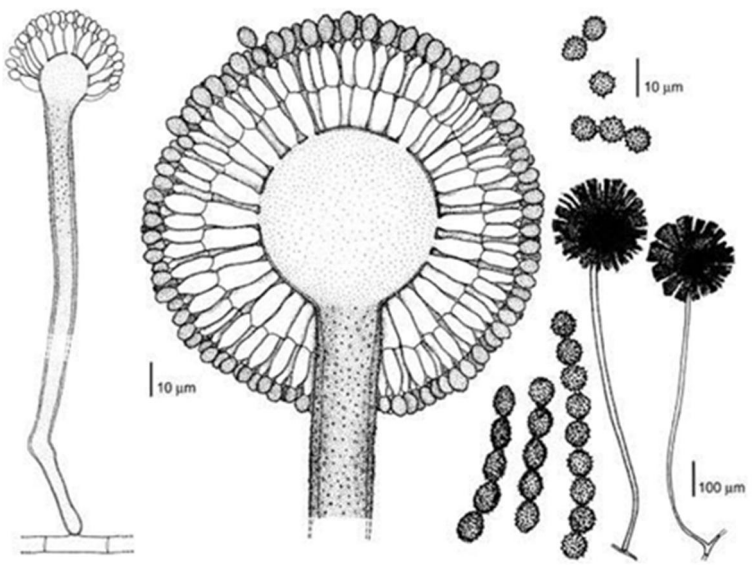

Figure 10. Reproductive structure of genus Aspergillus: conidiophore with uni-seriate and bi-seriate heads and conidia *(212)*.

Hedayati and co-Authors *(214)* reviewed the *A. flavus* complex and included 23 species or varieties, including two sexual species, *Petromyces alliaceus* and *P. albertensis.* On Czapek Dox agar, colonies of *A. flavus* are granular, flat, often with radial grooves, yellow at first but quickly becoming bright to dark yellow-green with age. Conidial heads are typically radiate, later splitting to form loose columns (mostly 300-400 µm in diameter), bi-seriate but having some heads with phialides borne directly on the vesicle (uniseriate) (Fig. 11). Conidiophore stipes are hyaline and coarsely roughened, often more noticeable near the vesicle. Conidia are globose to sub-globose (3-6 µm in diameter), pale green and conspicuously echinulated. Some strains produce brownish sclerotia.

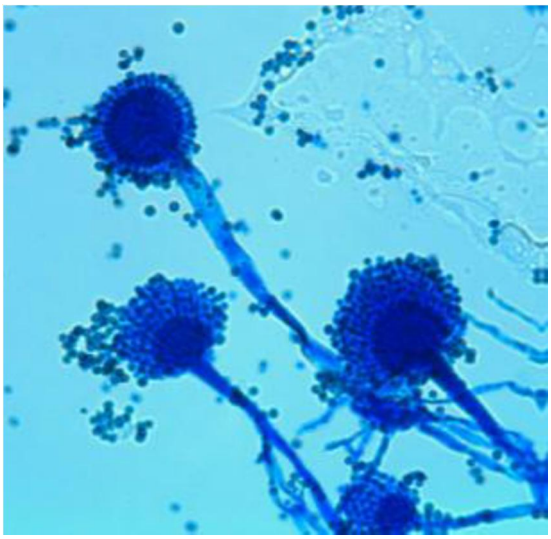

Figure 11. Typical uni-seriate and bi-seriate heads of *Aspergillus flavus.* Lactophenol-cotton blue wet mount. Total magnification: 400x.

A. flavus can be distinguished from *A. parasiticus* by its smooth spores and yellow-green colonies on Potato Dextrose agar (PDA) medium. *A. parasiticus* produces dark yellow-green conidia with nearly spherical vesicles that produce

roughened conidia. It can be readily distinguished from *A. flavus* by its rough-walled conidia *(202)*.

The synthesis of aflatoxins occurs through a series of highly organized oxidation-reduction reactions. Each genome of *A. parasiticus* and *A. flavus* has an approximate 70 Kb cluster, which includes 25 enzymes involved in aflatoxins biosynthesis. Fifteen of the 25 proteins encoded by the cluster are enzymes that catalyse oxidative reactions, six of which have the structural characteristics of cytochrome P450 mono-oxygenase. Most of the 25 genes in the aflatoxins biosynthetic pathway gene cluster are regulated by *aflR*, which encodes a zinc cluster motif (Cys-Xaa2-Cys-Xaa6-Cys-Xaa6-Cys-Xaa2-CysXaa6-Cys) similar to a family of fungal transcriptional activators associated with several metabolic pathways in fungi *(215)*. *AflR* binds to the palindromic motif 5′-TCGN5CGA-3′ in the promoter region of aflatoxin structural genes. The aflatoxin genes have one or two predicted *AflR* binding sites within 200 bp of the translation start site, although some binding sites occur further upstream. Some of the promoter regions of aflatoxin contain consensus-binding sites, involved in ambient pH regulation *(216)* and in nitrogen regulation *(217)*. This indicates that aflatoxin biosynthesis may be responsive to environmental conditions such as pH and nitrogen source. The regulation of fungal metabolism by pH involves a globally acting transcription factor encoded by *pacC* that is post-synthetically modified by a pH-sensing protease. It has been established that aflatoxin synthesis optimally occurs in the pH range of 3.4~5.5 *(218)*. In some isolates, nitrate suppresses aflatoxin synthesis, whereas nitrogen supplied as ammonium in media supports it *(219)*. The carbon source, and the availability of a precursor unit such as acetyl-CoA, may also affect aflatoxin biosynthesis.

At least 18 enzyme steps are required for the conversion of acetyl coenzyme A (acetyl CoA) to its final products, AFB1, AFB2, AFG1, and AFG2. Aflatoxin biosynthesis begins with the conversion of malonyl CoA to a condensed

polyketide noranthrone, by the products of two fatty acid synthase genes (*fas1* and *fas2*) and a polyketide synthase (PKS) gene. The *pksA* of *A. parasiticus* contains four conserved domains typical of other known PKS and FAS proteins, but it lacks two reductase domains, which are generally essential for fatty acid synthesis. The absence of ketoreductase in *pksA* is consistent with the lack of a reductive step in the PKS reaction that produces noanthrone. It is unclear whether the conversion of noranthrone to norsolorinic acid (NOR), the first stable metabolite, requires a specific enzyme. NOR is converted to averantin (AVN) mainly by a cytosol oxidoreductase. AVN is hydroxylated to form 5′-hydroxyaverantin (HAVN) by AVN mono-oxygenase, which shows strict stereospecificity for the 1′ carbon of AVN. The conversion of HAVN to averufin (AVR) occurs by two cytosilic enzymes: HAVN dehydrogenase catalyzes the first reaction from HAVN to a 5′-oxoaverantin (OAVN), and OAVN cylase, catalyzes the second reaction from the OAVN to AVR *(220).* AVR is converted to hydroxyversicolorone (HVN) by the *cypX* gene encoding a cytochrome 450 monooxydase, and HVN is converted to versiconal hemiacetal acetate (VHA) by the *moxY* gene encoding the HVN monooxygenase *(221).* VHA is converted to VAL mainly by the *estA* gene encoding an esterase. VAL is converted to VERB by the *vbs* gene encoding VAL cyclase. This is a key step in aflatoxin formation since it closes the bisfuran ring of the aflatoxin, which is required for binding to DNA, and gives aflatoxins their mode of action as mutagens. VERB is desaturated to versicolorin A (VERA) presumably by verB, a homolog of stcL in *A. parasiticus,* encoding the desaturase. The VERB contains a tetrahydro-bisfuran ring in its structure like AFB2 and AFG2, while VERA contains a dihdrobisfuran ring similar to AFB1 and AFG1. Thus, the branching step between AFB1/AFG1 and AFB2/AFG2 is the desaturation reaction from VERB to VERA *(222).* The conversion of VERA to demethylsterigmatocystin (DMST) has a structural change by more than one reaction, such as a combination of oxidative decarboxylation, hydrogenation, and two dehydrations. DMST and DHDMST are

methylated to ST and dihydrosterigmatocystin (DHST), respectively, by omtB, which is the O-methyltransferase I that catalyzes the transfer of the methyl groups from S-adenosylmethionine (SAM) to the hydroxyl groups of DMST and DHDMST. The further methylation of ST and DHST by omtA, Omethyltransferase II, results in O-methylsterigmatocystin (OMST) and dihydro-O-methylsterigmatocystin (DHOMST), respectively. The two O-methtyltransferases, omtB and omtA, show strict substrate specificity, because omtA cannot methylate DMST or DHDMST in spite of free 7-OH groups being present in these compounds *(223)*. OMST and DHOMST are converted to aflatoxins B1 and B2 respectively. Ehrlich and colleagues (2004) *(224)* demonstrated that the conversion of OMST and DHOMST to aflatoxins G1 and G2 requires two monooxygenases, ordA and cypA. This is consistent with the fact that *A. flavus* producing only aflatoxins B1 and B2 is missing portions of the gene *cypA*.

4.4. Ochratoxin-producing fungi

OTA was firstly purified in corn meal intentionally inoculated with *Aspergillus ochraceus*. Then, also *Aspergillus niger* var. niger, *Aspergillus foetidus* and *Aspergillus carbonarius* were found able to produce OTA *(225, 226)*. The OTA-producing *Aspergillus* species, *A. carbonarius* and the closely related *A. niger* which produces OTA more rarely, grow well at temperatures above 20 °C and produce pigmented hyphae and spores, making these species resistant to UV light: for these reasons these moulds are commonly found in grapes and similar fruits that mature in sunlight and at high temperatures *(227)*. In 2004 two new OTA-producing species of *Aspergillus* section *Circumdati*, *A. westerdijkiae* and *A. steynii*, were isolated from coffee, and in 2005, some other strains of *Aspergillus tubingensis* were found able to produce OTA in grapes *(228, 229)*. OTA can also be produced by some *Penicillium* species. In 1969, Walbeek and collaborators

(230) isolated OTA from *Penicillium viridicatum*, but due to considerable revisions in taxonomy of genus *Penicillium*, that OTA-producing strains of *P. viridicatum* corresponds to the current *P. verrucosum,* as indicated by Pitt *(231).* In 2001, *Penicillium nordicum* was determined and confirmed as the second OTA-producing *Penicillium* species *(232).* The two OTA-producing *Penicillium* species live in different ecological niches: *P. nordicum* generally contaminates food rich in NaCl and protein, such as cheeses and dry cured meats, while *P. verrucosum* usually contaminates cereals. Under many laboratory conditions, *P. nordicum* produces more OTA than *P. verrucosum* isolates *(233).*

As for other filamentous fungi, the species identification of *Aspergillus* and *Penicillium* genera is achieved through the observation of reproductive structures. *Aspergillus* section *Circumdati* includes species with rough walled stipes, biseriate conidial heads, yellow to ochre conidia and sclerotia that do not turn black (Fig. 12). Twenty species can be distinguished into the *Aspergillus* section of *Circumdati*. The taxonomy of this section remains in progress, and Frisvad and co-Authors *(228)* recently proposed the division of *A. ochraceus* into two species, *A. ochraceus* and *A. westerdijkiae.* Several species in the section *Circumdati* are able to produce OTA in culture medium, but the main species responsible for the presence of OTA in foods are *A. ochraceus* and *A. westerdijkiae (217). A. westerdijkiae* and *A. ochraceus* are very similar, and several isolates previously identified as *A. ochraceus* are now recognized as *A. westerdijkiae*, including the original OTA-producing strain (NRRL 3174). Amplification and sequencing of the ITS1-5.8S-ITS2 region from several Brazilian strains of both species showed specific nucleotide variations that distinguish *A. westerdijkiae* and *A. ochraceus (234).* In ITS1, all sequences of A. westerdijkiae differed from the *A. ochraceus* sequences by possessing a C instead of a T at positions 76 and 80. In addition, *A. ochraceus* has a deletion of a T at position 89. In ITS2, specific nucleotides at position 494–495 (AT) characterized the strains of *A. westerdijkiae*, compared to a TC at this position in *A. ochraceus*. Moreover, a T at position 487 is deleted

only in *A. ochraceus* strains. Similarly, Morello and co-workers *(235)* detected 39 species-specific single nucleotide polymorphisms within the b-tubulin genes from *A. westerdijkiae* and *A. ochraceus*, most of them (97.4%) in intronic regions.

Black aspergilli are one of the more difficult groups concerning classification and identification, and several taxonomic schemes have been proposed. The black aspergilli, *Aspergillus* section *Nigri,* include species with uniseriate or biseriate conidial heads, spherical to pyriform vesicles, smooth-walled stipes and black or near black-coloured conidia (Fig. 12). The differences between some species belonging to section *Nigri* are very slight and their discrimination requires molecular analysis. A total of 16 species are recognized in *Aspergillus* section Nigri: *A. aculeatus*, *A. brasiliensis*, *A. carbonarius*, *A. costaricaensis*, *A. ellipticus*, *A. ellipsoides*, *A. japonicus*, *A. foetidus*, *A. homomorphus*, *A. heteromorphus*, *A. lacticoffeatus*, *A. niger*, *A. piperis*, *A. sclerotioniger*, *A. tubingensis*, and *A. vadensis*, with the latter taxon recently described as a new species *(231). A. niger* sensu stricto, *A. tubingensis*, *A. foetidus* and *A. brasiliensis* are morphologically identical and collectively have been called the *A. niger* aggregate. Although the taxa included in the *A. niger* aggregate are morphologically indistinguishable, they differ in their ability to produce OTA and other metabolites. The ability of species other than *A. niger* "sensu stricto" within *A. niger* aggregate to produce OTA remain uncertain, probably due to the complexity of species identification.

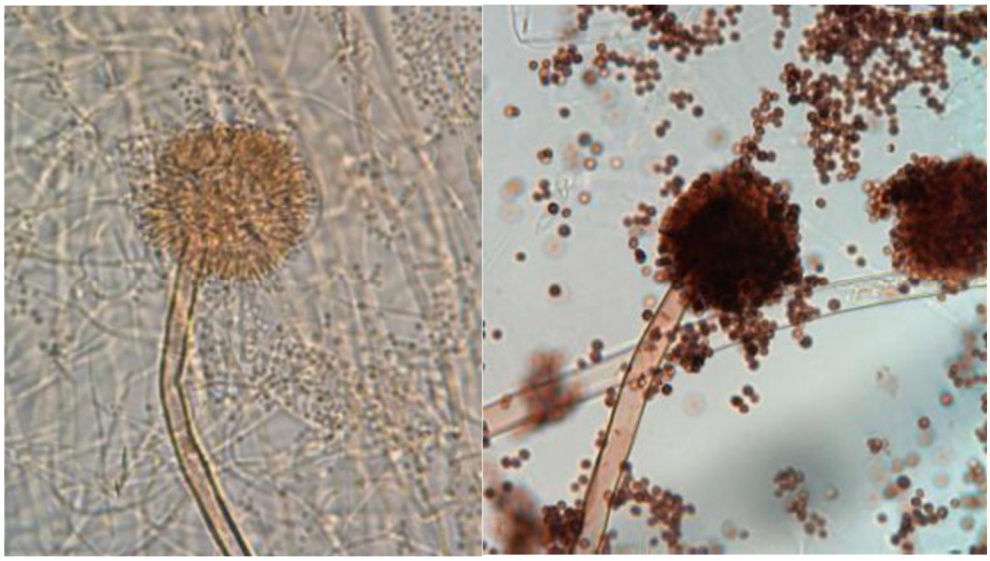

Figure 12. From the left: *Aspergillus ochraceus* and *Aspergillus niger* conidial heads. Lactophenol wet mount. Total magnification: 400x.

Penicillium taxonomy assignment is not easy for the inexperienced, and compared to *Aspergillus* it is a more diverse genus, in terms of numbers of species and range of habitats. All *Penicillium* are characterized by "brush-like" reproductive structures, with different branching patterns (Fig. 13). The conidiophores range from being simple to very complex patterns with multiple levels of branching resulting in overall symmetrical or asymmetrical patterns. Monoverticillate conidiophores have a terminal whorl of phialides and in some species, the terminal cell of the conidiophore is slightly swollen or vesiculate. Biverticillate conidiophores have a whorl of three or more metulae between the end of the stipe and the phialides; the metulae may be of unequal or equal length, vary in their degree of divergence, are usually more or less cylindrical but can also be clavate or slightly vesiculate. Terverticillate conidiophores have another level of branching between the stipe and the metulae, often just a continuation of the stipe axis and one side branch, sometimes a true whorl of three or more

branches. Quaterverticillate conidiophores are produced by only a few species, and have one extra level of branching beyond the terverticillate pattern. Presently, *P. verrucosum* and *P. nordicum* are the only OTA producers known and accepted in this genus, despite some reports on OTA production by other species. Nevertheless, different examples of incorrect citations of some *Penicillium* spp. producing OTA (*P. cyclopium*, *P. viridicatum*, *P. chrysogenum*) have been recently listed *(236).* It is worth bearing in mind that in the last century, OTA producers in this genus were classified as *P. viridicatum* for many years. *Penicillium verrucosum* and *P. nordicum* have common morphological characteristics, such as very similar colony diameters on many culture media or rough stipes. These are slow growing species of the subgenus *Penicillium*, which is by far the most difficult taxonomically, both because there are numerous species and because apparent differences between species are small. Both species have conidiophores hyaline, terverticillate, with all element appressed, stipes rough-walled and conidia globose, greenish and smooth-walled. Most of the isolates of *P. verrucosum* have a characteristic dark brown reverse colour on Yeast Extract Sucrose agar (YES), whereas almost all the *P. nordicum* strains show a pale, creamy or dull yellow reverse colour in this culture medium. The colony pattern of *P. viridicatum* is different on YES. Among other differences, Frisvad & Samson *(219)* considered *P. verrucosum* among the species always negative (no reaction) or occasionally producing a yellow reaction for the Ehrlich test, and *P. nordicum* among the species with a yellow reaction. These coloured reactions are related to the production of some alkaloids. Many species classified in this subgenus are morphologically similar, and identification using traditional morphological techniques remains difficult and a polyphasic approach, including a combination of DNA sequences, extrolites production and other phenotypical characters, is suggested for the species identification.

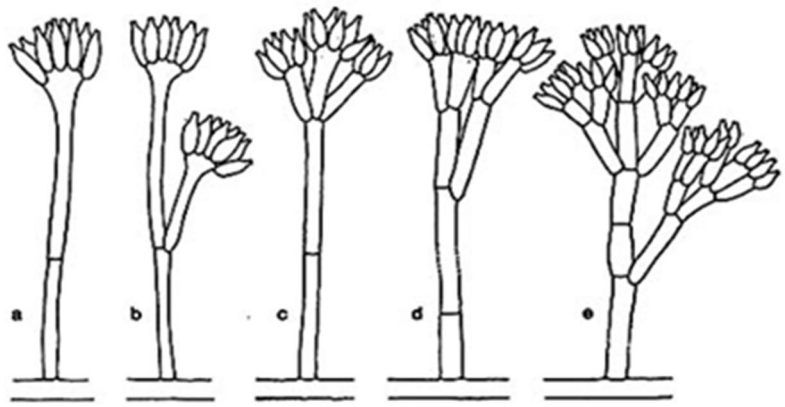

Figure 13. Reproductive structures of genus Penicillium. Conidiophores: a.b. monoverticillate; c. biverticillate; d. terverticllate; e. quaterverticillate. *(212)*

From the structure of OTA, its dihydrocoumarin moiety consists of a polyketide that is catalyzed by the enzyme polyketide synthethase (PKS). Then the enzyme non ribosomal peptide synthase (NRPS) links the polyketide moiety to L-phenylalanine through the carboxyl group *(237).* In *P. nordicum*, a putative gene cluster including a polyketide synthase (*otapksPN*) and a non-ribosomal peptide synthetase (*npsPN*), was recognised as responsible for OTA biosynthesis *(238).* OTA biosynthetic pathway had been proposed by Huff and Hamilton (1979) *(239).* They supposed that mellein, catalyzed by PKS, was oxidized to OTβ and then transformed to OTα by a halogenase/chloroperoxidase. Subsequently, OTα was esterified to ochratoxin C via link with the ethyl ester, and finally biosynthesized to OTA by a de-esterification reaction. However, they ignored the putatively ubiquitous intermediate, OTB, in some processes of OTA biosynthetic pathway. During the study on *pks* gene involved in OTA biosynthesis in *A. westerdijkiae* NRRL 3174, it was found that mellein played no role in OTA biosynthesis on analyzing the secondary metabolites *(240).* This was further supported by 14C-labelled precursor feeding experiments, which did not support

the intermediary role of mellein confirmed by Harris and Mantle *(241)*. In Harris and Mantle's study, they proposed that OTβ to OTα is catalyzed by a halogenase/chloroperoxidase and eventually to OTA via an amide bond with phenylalanine. Chlorination of OTα probably preceded the biotransformation from OTα to OTA. This indicated that chlorination was a penultimate biosynthetic step in OTA biosynthesis. Moreover, they proposed an alternative pathway in account of the role of OTB in which OTA was transformed through the synthetic step from OTβ to OTB via an amide group, but this did not explain the role of OTα involvement in OTA biosynthesis. It seems to support that OTB was not a by-product of OTA because the levels of OTA and OTB produced by *A. ochraceus* differed from one carbon or nitrogen source to another *(242)*. Gallo and co-Authors *(243)* hypothesized a OTA biosynthetic pathway similar to the alternative pathway of Harris and Mantle, in which OTα was mostly likely to be derived from the biodegradation of OTA in *A. carbonarius* ITEM 5010. For the first time they proved the involvement of an NRPS in the OTA biosynthetic pathway of an *Aspergillus* species. The absence of OTA, OTB, and OTα and the concomitant increase of OTβ concentration in the culture of *A. carbonarius* ITEM 5010 confirm the hypothesis that the bond between the phenylalanine and the polyketide dihydroisocoumarin, catalyzed by the synthetase, precedes the chlorination step, clarifying the order of reactions in the OTA biosynthetic pathway. Moreover, the non-OTA-producing keeps the capability to degrade OTA in OTα.

4.5. Analytical methods for the detection of aflatoxins and ochratoxin A

Aflatoxins and ochratoxin A contaminate different foods and foodstuffs in many steps of processing. Therefore, their presence in foods and feeds represents a constant health risk for animals and humans. Regulations relating to mycotoxins

have been established in many countries to protect the consumer from the harmful effects of these compounds. In several countries, these contaminants are subject to legislation that is based on the establishment of an Acceptable daily intake (ADI) or Tolerable daily intake (TDI). Different factors play a role in the decision-making process of setting limits for mycotoxins, including the availability of toxicological data and exposure data on mycotoxins, the knowledge of the distribution of mycotoxin concentrations within commodity or product lots, the availability of analytical methods and the legislation in other countries with which trade contacts exist. For the mycotoxins currently considered the most significant (AFB_1, B_2, G_1 and G_2; AFM_1; OTA; patulin; FB_1, FB_2, and FB_3, ZEA, T-2, HT-2 toxins and DON), the Joint Expert Committee on Food Additives (JECFA-Scientific Advisory Body of the World Health Organization WHO) and the Food and Agriculture Organization (FAO) has evaluated their hazard in several sessions *(244, 245)*. The correct evaluation of mycotoxin contamination and prevalence in foodstuffs depends principally on the degree of accuracy associated with the individual steps by which this information is obtained. Because the distribution of mycotoxins in the food matrix is often highly heterogeneous, taking a representative sample is the most critical stage. Thus, the error associated with sampling procedures is notably higher than that associated with subsampling or analysis. In most cases, successful sampling includes two steps: the primary sampling consists of taking the decision on "why, where and when" to collect the samples, then the secondary sampling consists of establishing how samples should be collected in order to be representative of the lot under investigation. The European legislation has approved a regulation dealing with sampling and methods of analysis of mycotoxins for official control (2006) to limit the sources of errors in evaluating the impact of mycotoxins on human health.

A part from sampling, reliable analytical methods are necessary to detect mycotoxins in daily practice. In addition to reliability, simplicity is desired, as it will affect the amount of data generated and the practicality of the ultimate

measures taken. The limits of determination of the methods need to be low enough for precise and accurate determination of the mycotoxins of interest at regulatory levels. Methods were also developed and validated for toxin–matrix combinations for which there were no regulations. In Europe, ten mycotoxin methods have been standardized by the CEN (European Committee for Standardization), and this number will grow substantially in the years to come. Although CEN mycotoxin methods are not mandatory for official food control in the EU, all CEN mycotoxin methods can be used in the EU for official food-control purposes, because their performance characteristics fulfill the criteria established by the EU regulation for sampling and analysis *(7).* Most mycotoxins are characterized by a low molecular mass and are soluble in a range of organic and aqueous organic solvents. For this reason, they can be separated and detected by chromatographic methods such as thin-layer chromatography (TLC), liquid chromatography (LC) and gas chromatography (GC) *(246).*

Before the separation and the detection, mycotoxins must be exhaustively extracted from sample. The purpose of the extraction step is to remove as much of the mycotoxin from the solid sample as possible, and distribute it into a liquid phase. Extraction can be performed by liquid-liquid extraction (LLE) by using two immiscible phase solvents, or solid phase extraction (SPE) by using a solid and a liquid phase (246).

TLC is one of the first methods used for detecting mycotoxins. This technique enables screening of large number of samples, and it is easy to perform and cost-effective. After extraction and clean-up, each sample is applied to a silica gel layer, or in alternative to phenyl non-polar bonded, silanized and polyamide layers, and separated using organic solvent. Mycotoxins are visualized on the TLC plate by observing under UV light and comparing with a standard, or by spraying chemicals that react with mycotoxins enhancing their fluorescence (or producing coloured derivatives). Aflatoxins and ochratoxin A are naturally

fluorescent compounds; hence, they are identified based on their fluorescent properties. Semi-quantitative analysis has been carried out for mycotoxins by TLC; however, this method shows low sensitivity. The TLC method has been improved in High-Performance Thin Layer Chromatography (HPTLC) to enhance the resolution and accuracy *(247)*.

HPLC, especially with UV and fluorescence detectors, is the one of the most frequently used both for routine analyses and as a confirmatory method for novel or screening techniques. Most mycotoxins are relatively small polar compounds and can be separated by reverse-phase HPLC using a mobile phase made from the composition of water, acetonitrile or methanol. The stationary phase is made by silica particles of small size, modified with a hydrophobic layer, mostly is C18. Due to selectivity and sensitivity, a fluorescence detector is preferred for mycotoxin analysis, whereas UV is used for mycotoxins like patulin and moniliformin, because of their strong UV absorption. Ochratoxin A in wine has been accurately detected by HPLC following immunoaffinity clean-up, with a detection limit of 0.01 ng/mL *(248)*. HPLC with fluorescence detector has been used for detecting aflatoxin B1 and ochratoxin in rice, and a detection limit of 0.07 and 0.08 µg/ kg, respectively, for these mycotoxins was reported by Nguyen and collaborators *(249)*.

To eliminate the need for sample derivatization for fluorescent activity, methods of Liquid Chromatography coupled with Mass Spectrometry (LC-MS) were developed. Spanjer and colleagues *(250)* had developed an LC-MS/MS method to detect 33 mycotoxins, including aflatoxin B1, B2, G1 and G2, and ochratoxin A with a limit of quantification of 1 µg/kg, simultaneously in various food materials. After separating the sample into chemical compounds by HPLC, a mass spectrometer will ionize, sort and identify these compounds based on the mass-to-charge ratio (m/z). Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are ionization techniques that are widely used for

different molecular weights and polarity of compounds. Furthermore, the most important mass analysers in mycotoxins analysis are Triple Quadrupole, Ion-trap and Time of flight. After selecting an appropriate mass analyser, all MS parameters such as cone voltage, capillary voltage, nebulizer gas, cone gas flow, desolvation temperature, mass resolution and collision energies have to be set for target mycotoxins. Several LC-MS methods for multiple mycotoxin analysis have been reported, which include extraction, sample pre-treatment and reverse-phase LC-MS quantification. An ultra-high-performance liquid chromatography combined with electrospray ionization triple quadrupole tandem mass spectrometry (UHPLC-ESIMS/MS) has been developed to determine aflatoxin M1 and ochratoxin A in milk. The limits of quantification of these toxins were reported to be in the range 0.003 to 0.015 μg/kg *(251)*.

For the detection of mycotoxins volatile at the column temperature (or can be converted into volatile derivatives), like trichothecenes, Gas Chromatography (GC) is often exploited. Electron Capture Detection (ECD), Mass Spectrometry (MS) and flame ionization are the common detectors used with GC. There are a few disadvantages with this method, such as the need for derivatization and thermal stability of mycotoxins, where heating degrades the samples.

All these chromatographic methods are time consuming for sample preparation and need expensive instruments. Therefore, rapid and cheap methods based on immunoassays, such as the Enzyme-Linked Immunosorbent Assay (ELISA), are preferred for mycotoxin screening nowadays. The direct competitive ELISA *(252)* is commonly used in mycotoxin analysis. Briefly, mycotoxin is extracted from a ground sample with solvent, a portion of the sample extract and a conjugate of an enzyme coupled to the mycotoxin are mixed, and then added to the antibody-coated microtiter wells. After washing, an enzyme substrate is added and colour develops. The intensity of the colour, in this case, is inversely proportional to the concentration of mycotoxin in the sample. Due to low sample volume

requirements and the fact that a clean-up step is not needed, ELISA test kits are more used than TLC or HPLC. ELISA technique has been used for determining aflatoxin in a large number of foods: Reddy and co-workers *(253)* have used indirect competitive ELISA for detecting aflatoxin B1 in rice with a detection limit of 0.02 ng/kg.

Alternative methods for the detection of mycotoxins exploit fluorescence properties characteristic of several mycotoxins. A novel biosensor based on surface plasmon-enhanced fluorescence spectroscopy has been developed for detecting aflatoxin M1 in milk with a detection as low as 0.6 pg/mL *(254)*. Also the use of infrared spectroscopy has proved to be a promising technique for the fast and non-destructive detection of mycotoxins in food grains. Near-infrared spectroscopy technique has also been used to detect aflatoxin B1 and ochratoxin A in red paprika in Spain *(255)*. Lateral flow or dipstick immunoassays, developed using the principal of ELISA, are successfully used for detecting mycotoxins. This technique was used for screening aflatoxin B1 and ochratoxin A simultaneously in chili samples, with limit of quantitation of 2 and 10 μg/kg, respectively *(256)*.

Table 5. Advantages and disadvantages of traditional and emerging methods for mycotoxin analysis *(data from 247, 250, 252)*.

Method	Advantages	Disadantages
Gas Chromatography	Simultaneous analysis of mycotoxins, good sensitivity, may be automated (autosampler), provides confirmation (MS detector).	Expensive equipment, specialist expertise required, matrix interference problems, non-linear calibration curve, carry-over effects from previous sample.
High Performance Liquid Chromatography	Good sensitivity, selectivity and repeatability, may be automated, short analysis times, official methods available.	Expensive equipment, specialist expertise required, may require derivatization.
Liquid Chromatography/ Mass Spectrometry	Simultaneous analysis of mycotoxins, good sensitivity, provides confirmation, no derivatization required.	Expensive, specialist expertise required, sensitivity relies on ionization technique, matrix assisted calibration curve (for quantitative analysis), lack of internal standards.
Enzyme-Linked Immunosorbent Assay	Inexpensive sample preparation and equipment, high sensitivity, simultaneous analysis of multiple samples, suitable for screening, limited use of organic solvents.	Cross-reactivity with related mycotoxins, matrix interference problems, possible false positive/negative results, confirmatory LC analysis required.
Lateral Flow Device	Rapid, no clean-up, no expensive equipment, easy to use, no specific training required.	Semi-quantitative, cross-reactivity with related mycotoxins, validation required for additional matrices.
Biosensors	Rapid, no clean-up procedure	Cross-reactivity with related mycotoxins, extract clean-up needed to improve sensitivity, variation in reproducibility and repeatability.

Removal of mycotoxins from all kind of foodstuffs and raw products is currently not feasible without affecting sensorial and nutritional properties at reasonable affordable costs. Physical, chemical and enzymatically-based removal work on grains and cereals, but they are not applicable for many other animal-derived foods *(19, 257)*. Therefore, prevention (in field and after harvest, as well as during processing and preservation) remains the most reliable strategy to limit and manage risks for the consumers, also limiting economical losses due to the destruction of contaminated foods. Approaches for early detection of the toxigenic fungi colonizing foods is increasingly taken in account to prevent OTA entering the food chain.

Traditionally, fungi can be recognised by the observation of characteristics (morphology, phenotypic traits) that are generally developed and exposed following the cultivation onto certain (specific or not specific) media. Microscopic characteristics like the hyphal structure, the morphology of reproductive structures and the presence of particular resistance structures like sclerotia, are examined under a light microscope. The traditional cultivation-dependent methods for the isolation and identification of *Aspergillus* and *Penicillium* from food samples are time-consuming and require a high knowledge of fungal taxonomy. Even with taxonomic expertise, identification is commonly difficult in some genera of fungi that contain a large number of closely related species. Therefore, the application of molecular biology techniques is a good alternative to traditional identification techniques, because it can reduce the time for fungal detection and identification, it is sensitive and specific and it potentially allows accurate identification of fungal species without the need for isolating pure cultures. The selection of target sequence specific for a given mycotoxin-producing fungus is a key process in the development of a PCR-based diagnostic assay. These target sequences used for designing PCR primers may be divided into two groups: i) “anonymous” DNA sequences identification at species level, and ii) functional genes related to a particular ability of a species (or a group of

species). Anonymous DNA sequences of genomic DNA may or may not contain functional genes and can be used ad targets for PCR primers to detect mycotoxigenic fungi. On the other hand, genes encoding enzymes involved in mycotoxin synthesis can be used to identify all fungi that share the ability to produce a mycotoxin.

Real-time Polymerase Chain Reaction (Real-time PCR) significantly reduces time and manual work, making it appropriate for large-scale analyses. Real-time PCR is a laboratory technique based on the polymerase chain reaction, amplifying and simultaneously quantifying a targeted DNA molecule. Unlike classical end-point PCR, real-time PCR is more sensitive and does not require gel electrophoresis. By using real-time PCR it is possible to detect an increase in fluorescence emission during the reaction, which is proportional to the initial copy number of the target sequence. The initial amount of template DNA is inversely proportional to a parameter measured for each reaction, which is called threshold cycle (Ct). The Ct value is the PCR cycle when the fluorescence signal increases above the background threshold. The application of this method to natural samples can provide an estimate of infection by a given species, but the presence of a mycotoxigenic fungus in a food sample does not ultimately indicate the production of OTA. The formation of OTA depends strongly on environmental conditions such as substrate, water activity, pH, and temperature.

The OTA biosynthetic pathway has not been entirely characterized in any of the OTA-producing species; consequently, the genes that encode enzymes involved in the biosynthesis of this secondary metabolite are poorly known. Because of this, in several PCR-based assays genes that were not associated to the ochratoxin biosynthesis, such as ribosomal RNA, and in particular ITS (Internal Trascribed Species) regions, β-tubulin, and calmodulin genes have been employed *(235, 258, 259).* A real-time PCR system based on the *otapksPN* sequence was used to monitor the growth and OTA production of *P. nordicum* in wheat *(260).* A strong

correlation between the copy numbers of the *otapksPN* gene and colonies forming units was observed.

Another different molecular approach can be explored for differentiating foods contaminated by mycotoxigenic fungi from safe food. If there is no information available about genes common to all mycotoxins biosynthetic pathway, Random Amplified Polymorphic DNA (RAPD) analysis can be exploited. RAPD proved to be a suitable molecular technique to study the genetic similarity or diversity among population, and resulted useful to determine the geographical origin of certain crops, as well as to identify polymorphism, enhancing the presence of single base mutations. Instead of using two primers that are designed based on pre-existing knowledge of the target sequence, RAPD profiles are generated through the random amplification of genomic DNA using a pool of single short primers (decamers) and the electrophoretic separation of the obtained fragments. Therefore, differences in the nucleotide sequence at the site complementary to the primer, cause the lack of annealing of the primer, and the consequent lack of the amplicons production, resulting in a different pattern of amplified DNA segments on the gel. RAPD technique has been exploited since it is simple, rapid and cost-effective *(261, 262)*.

5. DNA-based methods for cultivar identification

Plant variety and cultivar identification is one of the most important aspects in agricultural systems. The large number of varieties among crop plants has made difficult to identify and characterize varieties solely based on morphological characters because they are non-stable since they are an outcome of adaptation to environmental and climatic conditions. Molecular markers together with statistical tests and softwares are essential to effectively discriminate between closely related cultivars and characterize the required plant cultivars or varieties for cultivation, breeding programs as well as for cultivar-right-protection *(263)*. Hence, DNA-based techniques have become the main tool for cultivar genotyping, allowing both appropriate management of reference germplasm collections and effective varietal traceability during certified production.

Many of molecular markers do not require prior knowledge of genome sequence for primer design, such as Randomly Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) markers, while for other marker systems such as microsatellites, Sequence-Characterized Amplified Region (SCAR) and Single-Nucleotide Polymorphism (SNP), the target nucleotide sequences that must be known. Over the last few years, a rapid improvement occurred due to the development of several new high-throughput methods for microsatellite analysis and SNP genotyping that are suitable for automation, including High-Resolution Melting (HRM). These technologies greatly reduced the operating times and opened new research perspectives by allowing the analysis of a large number of markers in a large number of samples. All the main classes of molecular markers have been used for cultivar identification and traceability purposes *(264)*.

RAPD markers have been broadly employed for cultivar identification and certification purposes. RAPDs are generated by polymerase chain reaction (PCR) using 10-mer random primers *(265)*. These primers randomly anneal to several loci at relatively low temperatures and lead to the amplification of many sequences, potentially providing many markers per assay. However, the low annealing temperature may cause problems with reproducibility, necessitating preliminary selection of the most repeatable primers and regular comparisons of amplified profiles with reference samples. Fabbri and colleagues *(266)* screened 17 olive cultivars from the Mediterranean basin using 40 primers and all cultivars could be differentiated with a few primers, demonstrating the effectiveness of RAPD markers. Some RAPD-based studies, oriented towards restricted geographical areas, aimed to determine the origin of specific cultivars, give insight into local varietals or establish the geographic origin of samples *(267-269)*.

AFLP markers involve the use of restriction enzymes (using a frequent cutter combined with a rare cutter) to specifically digest sample genomic DNA: the obtained restriction fragments are then ligated to specific adaptor sequences that serve as target sites for subsequent PCR amplification with adaptor-directed primers. The amplicons are separated in gel electrophoresis and the size patterns are visualized. In spite of being more laborious and less easy to handle than RAPDs, AFLPs have been employed in several studies since the end of the 1990s as an alternative tool for revealing inter- and intra-cultivar variability (Table 6) *(270, 271)*.

SCARs are specific molecular markers that can be obtained by sequencing polymorphic amplified fragments generated by other marker systems (such as RAPD or AFLP) so as to design specific flanking primers. Though less polymorphic than other markers, SCARs are useful for cultivar identification.

Among the marker which require the knowledge of the genome sequences, microsatellites or simple sequence repeats (SSRs) are very exploited. This hypervariable short tandem repeats of DNA can be repeated different times in different individuals and the polymorphism is due to variations in the number of repeats. The development of microsatellite markers requires a laborious and expensive procedure involving the construction of a genomic library, cloning, sequencing and primer design. These markers, unlike RAPDs and AFPLs, require specific primers, significantly lowering the risk of non-specific amplifications. Hence, the advantages of highly reproducible and polymorphic markers largely compensate for the initial efforts by simplifying the successive analyses. Being by far the most reproducible class of molecular markers, microsatellites have been considered the markers of election with the aim of developing a robust method for cultivar identification. PCR using the unique sequences of flanking regions as primers and exploiting either dye-based or the fluorescent report probe detection are used to detect microsatellites.

PCR-based techniques are used also to identify SNPs, variations in a single nucleotide that occurs at a specific position in the genome. In both plant and animal genomes the extraordinary abundance of these markers has been estimated as one SNP every 100–300 bp. They may be found in both the non-repetitive coding or regulatory sequences and the repetitive non-coding sequences. While the polymorphism of microsatellite markers is due to length variations, SNPs occur when a single base pair shows different nucleotide alternatives in the genomic DNA. The principle of detection is to distinguish a perfect match from a single base mismatch, at the SNP site, between a probe of known sequence and the target DNA *(272, 273)*. A limited number of SNP markers has been developed in the initial studies, based on known gene sequence analysis. Since SNP markers need only a plus/minus assay, several more effective high-throughput approaches suitable for automation have been successively set

up for their analysis. High-density oligonucleotide arrays on DNA chips have been developed to analyse genotypes for SNPs. These chips use nucleic acids immobilized on solid-state surfaces, to be hybridized with the sample, without requiring the time-consuming and labour-demanding assays that are needed for most molecular markers (RAPD, AFLP and SSR), essential for size separation of multiple DNA fragments. Microsatellite analysis can be performed on agarose or polyacrylamide gels or, more conveniently, by capillary electrophoresis on automated sequencers.

Table 6. Comparison of selected genetic markers employed in plant *identification (data from 265, 270-272).*

Feature	**SSR**	**RAPD**	**AFLP**
Maximum tumber of possible loci in analysis	Limited by the size of genome and number of simple repeats in a genome (tens of thousands)	Limited by the size of genome, and by nucleotide polymorphism (tens of thousands)	Limited by the restriction site (nucleotide) polymorphism (tens of thousands)
Reproducibility	Medium to high	Low to medium	Medium to high
Amount of sample required per sample	10–20 ng DNA	2–10 ng DNA	0.2–1 µg DNA
Ease of assay	Easy to moderate	Easy to moderate	Moderate to difficult
Automation/multiplexing	Possible	Possible	Possible
Development	Expensive	Inexpensive	Moderate
Assay	Moderate	Inexpensive	Moderate to expensive
Equipment	Moderate to expensive	Moderate	Moderate to expensive

More recently, HRM closed-tube post-PCR analysis allowed high sample throughput for varietal certification. HRM is based on monitoring the fluorescence decrease due to the release of intercalating fluorescent dyes during thermal denaturation (melting) of double-stranded DNA. This technique allows the detection of polymorphism between samples based on differences in the melting temperature of PCR products, as low as in a single base. In the case of

microsatellites, HMR can reveal more polymorphisms than conventional electrophoresis owing to its ability to detect SNPs in the flanking region of the microsatellite repeats. Hence, HRM curves can be different in shape even in the case of monomorphic markers, and this magnifies the polymorphism degree of microsatellites *(274)*.

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Chapter 2: Outline

The safety of foods and feeds has become of increasing concern for consumers, governments and producers, and especially biological hazards pose the greatest threat. The capacity of foodborne bacteria to cause large outbreaks of acute illness is a constantly present alarm in the food supply chain. Moreover, the presence of certain microbial metabolites in food, especially toxins, can cause acute and chronic diseases.

Salmonella, one of the most studied foodborne pathogen, can be transmitted to humans through consumption of contaminated animal-derived food, especially eggs and raw meat from pig, turkeys and poultry. Although a coordinated approach has reduced human cases of salmonellosis in the EU over 100,000 human cases of salmonellosis are still reported each year, and economic losses linked to this problem are estimated as high as 3 billion euro a year. Therefore, it is crucial to distinguish cases of *Salmonella* infection that have originated from one source (an "outbreak") from cases originated from other sources, and to correlate the food source of infection to the strains isolated from patients, in order to monitor and track the outbreaks. Traditional typing systems based on phenotypes, such as serotype or phage-type, have been used for many years. However, more recent methods that examine the relatedness of isolates at a molecular level have improved the ability to differentiate among bacterial types and subtypes.

In addition to the consumption of food contaminated by bacterial pathogens, also the contamination by bacterial toxins is a major issue. Staphylococcal foodborne disease is very common worldwide, resulting from the contamination of food by Staphylococcal Enterotoxins (SEs). Although several SEs have been identified, SE type A (SEA), a highly heat-stable protein toxic even in few micrograms, is the most frequently isolated. Currently, the methods for the

detection of SEs exploit the recognition of the enterotoxin by specific antibodies in ELISA immunoassays, but alternative high-throughput and rapid methods seem to be promising.

Considering globally food contaminants from biological origin, also mycotoxins (toxins produced by different filamentous fungi) have a significant impact on human and animal health and economies. Among the major mycotoxins, aflatoxins and ochratoxin A (OTA) are the most studied because of their toxicity and their impact. Despite efforts to control fungal contamination, when fungal spores naturally present in the environment have the chance to germinate on foods, mycotoxins can be synthesized and accumulated. The presence of mycotoxins above the limits suggested for each food may result in downgrading or destruction of the contaminated food, because the toxins removal is difficult in plant foods and unfeasible on many animal-derived foods. The early detection and identification of fungi growing on foods helps to predict the risk of mycotoxin contamination, even when the toxin synthesis has not started yet. The detection of mycotoxin producing fungi achieved through DNA-based methods allow to recognize whether the isolates are able to produce the toxin, since often not all strains of the same species possess this ability. These molecular methods provide faster alternative to the morphological species identification.

Because of the globalization, the import of foods from extra EU countries with more permissive regulation about mycotoxins is growing. DNA-based approaches focused on the identification of the geographical origin of foods can be exploited to recognize crops grown in areas in which warm and humid climate promote the mycotoxin accumulation, thus preventing them to enter the food chain. The genetic diversity between crops populations at molecular level is often evaluated using Random Amplified Polymorphic DNA (RAPD), for its rapidity and low cost. This approach could be used to confirm the geographical origin of hempseeds (*Cannabis sativa*), considered in this research as model food often

imported from Asia. The consumption of hemp seeds (and derived oil and flours) has recently increased in Italy, since they are rich in proteins, fibers, omega-3 and omega-6 fatty acids. Although the cultivation of hemp in Italy has increased too, the local production do not cover the whole request of the market, leading to the importation from foreign countries. Several hemp cultivars are currently grown: DNA analysis can functionally work to establish the origin of the germoplasm (particularly when coupled with more specific techniques, e.g. isotopic ratio).

Aim of the thesis

The main aims of this research were:

- to investigate the relationship among *Salmonella* ser. Typhimurium and monophasic variant of *Salmonella* Typhimurium strains isolated from 3 pork-related foodborne salmonellosis outbreaks. This work is inserted within a broader Project performed in collaboration with the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Department of Pavia.

- to set up a Real Time PCR system to detect OTA-producing fungi. This method, together with culture-dependent method and LC/MS, has been applied in a monitoring study of the presence of OTA and OTA-producing fungi in salami. This work was performed in collaboration with IZSLER Department of Pavia.

- to select DNA markers useful for the identification and clustering of some Italian ecotypes of hemp (*Cannabis sativa* L.) from cultivars grown in China and Canada, the world-leading hemp producers, considering also the rate of occurrence of aflatoxins and OTA contamination (evaluated by HPLC-MS in collaboration with University of Parma).

- to set up a rapid and sensitive method for the detection of SEA in milk and milk derived products, exploiting Luminex xMAP and SPR-based technologies. The

work is a part of a project performed in collaboration with the RIKILT Department of the University of Wageningen (The Netherlands).

Part of the activities of the PhD have been financially supported by two Research Projects:

i) Progetto Ricerca Corrente, funded by Italian Minister of Health (Rome, Italy) (PRC 2013021). This project aimed to monitor the presence of OTA and OTA-producing fungi in salamis manufactured in Northern Italy, also evaluating a novel PCR-based approach for early detection of OTA producing fungi in salamis.

ii) Ricerca Finalizzata Sanitaria Nazionale, funded by the Italian Minister of Health (Rome, Italy) (RF-2011-02348684). The main aim of this Project is to develop rapid methods for the detection of enterotoxins, exploiting different advanced analytical approaches, comparing them with ELISA method.

Chapter 3: Published results

Foodborne salmonellosis in Italy: Characterization of *Salmonella enterica* Serovar Typhimurium and Monophasic Variant 4,[5],12:i- isolated from salami and human patients

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ABSTRACT

Salmonella enterica serovar Typhimurium (STm) and its monophasic variant 4,[5],12:i: (VMSTm) have been responsible for an increased number of foodborne infections in humans in Europe in recent years. The aim of this study was to investigate the origin of three foodborne salmonellosis outbreaks that occurred in Pavia Province (Lombardy region, northern Italy) in 2010. Phenotypic and genetic characteristics of the STm and VMSTm isolates from patients and from food that were recovered in the framework of the three outbreaks were evaluated through serotyping, phage typing, antimicrobial susceptibility testing, pulsed-field gel electrophoresis (PFGE), and multiple-locus variable-number tandem repeat analysis (MLVA). Salami from three artisan producers, which had all purchased meat from the same slaughterhouse, was the food source of infection in outbreak I. STm isolates were recovered from salami and patients with symptoms of gastroenteritis. These isolates had the same PFGE type and the same rare MLVA profile (3-18-9-NA-211). The same molecular profiles were found in an STm isolate from a salami, which likely was the source of another family outbreak (II). A VMSTm strain with common phenotypic and molecular profiles was isolated from three hospitalized patients and identified as the cause of another putative outbreak (III). During the following 3 years (2011 through 2013), 360 salami produced in Pavia Province were monitored for the presence of S. enterica. In 2011, no STm and VMSTm isolates were recovered from 159 salami tested. During 2012 and 2013, 13.9% of 201 tested salami harbored *S. enterica*, and half of the isolates were VMSTm, mainly in salami from those artisan producers involved in the previous outbreaks. These isolates were genetically variable, especially in terms of MLVA profiles. The data collected suggest that from 2012, VMSTm has replaced STm in the environments of the salami producers monitored in this study, and these data confirm the dominance of this emergent serovar along the pork supply chain.

Keywords: *Salmonella* Typhimurium, Monophasic Variant 4,[5],12:i-, foodborne outbreak, salami

INTRODUCTION

Salmonellosis is the second most commonly reported gastrointestinal infection and an important cause of foodborne outbreaks in the European Union (EU) and the European Economic Area (EEA).

Salmonellosis rates have shown a significant five-year decreasing trend in the EU/EEA, which is largely attributed to the implementation of successful veterinary control programs in poultry farms. In 2013, *Salmonella enterica* Serovar Typhimurium (STm) and the Monophasic Variant of *Salmonella enterica* Serovar Typhimurium (VMSTm) were the second and the third most commonly reported serotypes, respectively, isolated from humans in the EU. The reservoir of *Salmonella enterica* is the intestinal tract of wild and domestic animals and humans are usually infected through the consumption of contaminated raw or undercooked food *(1)*. Contamination often occurs when organisms are introduced into preparation areas and are allowed to replicate in food, due to inadequate storage temperatures and/or cooking or cross-contamination of ready-to-eat food *(2)*. In Italy, as in other countries, STm have been the highest ranking cause of human infection since 2001 *(3)* and VMSTm the second-highest since 2009 *(4)*. Together, these serovars account for more than 50% of the human *Salmonella enterica* isolates during the period 2009-2011 *(5)*.

Among all animal-derived strains notified in Italy to the National Reference Laboratory for *Salmonella enterica* in the framework of the Enter-vet network between 2002 and 2010, the proportion of VMSTm increased from 3.5% to 10.3%. In 2010, VMSTm was the most common serovar isolated from food and animal samples *(6)*. The vast majority of these monophasic strains were recovered from pigs and pork products *(7)*.

In Italy, pork products, and in particular salami, are widely consumed. Although these dry fermented sausages are traditionally considered safe due to their low pH, low water activity and high salinity, *Salmonella enterica* can survive fermentation and drying procedures, especially if the manufacturing process or fermentation periods are inadequate *(8)*. In Italy, salami contaminated with different clones of STm have been responsible for a few documented human outbreaks. Reported cases include an episode caused by STm DT 193 in 1995 *(9)*, another in 2004 due to STm DT 104A *(10)*, and an outbreak involving Italy and Sweden in 2005 caused by Not-Typeable (NT) STm *(11)*.

The aim of this study was to investigate three pork-related foodborne salmonellosis outbreaks caused by STm and VMSTm which occurred in Pavia Province (Lombardy Region, Northern Italy) in 2010. In addition, we implemented a three year (2011 – 2013) *Salmonella* monitoring program in Pavia Province among salami producers either involved or not involved in the three outbreaks, in order to monitor contamination over time.

MATERIAL AND METHODS

Outbreak description

Three human salmonellosis outbreaks (hereafter identified as I, II and III) occurred in Pavia Province, in the Lombardy Region of Northern Italy, in November 2010. Outbreak I involved about thirty guests from one restaurant over a period of one month. Guests had become ill with symptoms of gastroenteritis and the epidemiological investigation revealed that all had eaten salami at the restaurant. Outbreak II involved the five members of a family who consumed salami bought from a local salami producer (p3). All five family members had symptoms of gastroenteritis. *Salmonella enterica* belonging to the White-Kauffmann-Le Minor group B was isolated from four family members, among which, one was hospitalized and *Salmonella enterica* was serotyped. The epidemiological investigation of the two outbreaks identified four artisan

producers of salami (p1, p2, p3 and p6), which had all purchased meat from the same slaughterhouse A, as potentially responsible for these outbreaks. In both outbreaks, each guest ate approximately 50 g of pork products, of which, about half was salami.

In the same period, San Matteo Hospital in Pavia collected three *Salmonella enterica* isolates among patients with symptoms of gastroenteritis. In this case, the epidemiological investigation did not ascertain a correlation between the foodborne episodes and the exact source of infection. Based on the subtyping approaches used, which identified these three strains as indistinguishable, they have beenconsidered as belonging to another, putative, outbreak (III).

Sample collection and Salmonella enterica isolates

Twenty-two samples of salami were collected from four involved producers (p1, p2, p3, p6) in the context of the epidemiological investigations of outbreaks I and II from November 2010 to February 2011. All producers hadpurchased raw meat for salami production from artisan slaughterhouse A; in addition, producer p3 had purchased meat from industrial slaughterhouse B.

From March 2011 to December 2011, within a Provincial Surveillance Program carried out to investigate the presence of *Salmonella enterica* in salami from different producers, twenty-five salami from producers p1, p2, p5 and p6, which had purchased meat from slaughterhouse A, were sampled according to the criterion of one salami per production batch. In the same period, 134 salami were collected from epidemiologically unrelated artisan producers (91) and slaughterhouses, other than slaughterhouse A, according to the same sampling criterion.

In 2012 and 2013, within a Regional Surveillance Program conducted in Lombardy for the same purpose, 52 samples of salami were collected from 6 artisan producers: the four producers (p1, p2, p3 and p6) involved in the outbreaks of 2010 and two additional producers (p4 and p5), which had purchased raw meat

from slaughterhouse A. The sampling plan of the Regional Surveillance Program was conducted according to the Commission Regulation (EC) No 2073/2005 *(12)*, collecting five sampling units per batch of salami. Finally, over the same period, 149 additional samples collected from epidemiologically unrelated producers, both artisan (48) and industrial producers (31), and from slaughterhouses other than A and B in Pavia Province were analyzed for the same purpose. The distribution of *Salmonella enterica* isolates from salami by scope of sampling, period, producer, and slaughterhouse is reported in Table 1. In the context of the three outbreaks, a total of eight *Salmonella enterica* isolates were recovered from human feces, namely four from outbreak I, one from outbreak II and three from outbreak III (Table 2).

Salmonella enterica detection and serotyping

Salmonella enterica was isolated from salami according to method ISO 6579:2002/Cor1:2004 *(13)* at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER) laboratories. Suspect *Salmonella enterica* colonies were tested for biochemical properties with API® 20E micro-substrate system (bioMérieux). *Salmonella enterica* serotyping was performed according to the White-Kauffmann-Le Minor scheme by slide agglutination with O and H antigen specific sera (Staten Serum Institute, Copenhagen, Denmark). VMSTm were definitively identified and differentiated from STm using a previously described PCR protocol *(14)*.

Molecular characterization of ST and VMSTm isolates

Pulsed-field gel electrophoresis (PFGE) was performed according to the PulseNet standardized protocol using restriction enzyme *XbaI* (http://www.cdc.gov/pulsenet/PDF/ecoli-shigella-salmonella-pfge-protocol-508c.pdf). *XbaI* digested DNA from *Salmonella* Braenderup H9812 was used as molecular size marker (*15*). Gel images were analyzed by BioNumerics 6.6 software package (Applied Maths, Saint-Martens – Latem, Belgium). PFGE

pulsotypes differing by one or more fragments were considered as distinct *(16)*. The pulsotypes of the isolates were named according to the IZSLER coding system. Comparison of the profiles was performed by cluster analysis based on the Dice's Similarity Index and a dendrogram was obtained by using the unweighted pair group method with arithmetic average (UPGMA) (Tolerance: 1%, Optimization: 1%).

Multiple-locus Variable-number Tandem-repeat Analysis (MLVA) was performed according to the protocol previously described *(17)*. The size measurements for each locus were estimated using a CEQ™ 8000 Genetic Analysis System (Beckman Coulter, USA). MLVA profiles were assigned as a string of five numbers (STTR9–STTR5–STTR6–STTR10pl–STTR3), representing the Variable Number of Tandem Repeats (VNTR) at the corresponding locus, or with NA (no amplification) in case of negative PCR amplification, according to the MLVA nomenclature suggested by Larsson et al. *(18)*.

For each isolate, the corresponding VNTR profile was imported as character data into the BioNumerics 6.6 software package (Applied Maths, Saint-Martens – Latem, Belgium).

Phage-typing and antimicrobial susceptibility testing

The STm and VMSTm isolates were phage-typed by the National Reference Laboratory for *Salmonella* (Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy) using the protocol reported by Anderson et al. *(19)* and following the interpretative guidelines defined for STm by the Public Health England (Colindale, London, UK) *(6)*. Isolates that did not react with any of the typing phages were considered NT.

Antimicrobial susceptibility testing of human and salami STm and VMSTm isolates was performed using the disk diffusion method according to the criteria established by the Clinical and Laboratory Standard Institute *(20)*. The

antimicrobials (Becton Dickinson) tested included the following antibiotic disks: ampicillin (A, 10 μg), cefotaxime (Ctx, 30 μg), chloramphenicol (C, 30 μg), ciprofloxacin (Cp, 5 μg), gentamicin (G, 10 μg), kanamycin (K, 30 μg), nalidixic acid (Nx, 30 μg), streptomycin (S, 10 μg), sulfonamides (Su, 250 μg), tetracycline (T, 30 μg), and trimethoprim–sulfamethoxazole (SXT, 1.25 / 23.75 μg). *Escherichia coli* ATCC 25922 was used as a control strain in the tests.

Statistical analysis

Fisher's test was used to evaluate if the difference between the proportions of positive salami samples from the producers, related and not related to the 2010 outbreaks within the Regional Surveillance Program, were statistically significant. The level of significance was set at $p < 0.01$.

Table 1: Distribution of *Salmonella enterica* isolates from salami by scope of sampling, period, producer, slaughterhouse and serotype.

Epidemiological investigation of the outbreaks (November 2010 – February 2011)						
Producer	**Slaughterhouse**	**Sampled salami (n)**	**Positives for *Salmonella enterica* (n)**	**STm (n)**	**VMSTm (n)**	**Other serotypes (n)**
p1	A	3	2	2	/	
p2	A	10	5	3	/	2 (1 Derby; 1 Ohio)
p3	A	6	5	5	/	
p3	B	2	2	2	/	
p6	A	1	/	/		
Total		**22**	**14**	**12**	**0**	**2**
Pavia Province surveillance program (March 2011 - December 2011)						
Producer	**Slaughterhouse**	**Sampled salami (n)**	**Positives for *Salmonella enterica* (n)**	**STm (n)**	**VMSTm (n)**	**Other serotypes (n)**
p 1	A	6	1	/	/	1 (Derby)
p 2	A	17	/	/	/	/
p 5	A	1	/	/	/	/
p 6	A	1	/	/	/	/
Total		**25**	**1**	**0**	**0**	**1**
Other producers (91)	other slaughter-houses	**134**	**1**	/	**/**	**1** (Derby)
Regional surveillance program in Pavia Province (January 2012 – December 2013)						
Producer	**Slaughterhouse**	**Sampled salami (n)**	**Positives for *Salmonella enterica* (n)**	**STm (n)**	**VMSTm (n)**	**Other serotypes (n)**
p 1	A	1	/	/	/	/
p 2	A	36	7	/	6	1 (NT°)
p 3	B	2	1	/	1	
p 4	A	8	2	/	2	
p 5	A	3	2	/	1	1 (Infantis)
p 6	A	2	1	/	1	
Total		**52**	**13**	**0**	**11**	**2**
Other producers (79)	Other slaughterhouses	**149**	**15**	/	**3**	**12** (8 Derby, 2 Rissen, 1 Infantis, 1 NT°)

Table 2: Phenotypic and genetic characterization of 17 STm and 17 VMSTm. Phage-type NT indicates Non Typable strains, R-Type S strains susceptible for all the tested antibiotics and ND strains not tested for that characteristic.

Origin	Producer	Slaughterhouse	Source	No. of isolates	Serotype	PFGE XbaI profile	MLVA STTR profile[a]	Phage type (no. of isolates)	Resistance type[b]
Outbreak I (Nov. 2010)			Human	4	STm	STYMXB_BS.0043	3-18-9-NA-211	DT120 (3), NT[c] (1)	Susceptible
Outbreak I epidemiological investigation (Nov. 2010–Feb. 2011)	1	A	Salami	2	STm	STYMXB_BS.0043	3-18-9-NA-211	DT120	Susceptible
	2	A	Salami	3	STm	STYMXB_BS.0043	3-18-9-NA-211	DT120	Susceptible
	3	A	Salami	4	STm	STYMXB_BS.0043	3-18-9-NA-211	DT120	Susceptible
		B		2	STm	STYMXB_BS.0043	3-18-9-NA-211	DT29	Susceptible
Outbreak II (Nov. 2010)			Human	1	STm	ND[d]	ND	ND	ND
Outbreak II epidemiological Investigation (Nov. 2010–Feb. 2011)	3	A	Salami	1	STm	STYMXB_BS.0043	3-18-9-NA-211	DT120	Susceptible
Outbreak III (Nov. 2010)			Human	3	VMSTm	STYMXB_0131	3-13-10-NA-211	NT	ASSuT
Regional Surveillance Program (2012)	2	A	Salami	6	VMSTm	STYMXB.0131	3-14-9-NA-211	NT (2) DT120 (1) DT193 (3)	Susceptible
	6	A	Salami	1	VMSTm	STYMXB.0131	3-14-9-NA-211	DT193	Susceptible
	4	A	Salami	2	VMSTm	STYMXB.0131	3-13-10-NA-211 3-11-9-NA-211	NT	ASSuT
	5	A	Salami	1	VMSTm	STYMXB.0131	3-14-10-NA-211	NT	ASSuT
	3	B	Salami	1	VMSTm	STYMXB_PR.0538	3-11-14-NA-211	DT120	ASSuT
	7	C	Salami	1	VMSTm	STYMXB_PR.0550	3-12-9-NA-211	DT120	ASSuT
	8	D	Salami	1	VMSTm	STYMXB_PR.0553	3-12-10-NA-211	DT193	Susceptible
Regional Surveillance Program (2013)	8	D	Salami	1	VMSTm	ND	ND	ND	ND

a MLVA profile numbers are STTR9, STTR5, STTR6, STTR10pl, and STTR3. NA, no amplification (no PCR results).
b ASSuT, resistant to ampicillin, streptomycin, sulfonamides, and tetracycline. c NT, nontypeable. d ND, not done.

RESULTS

Salmonella detection and serotyping

The four human isolates from outbreak I were serotyped as STm. From outbreak II, the single isolate available was serotyped as STm. The three isolates from outbreak III were serotyped as VMSTm (Table 2).

During the epidemiological investigations of outbreaks I and II, 14 out of 22 (63.6%) salami from producers p1, p2 and p3, connected to slaughterhouse A, harbored *Salmonella enterica*. Of these 14 isolates, 12 isolates (85.7%) were serotyped as STm, while two belonged to different serotypes (Table 1). Within the Provincial Surveillance Program conducted from March to December 2011 one salami, out of 25 salami from producers p1, p2, p5 and p6, was positive for *Salmonella* Derby. In the same monitoring program, one salami harbored *Salmonella* Derby out of the 134 salami sampled from producers and slaughterhouses which were epidemiologically unrelated to the 2010 outbreaks. In 2012-2013, within the Regional Surveillance Program, the producers involved in the 2010 outbreaks and/or which purchased meat from the slaughterhouses involved, were studied. In this program, 13 salami out of 52 (25%) harbored *S. enterica* (Table 1). All producers but p1 had at leastone positive sample. Eleven of the 13 isolates were serotyped as VMSTm. In the same period, 15 out of the 149 salami (10.1%) from other producers and slaughterhouses were *S. enterica*-positive, and three isolates proved to be VMSTm. The difference between the proportions of *S. enterica*-positive salami from the producers involved and those not involved in the 2010 outbreak was statistically significant.

Molecular characterization of ST and VMSTm isolates

The PFGE and MLVA profiles of all STm and VMSTm isolates are reported in Table 2 and the genetic similarity of PFGE profiles is shown in the dendrogram of Figure 1.

The four human STm isolates from outbreak I were PFGE type STYMXB_BS.0043 and MLVA profile 3-18-9-NA-211. The only human STm isolate from outbreak II was not available for genotyping, and so for this isolate, ND (Not Done) is reported in Table 2. All STm isolates from salami, sampled during the epidemiological investigation of outbreaks I and II from the producers involved, had the same PFGE (STYMXB_BS.0043) and MLVA (3-18-9-NA-211) profiles as the outbreak I human isolates. This corroborated the hypothesis that salami from these particular producers was involved in the outbreaks. These findings were also supported by food questionnaires collected during the epidemiological investigation, from which salami emerged as the suspect food. The three VMSTm human isolates from outbreak III were PFGE type STYMXB_0131 and MLVA profile 3-13-10-NA-211.

With regard to the Regional Surveillance Program of 2012-2013, seven VMSTm isolates from producers p2 and p6, both of which had purchased meat from slaughterhouse A, shared the same PFGE (STYMXB_0131) and MLVA (3-14-9-NA-211) profiles. In addition, three isolates from producers p4 and p5 (slaughterhouse A), were of the same PFGE type (STYMXB_0131), but three different MLVA profiles (3-13-10-NA-211, 3-11-9-NA-211, 3-14-10-NA-211). Notably, MLVA profile 3-13-10-NA-211 from producer p4 was the same type as the isolates from outbreak III, which had occurred two years previously. Three VMSTm isolates from producers p3, p7 and p8 (slaughterhouses B, C, D) differed by both PFGE and MLVA profiles: three unique MLVA profiles (3-11-14-NA-211, 3-12-9-NA-211, 3-12-10-NA-211) corresponded to different PFGE types (STYMXB_PR.0538, STYMXB_PR.0550, STYMXB_PR.0553). Lastly, for the single VMSTm isolate of 2013, only serotyping was available.

Phage-types and antimicrobial susceptibility

Detailed results of phage-typing and antimicrobial susceptibility testing are reported in Table 2. Three out of four human STm isolates, apparently

representing the same clone, were phage-typed as DT120, while the fourth was NT. Ten of twelve STm isolates from salami were phage-type DT120, while the remaining two isolates were phage-type DT29.

Three human VMSTm isolates were NT. VMSTm isolates from salami were phage-typed as DT120 (n=3), DT193 (n=5) and NT(n=5).

With regard to antimicrobial resistance, all STm isolates were pansusceptible to the panel of antimicrobials tested. Eight VMSTm isolates were fully susceptible and eight showed the resistance to ampicillin, streptomycin, sulfonamides and tetracycline (R-Type: ASSuT) typical of this serovar, including the three human isolates of outbreak III and the isolate from salami from producer p4 which had the same PFGE and MLVA profiles as the outbreak strain. For the only human STm isolate from outbreak II and for the single VMSTm isolate from the 2013 Regional Surveillance Program, no phenotypic data was available. This is reported in Table 2 as Not Done (ND).

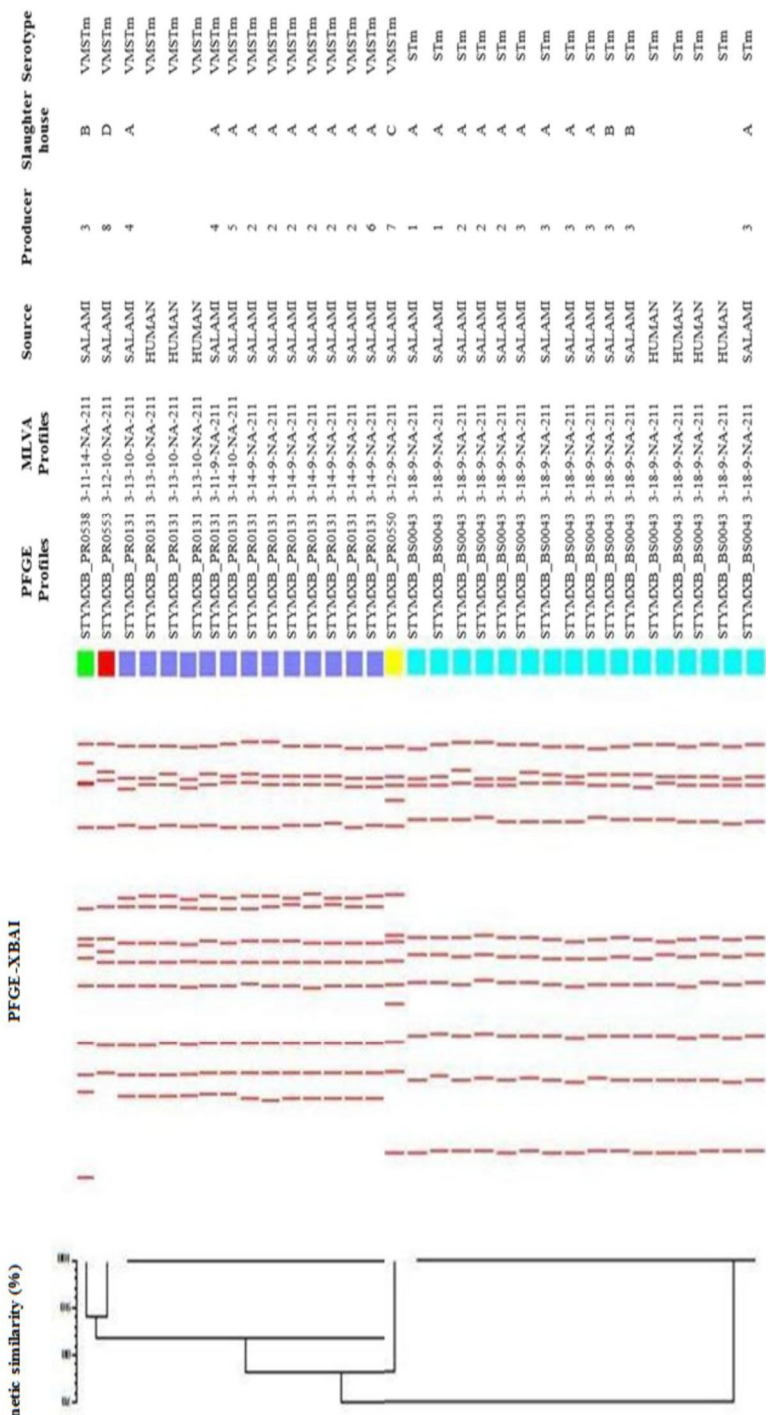

Figure 1: Dendrogram of genetic similarity among the PFGE profiles of 32 human and salami *Salmonella enterica* serovar Typhimurium (STm) and 4,[5],12:i:- (VMSTm) isolates. Similarity analysis was performed using the Dice Coefficient (opt: 1.00%; tol: 1.00%) and clustering was by the unweighted pair group method with arithmetic average (UPGMA).

DISCUSSION

The epidemiological investigations of three human salmonellosis outbreaks by STm and VMSTm that occurred in November 2010 in Lombardy, Italy, led to the isolation of *Salmonella enterica* from salami. As a consequence, the salami manufactured by producers in the area were monitored over time for the presence of *Salmonella enterica*.

A high proportion of the salami manufactured by the artisan producers involved in outbreaks I and II harbored *S. enterica*. More than 60% of sampled salami contained *Salmonella enterica.* Eighty-six percent (12 out of 14 samples) were contaminated with STm sharing the same PFGE and MLVA profiles (STYMXB_BS.0043 and 3-18-9-NA-211, respectively) as the human STm isolates recovered within outbreak I.

Interestingly, 3-18-9-NA-211 is a rare MLVA profile, having been found only among these isolates out of three thousand isolates examined by MLVA in the IZSLER database (unpublished results) and, to our knowledge, never having been reported in the literature worldwide so far, reinforcing the evidence of a link between these salami and the human cases. The fact that STm with a very rare MLVA profile was recovered from salami manufactured by three different producers, prepared in different environments and conditions, during a short timeframe (four months), suggests a common source of infection. In fact, the source of infection was identified as the artisan slaughterhouse A, from where the producers had purchased the meat. The single finding of STm with that rare MLVA profile in a batch of salami produced with meat from slaughterhouse B, by producer p3, could actually have been the consequence of cross-contamination at the manufacturer's premises, with meat from slaughterhouse A. This slaughterhouse also supplied meat to producer 3.

The same PFGE, MLVA and phenotypic profiles were also shared by a STm isolate recovered from a salami related to outbreak II and purchased from producer p3. This finding supports the salami as the speculative source of

outbreak II, although this was impossible to confirm due to the lack of subtyping data from the one available human isolate associated with this outbreak. The STm clone identified in outbreaks I and II was susceptible to the antimicrobials examined, and the isolates shared identical PFGE and MLVA profiles, although heterogeneity emerged with phage-typing (three phage types were associated with the clone; Table 2). Furthermore, the main phage-type identified within the clone (DT120) is quite common among animal-derived STm strains *(6)*. On one hand, this evidence is indicative of a possible lack of reproducibility of phage-typing. On the other hand, in the case of outbreaks I and II, the use of phage-typing alone would not have allowed us to unequivocally link the human cases and the sources of infection, due to the common occurrence of DT120.

Human strains associated with outbreak III belonged to VMSTm and shared the PFGE (STYMXB_0131) and MLVA (3-13-10-NA-211) profiles, as well as resistance pattern (ASSuT). This phenotypic and genetic profile combination is common among VMSTm isolated during IZSLER surveillance, and it is also quite common among Italian human VMSTm isolates, as reported by Luzzi et al. (*5)*. For outbreak III, it was not possible to ascertain the source of infection. Nevertheless, a VMSTm isolate with the same PFGE and MLVA profiles (STYMXB_0131 and 3-13-10-NA-211, 10 respectively), and the same ASSuT resistance pattern, occurred in a salami manufactured two years after outbreak III. This suggests that salami or pork meat could have been the source of the three human outbreak III cases.

In the monitoring conducted during March-December 2011, 1.3% of salami (2 out of 159) from producers related and unrelated to the outbreaks of 2010 harbored *Salmonella enterica* and no STm or VMSTm were detected. Probably, the correct application of the Good Manufacturing Practices (GMP) and sanitation of the different areas of the slaughterhouses and production areas after the outbreaks was successfully implemented. In particular, the sanitation practices of slaughterhouses and production areas were improved by increasing the frequency

of sanitation and by separating the food processing areas. On the contrary, during the monitoring conducted in 2012 – 2013, 13.9% (28 out of 201) of salami harbored *Salmonella enterica.* It is noteworthy that a significantly higher proportion of the positive findings originated from artisan producers which had been involved in the 2010 outbreaks and/or purchased meat from artisan slaughterhouse A (12 out of 50), while only a minor proportion were from other producers (15 out of 149). Overall, during 2012-2013 monitoring, 50% (14 out of 28) of the isolates were VMSTm and no STm was detected.

In the context of our investigations, the ASSuT antimicrobial resistance pattern was detected only in VMSTm isolates and not in STm, which is partially in agreement with data from EFSA and the ECDC *(21)*, reporting that more than 70% of STm and VMSTm serotypes isolated from pig meat show multiresistant patterns.

In conclusion, the results obtained in this study confirm that in 2012-2013, VMSTm has replaced STm in the environments of Pavia salami producers. This is in keeping with the observation of an increase in the isolation of animal-derived VMSTm that occurred in Italy from 2010 *(6)*. The extended sampling method adopted in our study proved suitable for purpose, enabling us to detect *Salmonella enterica* subtypes in salami, thus likely preventing the onset of food-borne outbreaks due to the correct application of GMP and hygienic measures adopted as a result. Although the data presented in this study confirm the clear role of the pig as an important contributor to the burden of human salmonellosis, currently, a control program for *Salmonella* in the pig chain has not been implemented yet in Italy. The evidence collected in the present study and the results of the source attribution studies conducted in Italy *(22-24)* strongly support the need to urgently focus efforts on the swine supply chain through the application of control plans, as has been already done for the poultry sector, with the aim of further reducing salmonellosis at the national level. Moreover, further studies would be necessary to clarify the factors affecting the persistence of serovars including STm and

VMSTm in pig farms as well as in pig slaughterhouses and pork product establishments.

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Chapter 4: Unpublished results

Ochratoxin A and ochratoxin-producing fungi in traditional salami manufactured in Northern Italy

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ABSTRACT

Fungi have a crucial role in the correct maturation of salami, but the fungal metabolite ochratoxin A (OTA) is nephrotoxic, immunotoxic and carcinogenic. In a monitoring study conducted in Northern Italy, OTA was measured through liquid chromatography coupled with mass spectrometry above the limit of Italian regulation in 13 out 133 traditional salamis (9.8% of the total count). From the same salamis, 247 fungal isolates were collected and identified at a species level. The most commonly isolated species resulted *Penicillium nalgiovense, Penicillium solitum* and *Penicillium chrysogenum*, while no *Penicillium nordicum,* the OTA-producing species most frequently isolated from proteinaceous food, was found. In 3 cases, *Aspergillus westerdijkiae*, a recognised ochratoxigenic mould, was identified. The presence of a target DNA sequence common to all OTA-producing fungi (*otanpsPN*) and of a sequence specific for the ochratoxigenic strains of *P. nordicum* was evaluated by real time PCR. None of the tested strains, including the three *A. westerdijkiae*, showed to possess *otanpsPN*, and none of the *A. westerdijkiae* strains was proved able to produce OTA, when grown under conditions favourable to OTA biosynthesis. Conversely, the *otanpsPN* target gene was amplified from the DNA purified from the 3 salami casings harbouring both *A. westerdijkiae* and OTA, but also from other 11 salami casings where *A. westerdijkiae* was not isolated, suggesting that OTA-producing strains were no longer viable and isolable at the end of maturation. The DNA sequence *P. nordicum*-specific was also found in 11 out of 19 DNA samples purified directly from salami casings.

The amplification of targets specific for OTA-producing strains performed on DNA purified directly from the salami casing resulted a better approach to predict the presence of OTA presence than the traditional culture-dependent microbiology methods.

Two novel primer pairs were designed and tested in real time PCR in order to implement the system of monitoring. Both primer pairs gave amplicons in presence of the species *A. westerdijkiae, A. ochraceus* and *P. nordicum*.

Keywords:
Salami, *Aspergillus*, *Penicillium*, Ochratoxin A

INTRODUCTION

The colonization of salami casings by fungi is crucial for the correct maturation of salami. The growth of fungi on salami surfaces has antioxidative effects, prevents rancidity and keeps the correct colour. Fungal growth is responsible for the development of the characteristic salami flavour. In fact, fungi decompose lipids, allow the β-oxidation of fatty acids, the oxidation of lactate and the proteolysis *(1)*. Moreover, fungal flora competes with pathogens and spoiling microorganisms for nutrients, assuring a good microbiological quality *(2)*. On the other hand, any variation in temperature or humidity at each production stage can promote undesired and uncontrolled fungal growth, which causes alteration in appearance, smell and taste *(3, 4)*. Among fungal metabolites produced during fungal growth, mycotoxins represent a potential health hazard for consumers. Ochratoxin A (OTA) is the main mycotoxin found in meat *(5, 6)* and its accumulation in foods may affect human health, since it is nephrotoxic, immunotoxic, teratogenic and carcinogenic *(7)*.

OTA is synthesized by fungal species belonging to *Aspergillus* genus, section *Circumdati* (mainly *A. ochraceus*, *A. steynii* and *A. westerdijkiae*), and section *Nigri* (*A. carbonarius* and *A. niger*), and *Penicillium* genus section *Viridicata (P. nordicum* and *P. verrucosum) (8)*. In meat-derived products, OTA production is mainly due to the presence of *P. nordicum (9 - 11)*. It can occur at a frequency up to 11% of the fungal population on the surface of dry-cured ham *(5, 12)*, leading to OTA accumulation on the surface of the final product *(13)*. The production of

OTA is strain dependent, and requires *ad hoc* conditions of temperature, nutrients and water activity (a_w). Each fungal species has different optimal conditions for producing OTA: for example, OTA-producing strains of *A. westerdijkiae* synthesizes OTA when temperatures are between 24°C and 28°C and a_w is in the range of 0.96-0.99 *(14, 15).* As OTA can be produced on a great variety of crops, which are downgraded to animal feed when contaminated with OTA, this mycotoxin can accumulate in swine muscles, resulting in contaminated meat *(16).* Therefore, OTA detected in salami can originate both from contaminated meat and from the metabolism of fungi growing on the casings. To inhibit the undesired growth of fungal species responsible for the alteration of organoleptic features and for the production of mycotoxins, specific fungal starters are inoculated in large-scale before the starting of the maturation *(17).* In Italy, most of salami are produced by small and medium-sized factories, mainly located in the Northern Italy regions of Emilia Romagna and Lombardy *(18).* These factories usually follow their traditional recipe for the preparation of salami, so no fungal starter is added, allowing the proliferation on the salami casings of the mycobiota already present in the air of maturing rooms.

Currently, OTA is not routinely searched in salami, since its carry-over in animal products is not considered a major health concern *(19).* European commission has not set limits yet for OTA in meat and cheese, but in Italy, where the consumption of dry-cured meat is widespread, the Italian regulation has established the limit of 1 µg/kg (1 ppb) of OTA in meat and meat-derived products (Italian Ministry of Health, 1999) *(20).*

The aim of this work was to evaluate the presence of OTA and ochratoxin-producing fungal species in traditional salami produced in Lombardy and Emilia Romagna. The concentration of OTA in all salami was measured by mass spectrometry. The identification of fungal species isolated from salami was carried out through traditional microbiological methods. Molecular methods based on real time PCR were employed to verify the presence of OTA producing

strains, by checking for the positivity to non-ribosomal peptide synthetase (*otanpsPN*) gene, which encodes for a key enzyme for OTA biosynthesis in OTA-producing *Penicillium* and *Aspergillus* strains. The presence of OTA-producing strains of *P. nordicum* was checked by amplifying a species-specific fragment of polyketide synthase (*otapksPN*) gene *(12, 21- 23).*

MATERIAL AND METHODS

Sampling

One hundred and thirty-three salamis ready for the consumption produced from 2013 to 2015 were collected from 37 different producers located in Lombardy and Emilia Romagna (Fig. 1). Sampling for OTA quantification was performed by cutting out 20 g of each salami. For the strains isolation, 10 g of salami casing were homogenized in 90 ml of buffered peptone water 0,1%. Four 10-fold serial dilutions were subsequently prepared.

Figure 1. Salami contaminated by ochratoxin A and harbouring *Aspergillus westerdijkiae*.

Ochratoxin A detection

The OTA presence was evaluated in 133 salamis through an LC-MS/MS (liquid chromatography- tandem mass spectrometry) method based on a previously published work *(24).*

OTA was also searched and quantified in 50 ml of 7 fungal cultures (2 *A. westerdijkiae* isolates from salami, *A. westerdijkiae* CBS 112803, *A. ochraceus* CBS 108.08, *A. steynii* CBS 112812, *P. verrucosum* CBS 115508 and *P. nordicum* CBS 110769) in 2% Yeast Extract 20% Sucrose broth *(25)*. The *Aspergillus* species were incubated at 25±1°C for 15 and 30 days, while the *Penicillium* species at 22±1°C for 15 days.

Homogenized salami (20 g) was added to 100 ml of an extraction solution mix formed by acetonitrile:water (84:16, v/v) acidified with 2.5% of acetic acid. The mixture was stirred for 60 min. After filtration on a paper filter, an aliquot of 5 ml was purified through a Mycosep 229 Ochra. An aliquot of 2 ml was transferred in a tube and n-hexane (2 ml) was added. After stirring on a vortex (1 min) the solution was centrifuged at 3000 rpm (5 min). The hexan layer was discharged. Then the idroorganic (1 ml) layer was transferred in a LC vial and underwent to LC-MS/MS analysis.

LC-MS/MS analysis was carried out by a 6430 Triple Quad MS (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray interface. The separation was achieved on a ZORBAX SB-C18 (50 mm, 2.1 mm ID, 1.8 mm) column with a pre-column filter (0.3 mm). Gradient elution was performed using eluent A (0.1% formic acid in H2O) and eluent B (0.1% formic acid in CH3CN). The gradient was set as follows: the first step was a linear gradient from 95% A to 95% B in 5 min, followed by a 1 min isocratic step at 95% B. The column was reconditioned at 95% A for 1.5 min. The total run time was 7.5 min. The following parameters were set: flow rate 0.4 ml/min, column temperature 40°C and injection volume 10 µl. The retention time of the analyte was 3.41 min. Quantification was carried out by the external standard method in multiple reaction monitoring (MRM) mode (ESI-) using the following transitions: 402.1 ->358.0 (Quantifier) and 402.1 -> 211.0 (Qualifier). MS/MS parameters were set as follows: capillary 4000 V, gas temperature 300°C, gas flow 10 L/min, nebulizer

35 psi, dwell time 200 ms, fragmentor 143 V, collision energy 16 V (Quantifier), collision energy 36 V (qualifier) and cell acceleration voltage 7 V.

Isolation and culture-dependent identification of fungal strains

For the fungi isolation, each homogenate and its serial dilutions were plated on Dichloran 18% Glycerol (DG18) *(26)* and Dichloran Rose Bengala Chloramphenicol (DRBC) *(27)* agar media, depending on the water activity, as recommended by ISO 21527-1:2008 and 21527-2:2008. Each homogenate and its serial dilutions were also plated on Malt Extract Agar (MEA) and incubated at 25±1 °C for 5 days.

The isolates were transferred to Potato Dextrose Agar and incubated at 25±1 °C for 5 days. The species identification of each isolate was carried out through the observation of macroscopic and microscopic features expressed on MEA, DG18 and Czapek Yeast Autolysate agar (CYA) media for *Aspergillus,* while MEA, CYA, Yeast Extract Sucrose agar (YES) and Creatine sucrose agar (CREA) were used for *Penicillium*, as suggested by dichotomic keys *(28)*. To differentiate certain species of *Penicillium*, Erlich test was performed *(29)*. For the observations of the reproductive structures, microscopic mounts were prepared in lactic acid from colonies grown on MEA.

DNA purification

The isolates of *Aspergillus* and *Penicillium* were transferred to 2 ml of BHI (Brain Hearth Infusion) broth incubated for 3 days at 25 °C ± 1°C and then pelleted for 10 minutes at 16000 x g. Two grams of 19 salami casings from the total 133 salami samples (12 positives to OTA and 7 negatives) were washed with physiological saline, and the suspension of spore was pelleted by centrifugation for 10 minutes at 16000 x g.

Genomic DNA was purified using a commercial kit (Blood and Tissue kit – QIAGEN, Milan Italy), with minor modifications. To achieve the fungal wall lysis all pelleted cultures were incubated with 200 U per sample of Lyticase (Sigma) at

30 °C for 30 minutes. Genomic DNA was eluted in 200 µl of AE buffer. A single extraction was performed from each culture or salami casing.

Amplification of ITS regions

The universal primers ITS1 [5'-TCC GTA GGT GAA CCT GCG G-3'] and ITS4 [5'- TCC TCC GCT TAT TGA TAT GC-3'] were used to amplify a fragment of ITS region, to evaluate the integrity of DNA *(30).* Five microliters of fungal DNA (1µg/ml) were used for the PCR assay. The reaction was carried out in a total volume of 25 µl, containing 0.4 µM of each primer, 0.2 mM dNTPs (Euroclone, Milan Italy), 1 U of Hot Start Taq (QIAGEN, Milan Italy) and 1X PCR Buffer 1.5 mM $MgCl_2$ (QIAGEN, Milan Italy). The amplification cycle was as follows: an initial denaturation step at 94 °C for 1 min, 40 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min. A final extension at 72 °C for 10 min was added. The DNA fragments were length separated by electrophoresis through 2% agarose gels in Tris Borate EDTA (TBE) buffer and 0.5 µg/ml of ethidium bromide. Results were documented by using a UV transilluminator (Alliance 2.7, Uvitec, Cambridge).

Real time PCR on otanpsPN gene

The amplification of a fragment of the *otanpsPN* gene conserved in all OTA-producing strains was performed as described by Rodriguez and colleagues (2011) *(31)* on DNA samples isolated from: 113 cultures of *Aspergillus* and *Penicillium* spp. strains, considering at least two strains for each species and all *Penicillium* strains identified only at genus level; 19 salami casings. The single reaction mixture was composed of 1.975 µl of RNase free water, 12.5 µl of Go Taq PCR Master mix (Promega, Milan Italy), 1 µl of F-npstr (5'-GCC GCC CTC TGT CAT TCC AAG-3') (10µM), 1 µl of R-npstr (5'-GCC ATC TCC AAA CTC AAG CGT G-3') (10µM), 1.125 µl of NPSprobe (6FAM-CGG CCG ACC TCG GGA GAG A–BHQ1) (10µM), 0.4 µl of TaqMan Exogenous Internal Positive Control Mix (Applied Biosystems, Monza Italy), 2 µl of TaqMan Exogenous

Internal Positive Control (DNA), and 5 µl of DNA (1 µg/ml), in a final volume of 25 µl. DNA samples were loaded in triplicate on the plate. At each working session, a No Template Control (NTC), a negative control consisting of DNA from *Penicillium nalgiovense* and two positive controls consisting of DNA from *P. nordicum* CBS 110769 and *P. verrucosum* CBS 115508 were included. The amplification was carried out in a CFX96X Touch (BioRad, Milan Italy), programmed to perform a first denaturing step of 2 min at 95 °C and 35 cycles of 20 sec at 95 °C, 20 sec at 55 °C and 30 sec at 72 °C.

Real time PCR for the identification of OTA-producing strains of P. nordicum

DNA isolates from 39 *Penicillium* spp. strains, chosen considering at least one strain of each species plus other strains for which the species was not identifiable using the culture-dependant method, were also tested in real time PCR using *P. nordicum* specific primers and probe, targeting a species-specific fragment of *otapksPN* gene as described by Geisen (2004) *(32)*. The same reaction was carried out also on DNA isolates from 19 salami casings. The reaction mixture was composed of 3.08 µl of RNase free water, 10 µl of Go Taq Probe qPCR (Promega, Milan Italy), 1 µl of otapksPN1 (5'-CACGGTTTGGAACACCACAAT-3') (10µM), 1 µl of otapksPN2 (5'-TGAAGATCTCCCCCGCCT-3') (10µM), 1 µl of otapksPN probe (6FAM-CGTACCAATCCCCATCCAGGGCTC–BHQ1) (10µM), 0.32 µl of TaqMan Exogenous Internal Positive Control Mix (Applied Biosystems, Monza Italy), 1,6 µl of TaqMan Exogenous Internal Positive Control (DNA), and 2 µl of DNA (1 µg/ml) in a final volume of 20 µl. DNA samples were loaded in triplicate on the plate. In each working session, a NTC, a negative control consisting of DNA from *P. verrucosum* CBS 115508 and a positive control consisting of DNA from *P. nordicum* CBS 110769 were added. The amplification was carried out in a StepOne Plus Real Time PCR System (Applied Biosystems, Monza Italy), programmed to perform 2 min at 95 °C, 35 cycles of 20 sec at 95 °C, 20 sec at 55 °C and 30 sec at 72 °C.

End point PCR for the identification of A. westerdijkiae

The identification of *A. westerdijkiae* strains was confirmed by a species-specific reaction, as described by Gil-Serna and colleagues (2009) *(33)*. The PCR assay was performed using 5 µl of the DNA template (1 µg/ml) in a total reaction volume of 25 µl. The reaction mix consisted of PCR buffer [20 mM Tris - Cl, KCl, $(NH_4)_2SO_4$, $MgCl_2$ 1.5mM, pH 8.0] (QIAGEN, Milan Italy), 0.6 µM each of WESTF (5'-CTTCCTTAGGGGTGGCACAG-3') and WESTR (5'-CAACCTGATGAAATAGATTGGTTG-3') primers for ITS1-5.8S-ITS2 region of rDNA, 0.2 mM of dNTPs (Euroclone, Milan Italy), and 1 U of Hot Start Taq DNA polymerase (QIAGEN, Milan Italy). The reactions were performed in a thermocycler (Veriti 96 wells thermal cycler, Applied Biosystems). Thermal program included initial DNA denaturation at 95 °C for 15 min that followed by 40 cycles, consisting of denaturation at 95 °C for 30 sec, annealing at 63 °C for 30 sec, and extension at 72 °C for 40 sec, with a final extension at 72 °C for 5 min following the last cycle. The DNA fragments were length separated by electrophoresis through 2% agarose gels in Tris Borate EDTA (TBE) buffer and 0.5 µg/ml of ethidium bromide. Results were documented by using a UV transilluminator (Alliance 2.7, Uvitec, Cambridge).

Primer design for A. westerdijkiae confirmation

Since *A. westerdijkiae* was isolated, two specific pairs of primers for real time PCR were designed in order to detect this species. The available sequences of beta tubulin region of 10 *A. westerdijkiae* isolates (Genbank accession no.: EF150881.1, EF150880.1, EF150879.1, KT253229.1, KP329877.1, KP329876.1, KP329873.1, EF661329.1, EF661330.1 and JX535306.1) and of other 8 isolates of *Aspergillus* species were aligned on MultAlin *(34)*. The sequences considered for the alignment were: KC433701.1 (*A. acidus*), KF434634.1 and KF434635.1 *(A. carbonarius*), M38265 *(A. flavus),* AY048754.1 *(A. fumigatus)*, FJ828905.1 and FJ828914.1 *(A. niger)*, EF150882.1, EF150878.1, EF150877.1, KJ775051.1,

AY160979.1, EF661323.1, KJ767724.1, FJ608392.1, EU982088.1 and KJ136106.1 *(A. ochraceus)*, EF661346.1 *(A. steynii)* and L49386.1 (*A. parasiticus)*. The *in silico* specificity of the primer pairs was checked through Basic Local Alignment Search Tool (BLAST) *(35)* within the GenBank database. Primer sequences are shown in Table 1.

Table 1. Sequences 5'→3' of the two primer pairs and probes designed for Real Time PCR

Aspergillus westerdijkiae first pair (beta tubuline)	Awest1F	TCTACACGTTGGCATTG
	Awest1R	AACAAGTATCTAGGATTGAG
	Awest1probe	6FAM - TGGGCTATCTGAGATCATCTTTG – BHQ1
Aspergillus westerdijkiae second pair (beta tubuline)	Awest2F	AGAGATTGATTGTCTGATGGG
	Awest2R	TTGTACGGGGCGTTTCG
	Awest2probe	6FAM - TCCTCCGACCTTCAGCTGGAGC – BHQ1

Real time PCR for the identification of A. westerdijkiae

Two microliters of DNA isolated from fungal cultures (1 µg/ml) were used for the PCR assays, in a total volume of 20 µl, containing 6,2 µl of RNase free water, 10 µl of Promega Go Taq PCR Master mix (Applied Biosystems®), 0.4 µl of forward (10 µM) and 0.4 µl of reverse primer (10 µM), and 0.6 µl of probe (10 µM). For the first primer pair the thermal cycling was optimised as follows: the initial denaturation at 95 °C for 2 min was followed by 40 cycles (95 °C, 15 sec; 52 °C, 20 sec; 65 °C, 30 sec). For the second primer pair the initial denaturation at 95 °C for 2 min was followed by 40 cycles (95 °C, 15 sec; 55 °C, 20 sec; 65 °C, 30 sec). DNA samples were loaded in triplicate on the plate. In each working session, a NTC was also included.

Both primer pairs were tested on *A. westerdijkiae strain* CBS 112803 purchased from CBS KNAW (Utrecht, The Netherlands), *A. westerdijkiae* FUN LO SPV4 of Biobanking of Veterinary Resource from Istituto Zooprofilattico Sperimentale

della Lombardia e dell'Emilia Romagna (IZSLER, Brescia, Italy) and on 4 *A. westerdjkiae* strains isolated from salamis collected from 2011 to 2015. Aspecific annealing was evaluated on *A. ochraceus* CBS 108.08, *A. steynii* CBS 112812, *P. nordicum CBS 110769* and *P. verrucosum* CBS 115508, purchased from CBS KNAW; *A. fumigatus* FUN LO SPV2, *A. terreus* FUN LO SPV3, *A. candidus* FUN LO SPV1, *P. nalgiovense* FUN LO SPV7, *Penicillium solitum*, *Penicillium roquefortii* FUN LO SPV8, *Penicillium brevicompactum* FUN LO SPV6, from IZSLER's collection; *A. braziliensis* ATCC 16414 and *A. niger* ATCC 6275.

RESULTS

Prevalence of ochratoxin A in salami

Out of 133 salamis, 13 (9.8%) resulted positive to ochratoxin A. In Lombardy region 12/68 salamis (17,6%) were found positive, while in Emilia Romagna region only 1 out of 65 salamis tested (1,54%) was positive. In two cases high concentration of OTA were registered (691 µg/kg and 55,86 µg/kg). The concentration of OTA in positive samples is shown in Table 2.

Mycobiota of salami surface

From 133 salamis, a total of 247 fungal strains were isolated and identified at species level, in order to investigate the source of OTA. The genus *Penicillium* was the predominant one (185 strains), followed by the genus *Aspergillus* (33 strains). The remaining isolates belonged to species of *Mucor* (22 strains), *Cladosporium* (4 strains), *Geotrichum* (1 strain), *Eurotium* (1 strain) and *Fusarium* (1 strain). One hundred and eighty-five *Penicillium* colonies have been identified through culture-dependent method. Most of isolates (31.9%) were *P. nalgiovense*, followed by *P. solitum* (15.7%) and *P. chrysogenum* (12.4%). Other isolates were identified as *P. commune* (7%), *P. corylophilum* (6.5%), *P. citreonigrum* (4.9%) and *P. crustosum* (4.3%) (Fig. 2). For 7 *Penicillium* isolates it was not possible to identify the species using the dychotomic key. None of the

isolates was classified as *P. nordicum.* Regarding the 33 *Aspergillus* isolates, 30 (90.9%) were identified as *A. candidus* and 3 (9.1%) as the *A. westerdijkiae* (Fig. 3).

Table 2. Ochratoxin A concentration, molecular results about the presence/absence of *otanpsPN* gene and *P. nordicum*-specific *otapksPN* gene sequence in salami casings and viable fungal species isolated from 19 salamis.

OTA (µg/kg)	***otanpsPN* gene**	***P. nordicum*-specific *otapksPN* gene sequence**	**Isolated fungal species**
691	+	-	*A. westerdijkiae, P. chrysogenum*
7,74	+	+	*A. westerdijkiae, P. crustosum*
1,85	+	+	*Penicillium* spp.
1,14	+	+	*A. candidus, P. chrysogenum*
1,67	+	+	*P. solitum*
2,95	-	-	*A. candidus, P. nalgiovense*
1,32	+	+	*P. solitum*
-	+	-	*Penicillium* spp., *Mucor* spp.
12,48	+	+	*A. candidus, P. solitum*
1,47	+	+	*P. crustosum, P. nalgiovense, Penicillium* spp.
9,40	+	+	*A. westerdijkiae*
1,76	+	+	*A. candidus, P. nalgiovense, P. echinulatum*
5,67	-	-	*A. candidus, Mucor* spp.
-	+	-	*A. candidus, P. rugulosum*
-	-	-	*P. chrysogenum, Penicillium* spp.
-	+	+	*Micelia sterilia*
-	-	-	-
-	-	-	*A. candidus, P. cavernicola, P. chrysogenum, Penicillium* spp.
-	+	+	*P. nalgiovense, P. chrysogenum, Mucor* spp.

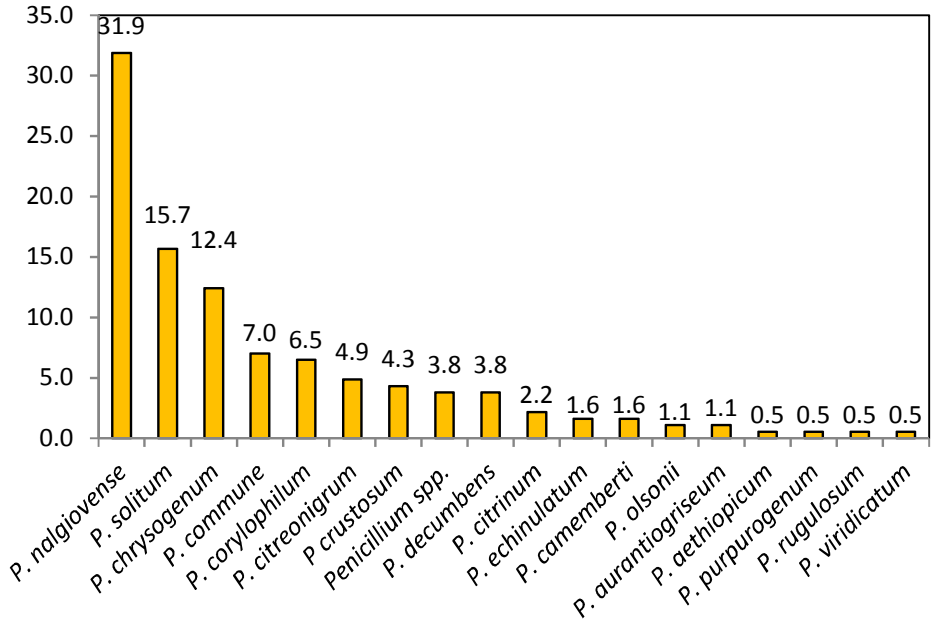

Figure 2. Distribution of *Penicillium* species. Percentage relative to the occurrence of each *Penicillium* species is shown.

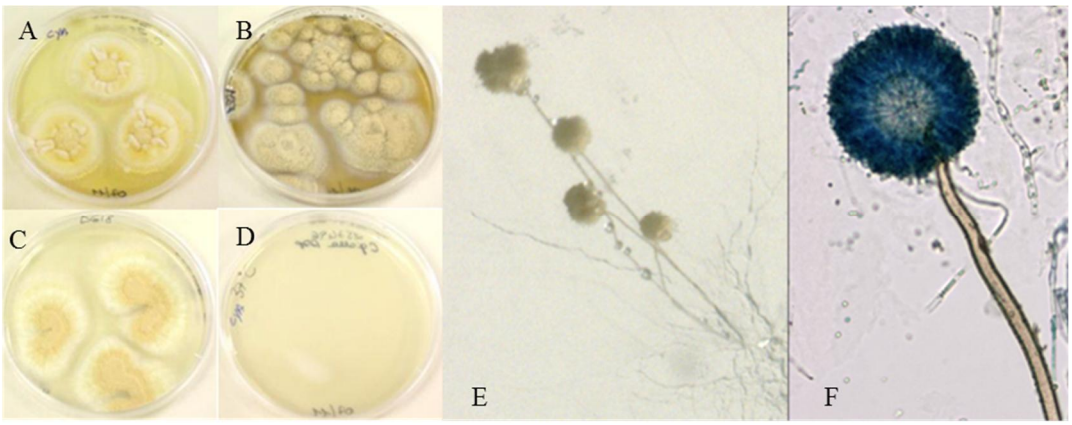

Figure 3. *A. westerdijkiae* grown on CYA (A), MEA (B) and DG18 (C) at 25±1 °C for 7 days. *A. westerdijkiae* on CYA was also incubated at 37 ± 1 °C for 7 days and no growth was observed (D). Conidial heads as visualized at stereomicroscope (E) and after lactophenol-cotton blue staining at optical microscope (total magnification 400X) (F).

PCR reactions for the detection of OTA-producing strains, ochratoxigenic P. nordicum and A. westerdijkiae from fungal isolates

Since only 3 (1.21%) strains of *A. westerdijkiae* potentially able to produce OTA were isolated, while 13 salamis (9.8%) presented OTA above the permitted concentration, a real time PCR-based approach was employed to verify the presence of DNA belonging to OTA-producing strains within 113 *Penicillium* and *Aspergillus* fungal cultures. Moreover, a real time PCR was also used to detect the presence of DNA of ochratoxigenic *P. nordicum* strains amongst 39 *Penicillium* strains, considering at least one strain per species and including also the 7 strains identified as *Penicillium* spp.

None of the DNA samples purified from the fungi isolated from the salami and tested in real time PCR confirmed the presence neither of the gene *otanpsPN*, nor of the species *P. nordicum*. The 3 strains microbiologically identified as *A. westerdijkiae* were confirmed molecularly through specific end-point PCR. However, when grown in the optimal conditions for the production of OTA, 2 out of these 3 strains were unable to produce the toxin, thus confirming the result of the real time PCR assay, while the remaining strain was impossible to test. Under adequate conditions for OTA synthesis, only the reference strains *P. nordicum* CBS 110769, *P. verrucosum* CBS 115508 and *A. ochraceus* CBS108.08 produced OTA, a positivity confirmed also in real time PCR for the presence of *otanpsPN* gene.

PCR reactions for the detection of OTA-producing strains and P. nordicum from salami casings

Since none of the isolated strains resulted to be the source of OTA in salamis, 19 salami casings, 12 of which belonging to salamis positive to OTA presence, were tested in real time PCR for the presence of OTA-producing strains and toxigenic *P. nordicum* strains. For the target *otanpsPN* gene, 14 DNA samples purified directly from the salami casings resulted positive, while for the target *otapksPN*

fragment specific for OTA-producing *P. nordicum* strains, 11 DNA samples out of 19 were positive (Tab.2).

PCR reactions for Aspergillus westerdijkiae

To test the specificity of the two primer pairs designed for the detection of *Aspergillus westerdijkiae*, 6 *A. westerdijkiae* strains, including the three strains isolated in this study, as well as 13 strains belonging to other *Aspergillus* and *Penicillium* species frequently isolated in food have been subjected to PCR. All strains of *A. westerdijkiae*, *A.ochraceus* and *P. nordicum* gave a positive amplification (Table 3). All other analysed food related fungi gave negative results.

Table 3. Positivity shown as Cycle Threshold (CT) to the two Real Time PCR reactions performed using the two primer pairs designed for *A. westerdijkiae.* The mean of triplicates of three independent experiments and their standard deviations are shown. The fungal DNA tested belonged to reference strains obtained from CBS-KNAW, IZSLER and ATCC collections or isolated during this project (*) or during a previous project (**).

Fungal species	**CT of Real Time PCR by using the first primer pair**	**CT of Real Time PCR by using the second primer pair**
A. westerdijkiae CBS 112803	33.58±0.16	29.99±0.01
*A. westerdijkiae PV1**	33.62±0.44	32.9±0.75
*A.westerdijkiae PV2**	33.42±0.72	34±0.19
*A.westerdijkiae MB**	35.48 ± 0.12	35.02±0.20
A.westerdijkiae FUN LO SPV4	28.5±0.06	28.13 ±0.32
A.westerdijkiae **	31.98±0.15	32.3±0.01
A. ochraceus CBS108.08	34.72±0.35	33.17±0.09
A. steynii CBS112812	-	-
P. verrucosum CBS 115508	-	-
P. nordicum CBS 110769	36.48±1.76	34.3±2.52
A. fumigatus FUN LO SPV2	-	-
A. terreus FUN LO SPV3	-	-
A. candidus FUN LO SPV1	-	-
A. niger ATCC 16414	-	-
A. braziliensis ATCC 6275	-	-
P. roquefortii FUN LO SPV8	-	-
P. brevicompactum FUN LO SPV6	-	-
*P. solitum***	-	-
P. nalgiovense FUN LO SPV7	-	-

DISCUSSION

The risk of OTA ingestion resulting from the consumption of salami is frequently underestimated, but in this work 9.8% of the salamis analysed were found contaminated with this toxin at levels higher than the limit of 1 µg/kg suggested by Italian regulation. In 3 salamis (2.25%) OTA concentration was even higher than 10 µg/kg, with a sample presenting a concentration 691 times over the limit. These data are comparable to what observed by other Authors: Pietri and colleagues (2006) *(36)* found OTA above the limit in 17% of salamis produced in Northern Italy, and in the 7% of cases OTA concentration was higher than 10 µg/kg. Armorini and colleagues (2016) *(37)* found 5 of a pool of 50 (10%) salamis produced in Veneto region of Northern Italy contaminated by OTA, and one sample where OTA had reached 103.69 µg/kg.

Even higher percentages of OTA contamination have been observed by several other Authors: Markov and colleagues (2013) *(38)*, during a monitoring study on Croatian salamis, found 54% of contaminated samples; Monaci and colleagues (2005) *(39)* reported OTA in 46.7% of salamis produced in Southern Italy. The high prevalence of OTA contamination registered by these studies could be explained by the climate conditions occurring in the areas investigated. In the present study, despite the high percentage of contaminated salamis, only 3 strains out of 247 identified through culture-dependent method confirmed to be *A. westerdijkiae* via PCR, a species potentially ochratoxigenic. *A. westerdijkiae* has been recently recognized as a major contaminant of meat products *(40)*, even though it is more known as major risk agent in carbon-rich food matrices of plant origin *(14, 41).* This species, together with *A. steynii,* derives from a taxonomical revision made within the *A. ochraceus* group, and it is acknowledged as a relevant OTA-producing species *(42).* Scaramuzza and collaborators (2015) *(43)* found it present in three Italian meat manufacturing plants, while Iacumin and co-Authors (2011) *(10)* reported in their study that 34% of casings of Italian sausages displaying high levels of OTA were contaminated with *A. ochraceus*.

In this monitoring study, performed on artisanal salami produced in Lombardy and Emilia Romagna regions of Northern Italy, other fungi belonging to the genera *Aspergillus* and *Penicillium* were isolated. Thirty isolates were identified as *A. candidus*, a species believed unable to produce ochratoxin A, however the most frequently isolated fungi were *P. nalgiovense*, *P. solitum*, *P. chrysogenum* and *P. commune*. These species are usually considered safe and are not able to produce ochratoxin A. *P. nalgiovense* and *P. chrysogenum* are even admitted by Italian legislation as fungal starters, and are commonly used in large-scale production. *P. solitum* and *P. commune* can produce metabolites which effects are not well known, in particular *P. commune* produces cyclopiazonic acid, an inhibitor of Ca^{2+} ATPase in skeletal muscle and in the sarcoplasmic reticulum *(44)*.

Data about mycoflora of salami obtained in this work are comparable to what observed by other Authors, and the most frequently isolated fungal species from salami constitute the typical flora of this food around the world. In a study conducted in Greece *(4) Penicillium* was the predominant fungal genus in sausages, and the species most frequently isolated were *P. solitum*, *P. nalgiovense*, *P. commune* and *P.olsonii*. Also in other two studies, led respectively in Spain *(45)* and in Argentina *(46)*, *P. nalgiovense*, *P. chrysogenum*, *P. olsonii*, *P. solitum* and *P. griseofulvum* resulted the most isolated species. *Penicillium* results the predominant genus also in dry-cured meat produced in Italy, with the species *P. nalgiovense*, *P. chrysogenum*, *P. olsonii*, *P. solitum* and *P. nordicum (47)*. The lower occurrence of *Aspergillus* respect to *Penicillium* in all studies is due to the temperature of salami maturing rooms, which ranges 10-22 °C, optimal for the growth of *Penicillium (28)*.

In order to support the results of the microbiology tests, and to further investigate the reason of the difference between the high prevalence of OTA in the salamis analysed in this study and the low occurrence of isolated OTA-producing species, a molecular assay has been set up using real time PCR. All the field isolates

analysed resulted negative for the *otanpsPN* gene, and all the *Penicillium* species were recognized as not *P. nordicum*. This outcome is in line to what observed using the culture dependent method. Interestingly, the 3 *A. westerdijkiae* strains resulted also unable to amplify the *otanpsPN* gene. The lacking of this gene, common to all OTA-producing strains, was also confirmed by the inability of these fungal cultures to produce OTA after 30 days of incubation at 25 °C. This is consistent with the fact that not all strains within a recognised potential OTA-producing species are able to produce OTA. Few data about the percentage of OTA-producing strains in *A. westerdijkiae* species are available, while for the most studied ochratoxigenic species contaminating grapes, *A. carbonarius*, these data are well known *(48)*. In a study conducted by Gil-Serna and colleagues (2011) *(49)*, 6 out of 8 *A. westerdijkiae* strains (75%) isolated from different foods and their close environment resulted able to produce OTA if grown on CYA medium. However, the low number of strains considered by both their and our study does not allow to throw any conclusion regarding the occurring prevalence of OTA-producing strains within this species. A more complete study should include a higher number of colonies isolated from the same salami and sharing the same morphologic characteristics, which should be tested for the presence of the *otanpsPN* gene, in order to define the variability occurring among strains belonging to the same species and growing simultaneously.

In our study the classical microbiological method coupled to the molecular assay used as confirmation tool failed to identify the origin of the OTA contamination. This could be due to the fact that the mycoflora of the early maturation stage, that colonizes the salami when the a_w is still high, is different from the flora present at the end of the maturation, and some of the strains responsible for the production of OTA might be no longer viable. To verify the presence of OTA-producing strains in any stage of salami maturation, the same real time PCR reaction for *otanpsPN* gene was conducted by testing total genomic DNA purified directly from the casing of 19 salamis, 12 of which found positive to OTA. Ten salamis

in which OTA was quantified above 1 μg/kg of meat had their salami casings positive to *otanpsPN* gene target. On the contrary, in two salamis positive for OTA, neither the *otapksPN* gene target nor its fragment specific for OTA-producing *P. nordicum* was amplified (Tab. 2). For these 2 cases either the molecular test failed at some level, or the toxin could come from the carry-over in swine tissues used to prepare salami, originating from the contaminated feeding. Among the 7 salami casings negative to OTA presence, 4 resulted positive to the amplification of *otanpsPN*. This discrepancy could be easily due to the fact that in these samples there have not been the conditions of temperature, a_w, pH and nutrients necessary for the OTA synthesis (Tab. 2). Moreover, in our study, no strain of *P. nordicum* was isolated, but 11 salami casings were positive to the amplification of the *otapksPN* gene fragment specific for this species. Since *P. nordicum* prefers high water activity, it belongs to the flora of early maturation stages and is probably not viable and isolable in late maturation stages. Finally, one of the salami casing positive for the two targets, but negative for OTA, was colonized only by one fungus, which did not show reproductive structures and was therefore impossible to classify (Tab. 2).

In the 3 salamis where the OTA-producing species *A. westerdijkiae* were isolated, high concentrations of OTA were measured (691 μg/kg, 9.4 μg/kg and 7.74 μg/kg), but none of the isolated strains were able to produce the toxin after the incubation for 30 days at 25 °C. Moreover, despite these isolates proved to lack the *otanpsPN* gene, all the DNA purified from the three casings were positive to this gene. Finally, although in two cases the DNA of *P. nordicum* was found, the salami with the highest OTA concentration did not display its presence (Tab. 2): this leads to the conclusion that most probably more than one strain of *A. westerdijkiae* were simultaneously present at some maturation stage, and that the one that was not isolated was responsible for the production of the toxin. Given the excess of toxin measured by LC-MS, the chances that the contamination was due to carry-over in swine tissues are in fact negligible. Moreover, because no

strains of *P. nordicum* were isolated from the environment of whole salami factory (data not shown), it is unlikely that this species was the primal source of contamination.

In this work, two primer pairs for the identification through real time PCR of *Aspergillus westerdijkiae,* the only potential OTA-producing species isolated in this monitoring, were designed. All *A. westerdijkiae* strains tested resulted positive for both the primer pairs, but both the primer pairs do not resulted species-specific, since they also work for the only strain of *Aspergillus ochraceus* and *Penicillium nordicum* tested. While aspecific amplification of *A. ochraceus* DNA can be caused by the fact the two species are closely related, as proven through partial beta tubulin sequencing by Frisvad and colleague (2004) *(29)*, aspecific amplification obtained with *P. nordicum* DNA was unexpected. Hence, both the primer pairs could be exploited for the rapid and early detection of the three species *A.westerdijkiae*, *A. ochraceus* and *P. nordicum*, which are recognized by literature the main OTA-producing species contaminating salami *(6, 9 - 11, 43)*. Further tests are required to verify if these primer pairs are able to recognise other strains of *A.ochraceus* and *P. nordicum* different from the one tested. Only the knowledge of the whole genome of *A. westerdijkiae* and all its closely related species will allow the design of species-specific primer pairs in other regions of DNA.

CONCLUSION

In this study, about 10% of the tested salamis resulted contaminated by ochratoxin A. None of the 247 strains isolated was ochratoxigenic, and only 3 belonged to the ochratoxigenic species *Aspergillus westerdijkiae*. These data suggest that the identification of viable strains at the end of maturation is not always predictive for the presence of OTA, since the information about the mycoflora present at early stages could be lost. Moreover, the isolation of the strains responsible for the production of toxins previously identified in a salami does not represent an

easy task, and often the microbiological search is not able to recognize the presence of these strains within the multitude of different fungal strains that colonize salamis at the end of the maturation. Therefore, not only the culture-dependent identification, but also the molecular tools performed on the fungal DNA as confirmation, do not result exhaustive indicators of the presence of OTA-producing strains.

On the other hand, this study highlighted how the use of molecular tools applied directly on the contaminated matrix, and thus by-passing the standard cultivation methods, might be more predictive of the presence of OTA, even with some limitations. In fact, if the molecular tool applied on the DNA purified from salamis recognised 14 salamis as presumably contaminated by OTA when in 10 salamis OTA was effectively detected, the culture-dependent identification has highlighted only 3 salamis as likely contaminated by OTA.

Although the amplification of predictive targets in salami casings does not allow to recognize the risk in salami in which OTA comes from contaminated meat, it helps foreseeing the production of the toxin in salami colonized by OTA-producing strains, and gives indications about the risk following the precautionary principle. Despite this method looks promising for predicting the OTA risk in salamis, it is worth to highlight the importance of using high yield purified DNA for the amplification of the selected targets. Natural casings are used for the manufacturing of traditional Italian salami, and their main component collagen can inhibit DNA amplification, as Kim and colleagues (2000) *(50)* reported for the amplification of bacterial DNA from sausage casings made with pig intestine. In our study two commercial methods for DNA purification from both the casings and the suspensions of spores obtained through the washing of casings were tested, in order to select the method allowing the highest purity (data not shown). Given the prevalence of OTA found in traditional salami, further wider monitoring studies, that include small-scale salami production plants as well, implemented with data about the presence of fungal spores in air and physical

parameters, like temperature and humidity, should be carried out. The correct identification of fungal species and strains colonizing salami achieved through rapid molecular methods is a promising tool for the rapid quality and safety assessment of food products and for HACCP (Hazard Analysis Critical Control Point) analysis, in order to avoid the exposition of salami colonized by OTA-producing fungi to favourable conditions for the synthesis of the toxin.

Even if the amplification of predictive targets in salami casings does not allow to recognize the risk in salami in which OTA comes from contaminated meat, it allows to foresee the toxin production in salami colonized by OTA-producing strains and gives an indication about the risk following the precautionary principle. Despite this method is promising for predicting the OTA risk in salamis, it is crucial to use purified DNA for the amplification of the selected targets. Natural casings are used for the manufacturing of traditional Italian salami and their main component, the collagen, can inhibit DNA amplification as Kim and colleagues (2000) reported for the amplification of bacterial DNA from sausage casings made with pig intestine. In fact, in our study two commercial methods for DNA purification from both the casings and the suspensions of spores obtained through the washing of casings were tested, in order to choose the method, which allow the highest purity (data not shown).

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Chapter 5: Unpublished results

Hemp traceability and safety: RAPD (Random Amplified Polymorphic DNA) and mycotoxin detection as integrated tools of control

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ABSTRACT

The demand for hempseeds (*Cannabis sativa* L.) has recently increased, due to nutritional properties of this food, rich in protein, fibres, Omega 3 and 6 fatty acids. Although the hemp cultivation in Italy has been reintroduced, the Italian production does not cover the whole request of the market thus requiring the importation of hempseeds from foreign countries. Fungi can grow on hempseeds during growth in field, transport and preservation, potentially leading the production of mycotoxins, entering in the food chain and representing a crucial risk for human health

Aim of this work was to exploit DNA RAPD markers to set up a method to recognize and cluster some Italian ecotypes of hemp from hempseeds grown in China and Canada, which are among the world-leading hempseeds producer. Twenty decamers were tested for the amplification of the 10 hemp ecotypes. The Random Amplified Polymorphic DNA (RAPD) technique by using selected 6 primers resulted an inexpensive and useful approach for germoplams identification at ecotype level. Data on mycotoxins show a low degree of contamination, both for Italian and Chinese samples. More deep investigation on larger samples is required to define the risk correlated to this food product. Finally, the combined DNA analysis for traceability and mycotoxins detection is a promising tool for the risk assessment of hemp-related products.

INTRODUCTION

Hemp (*Cannabis sativa* L.) is a source of fibers and cellulose, and its seeds are considered a heath protecting food. Hempseed typically contains over 30% of oil and about 20-25% of proteins, with a considerable amount of dietary fiber, minerals like iron and zinc, and vitamin E and B1 *(1)*. Hempseeds can be pressed to make oil highly stable to oxidation and particularly rich in linoleic acid (ω-6) and α-linolenic acid (ω -3) *(2)*. Hempseed oil has a high unsaturated/saturated fatty acid ratio, and a 3:1 ratio of omega-6 to omega-3 essential fatty acids, which

matches the balance required by human body, resulting useful to produce nutraceutical products *(3)*. Recent researches performed on hemp proteins showed that specific fractions obtained following enzymatic digestion and ultrafiltration show significant antihypertensive properties. In fact, hemp protein hydrolisate after digestion by the sequential action of pepsin and pancreatin (to mimic human gastrointestinal digestion), separated into peptide fraction by ultrafiltration, showed in vitro inhibition of the activities of angiotensin I-converting enzyme (ACE) and renin, the two main enzymes involved in hypertension *(4)*. Moreover, following a consecutive fractionation of this hydrolisate by reverse-phase HPLC followed by tandem mass spectrometry analysis of active peaks, 23 short-chain peptides were identified and characterized. Among these, two peptides (Trp-Val-Tur-Tyr and Pro-Ser-Leu-Ala) showed a significant anthypertensive capacity *(5)*. Recenlty, the production of the hemp oil is a key strategy to obtain alfa linolenic fatty acid-rich food products, particularly when cold pressed. This process allow to obtain a by-product, called hemp "cake", which can be milled to produce flour, starting base for products like pasta, cookies and energy bars. The hemp flour composition depends on the variety and planting locality, and it differs according to the defatting process *(6)*. High levels of proteins, particularly edestin and albumin, which are easily digested, characterize the hemp flour, with high amounts of essential amino acids *(7)*.

The hemp cultivation started in Asia in the Neolithic period and spread to Middle East and Europe, where some countries like Italy, Russia, France, Poland, Spain and United Kingdom developed a great tradition *(8, 9)*. Hemp production has declined in all Europe in the 20th century because of the spread of cheaper synthetic fibers, but during the second half of the '90s the European Community started to promote crops with low environmental impact, alternatives to crops produced in excess, and hemp cultivars with a low content of tetrahydrocannabinol (THC) were reintroduced. Currently, more than 30 Countries worldwide grow hemp, and, according to FAOSTAT, France has been

the world-leading hemp producer in 2014, followed by China *(10)*. In Italy hempseeds are grown on the 0,002% of its agricultural area *(11)*. Despite the weak spreading of hemp cultivation, the production has been focused on traditional Italian cultivars like Carmagnola, Carmagnola Selezionata (CS) and Fibranova. These are high-quality cultivars that had made Italy the second hemp producer in the world from the 19th century until the beginning of the 20th century.

The traceability of hempseeds, like other crops, has become essential to guarantee consumers about the geographical origin of a product, as well as to comply with safety by containing the risks. Contamination of cereal commodities with mycotoxins during long-lasting storage and transportation represents a significant hazard for consumer health: changes in temperature and relative humidity, which may occur during transportation, make these commodities likely to be damaged by molds growth, and potentially by mycotoxins accumulation, even if the more significant quantity of mycotoxins is generally formed during the pre-harvest period *(12, 13)*. Differences among the samples can be also correlated to the cultivar as well as to environmental or agronomical parameters (pre-cropping). Within the most frequently isolated mycotoxins in post-harvest phases, aflatoxins (aflatoxin B1, B2, G1, G2) and ochratoxin A (OTA) are particularly toxic. Aflatoxin B1 is the most potent chemical liver carcinogens known, and it is also immunosuppressive, teratogenic and mutagenic *(14)*. Kidney has been identified as target organ of OTA, but in sufficiently high concentrations also liver can be damaged by this mycotoxin *(15)*. Ochratoxin is carcinogenic in rats and mice, and it is suspected as possibly carcinogenic in humans too *(16)*. The maximum level of aflatoxins and ochratoxin A permitted in hemp oil and hemp flour is set respectively by European Commission (EC) Regulation No. 165/2010 *(17)* and European Commission (EC) Regulation No.1881/2006 *(18)*.

To prevent mycotoxins from entering the food chain, controlled storage conditions and safety controls aimed at monitoring their levels are required. Rapid, inexpensive and efficient analytical methods for crops traceability are

needed to protect products belonging to approved cultivars, which should be free from diseases, human pathogens, natural toxins and chemicals.

The genetic diversity between populations at molecular level is often evaluated in crops using various PCR-based techniques. The Random Amplified Polymorphic DNA (RAPD) technique has been successfully used in crop genetic analysis, to assess the phylogenetic diversity among 40 rice accessions from Africa *(19)* and to estimate the genetic diversity in a set of land rices in comparison to a representative sample of improved rice varieties *(20)*. Since RAPD analysis performs on both coding and non-coding regions in the genome, RAPD has been chosen as method to be tested for the classification of different genotypes within the same species *(21, 22)*. The RAPD is a simple, fast and inexpensive method, and it does not require the knowledge of genomic sequences. Since it can be considered as a primary low-budget approach to determine the geographical origin of crops, RAPD technique was used in this work in order to cluster some ecotypes of hempseed and particularly to discriminate Italian high-quality ecotypes intended for human consumption from foreign products, often intended for other purposes. Finally, we aimed to correlate the geographical origin to the mycotoxin content, trying to identify if this double approach can be used to estimate the safety as well as the quality of the hemps seeds.

MATERIAL AND METHODS

Samples

*Differen*t ecotypes of hempseeds coming from Canada, China and different parts of Italy (Alessandria, Asti, Carmagnola, Vezza d'Alba and Verona) were considered in this study. Seeds were first washed in NaOCl 2% for 2 minutes and treated with denatured alcohol to eliminate the microbial contamination on their surface.

DNA extraction

For each variety, 5 g of seeds were finely ground in liquid nitrogen with mortar and pestle. Total genomic DNA was then extracted and purified from 1 g of powdered seeds, using a commercial kit (NucleoSpin Plant II, Macherey-Nagel, Switzerland). DNA concentration was determined using Qubit fluorometer (Invitrogen, Thermo Fisher, USA), and only samples displaying 15 to 50 ng/µl were considered.

RAPD analysis

Preliminary RAPD experiments were performed to standardize DNA polymerase and Mg^{2+} concentrations. A set of 20 random decamer oligonucleotides (Table 1) (Invitrogen) was tested on 10 hemp ecotypes having different origins (5 Italian, 4 Chinese and 1 Canadian). For each primer, the reaction mixture was composed of 30 ng of DNA template, 1 U of DNA polymerase (Biotools), 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Biotools), 1X Buffer (Biotools), 0,6 µM primer, in a final volume of 20 µl. The amplification was carried out in an iCycler thermal cycler (BioRad, USA), programmed to perform a first denaturing step of 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 37 °C, 1 min at 72°C, and terminating with 7 min at 72 °C.

The RAPD amplicons were separated on agarose gels 2.5% in TBE buffer 1X (8.8 mM Tris-HCl, 8.8 mM boric acid, and 0.2 mM EDTA) for 200 min at 80 V. The gels were stained with Atlas ClearSight (Bioatlas) and photographed under ultraviolet light using the Alpha Imager HP detector (Alpha Innotech).

Table 1. Sequences of the arbitrarily selected random primers used for RAPD-PCR reactions.

Primer	**Sequences 5'→3'**	**Primer**	**Sequences 5'→3'**
A10	GTGATCGCAG	C4	GATGACCGCC
A11	CAATCGCCGT	D2	GGACCCAACC
A12	TCGGCGATAG	D5	TGAGCGGACA
A14	TCTGTGCTGG	E12	TTATCGCCCC
A15	TTCCGAACCC	E18	GGACTGCAGA
B1	GTTTCGCTCC	F3	CCTGATACC
B2	TGATCCCTGG	F13	GGCTGCAGAA
B6	TGCTCTGCCC	J20	AAGCGGCCTC
B12	CCTTGACGCA	M11	GTCCACTGTG
B20	GGACCCTTAC	U2	CAATCGCCGT

Data analysis

RAPD amplicons were scored in a data matrix for each primer and genotype, exploiting a previous approach used for the analisys of other matrices *(23)*, assigning "1" value in case of presence of a visible band (amplicon) and "0" value when absent. Only clear major bands were scored.

A classification analysis was carried out using Dice's *(24)* index of similarity for all pair-wise comparisons, based on the proportion of shared bands produced by each primer. Hierarchical cluster analysis of the molecular data sets was performed using the statistical software R (R Core Team) *(25).* The hierarchical cluster analysis uses a set of dissimilarities for the objects to be clustered. Initially, each object is assigned to its own cluster and then the algorithm proceeds iteratively, at each stage joining the two most similar clusters, continuing until there is just a single cluster. At each stage distances between clusters are recomputed by the Lance–Williams dissimilarity update formula.

Aflatoxin B1, B2, G1 and G2 determination in hemp samples

- *Sample preparation.* Ground sample (2 g) was weighted in a 50-mL centrifuge tube and then blended in a high-speed blender (Ultraturrax T18 Basic; IKA, Stauffen, Germany) with 8 ml of methanol/water (60:40 v/v) for 3 min. After centrifugation (4000 rpm, 20 min), 4 ml of the supernatant were diluted with 8 ml of PBS buffer (10 mM, pH 7.4), until the content of methanol was lower than 20% (v/v). This solution was filtered through membrane filter (0.45µm) and applied onto the immunoaffinity column (AflaStar, RomerLabs, Tulln, Austria). After that the analytes were eluted with 2 ml of methanol. The solvent was dried down under a gentle stream of nitrogen and the residue was derivatized as follow: 200µL of hexane were added to the vial, mixed by vortex for few seconds and then 50µL of trifluoroacetic acid was added. The sample was mixed again for 30s. The reaction time was 5 min. Finally, 500µL of water/acetonitrile (90:10 v/v) was added, mixed and leaved for about 10 min to be separated well the layers. The aqueous layer was analysed by HPLC/FLD.

- *HPLC/FLD analysis.* HPLC-FLD analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a Waters™ 474 Scanning Fluorescence Detector. Chromatographic conditions were the following: the column was a Waters C18 XTerra (250 mm × 2.1 mm, 5 µm), The flow rate was 0.250 mL/min; the column temperature was set at 30°C; the injection volume was 20 µL; isocratic elution was performed using bidistilled water (eluent A) and methanol (eluent B) in proportion: 70:30 for 35 min. The fluorescence detection was obtained with the following conditions: $\lambda_{ex} = 365$ nm, $\lambda_{ex} = 425$ nm, gain = ×100, attenuation = 32, band width: 40 nm. For each sample, the entire procedure was performed in duplicate (n = 2). Calibration curves were performed in the calibration range 0.25-2 µg/kg.

Ochratoxin A determination in hemp samples

- *Sample preparation.* Ground sample (1 g) was weighted in a 50-ml centrifuge tube and extracted with a solution of 10 ml chloroform acidified with 75 µl of 85% *o*-phosphoric acid solution, by homogenising with an Ultraturrax (Ultraturrax T18; IKA-WERKE, Stauffen, Germany) for 2 min. After filtration on a paper filter, the whole volume was collected in a 15-ml centrifuge tube. Then, ochratoxin A was extracted by washing the organic phase with 1 ml of 0.2 M Tris-HCl buffer (pH 8.5). To expedite emulsions breakage and the separation of aqueous and organic phases, samples were centrifuged at 4000 rpm for 5 min. Finally, 450 µl of the upper aqueous phase were transferred in a 2-ml septum vial. To avoid the growth of OTA-degrading microorganisms in the aqueous phase, a volume of CH_3CN was added to achieve a 0.2 M Tris-HCl: CH_3CN (90:10, v/v) ratio. An aliquot (10 µl) of this solution was analysed by HPLC/FLD.

- *HPLC/FLD analysis.* LC-FLD analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a Waters™ 474 Scanning Fluorescence Detector, under isocratic conditions at 30°C, with an aqueous NH_3/NH_4Cl (20 mM, pH 9.8):CH_3CN (85:15) mobile phase; the column was a C18 XTerra (250 mm × 2.1 mm, 5 µm), the flow rate was 0.250 ml/min and the injected volume was 10 µl. The fluorescence detection was obtained with the following conditions: $\lambda_{ex} = 380$ nm, $\lambda_{em} = 440$ nm, gain = ×1, EUFS = 100, band whidt: 40 nm. For each sample, the entire procedure was performed in duplicate (n = 2). Calibration curves in 0.2 M Tris–HCl: CH_3CN (90:10, v/v) were used (calibration range 0.5-7 µg/kg).

RESULTS

Six primers to cluster hempseeds depending on their geographical origin

The aim of this work was to verify whether a simple molecular tool like RAPD could be applied for the easy identification of hempseed samples cultivated in different countries (Italy, China and Canada).

Out of the 20 RAPD primers used for the amplification of the 10 hemp ecotypes, 5 primers (A14, B1, E12, E18 and U2) did not give any band for almost all ecotypes. Among the other 15 primers, 6 primers (A10, A11, B20, C4, F3 and J20) produced reproducible fingerprints, characterized by the presence of polymorphic amplicons in some ecotypes. A total of 48 distinct bands were obtained, 33 of which (68.75%) were polymorphic.

By studying all of the 48 RAPD products, a hierarchical clustering with a good separation was obtained (Fig. 1); the 10 ecotypes clustered into two major groups, with Dice's similarity coefficient ranging from 0.30 to 0.91. The Chinese samples were clearly separated from samples cultivated in Italy and Canada, with the Italian ecotypes clustering together. The Canadian ecotype shared the same root with the Italian samples.

The most similar clustering obtained by using less than 6 primers was obtained considering 38 bands, corresponding to the amplification of 5 selected RAPD primers (A11, B20, C4, F3 and J20). The primer A10 appeared to not add information when used in combination with the other five, but it is essential when a lower number of primer were used.

Comparisons between ecotypes were calculated considering each combination of 2 primers. Nine pairs of primers out of fifteen possible combinations containing either primer A10 or A11 resulted useful in separating each ecotype. Amongst these 9 combinations, 5 combinations (A10 and B20, A11 and B20, A10 and C4, A10 and F3, A11 and F3) allowed the distinction between Chinese and Italian ecotypes (Figure 2). On the contrary, none of the primer, when used alone, was capable to form separate clusters, even if the primers A10 and A11 resulted able to separate each ecotype from the others. A very clear and distinguishable cluster for ecotypes/samples obtained from China is recoverable (Fig. 2).

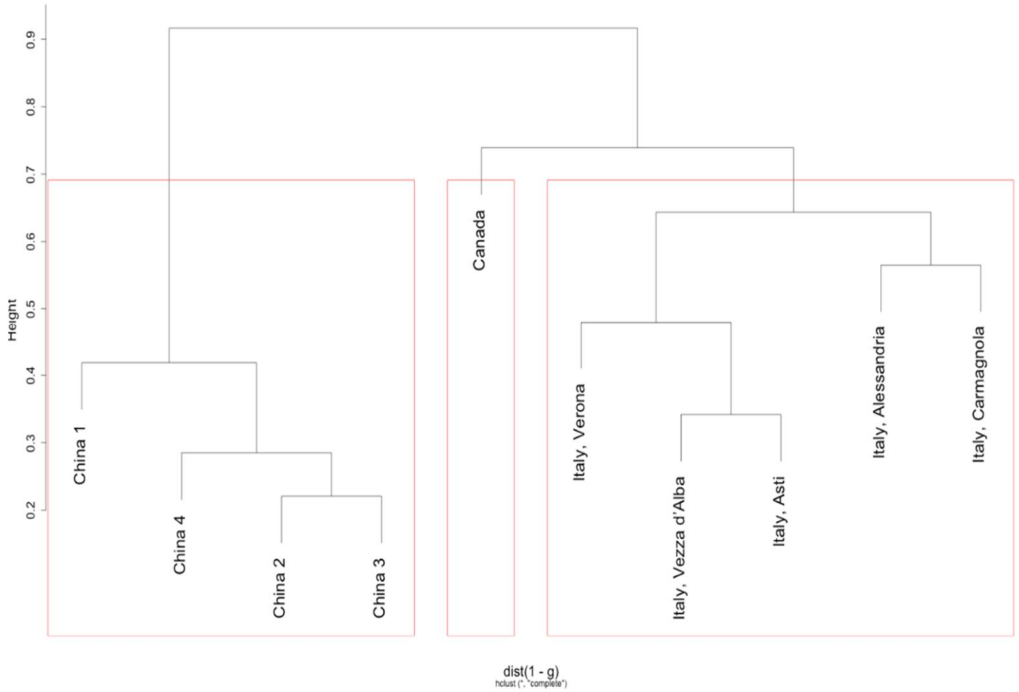

Figure 1. Hierarchical clustering obtained from the 48 selected polymorphic RAPD-bands generated by primers A10, A11, B20, C4, F3 and J20; the height is derived from the distance matrix values. The geographical origin is shown for each ecotype.

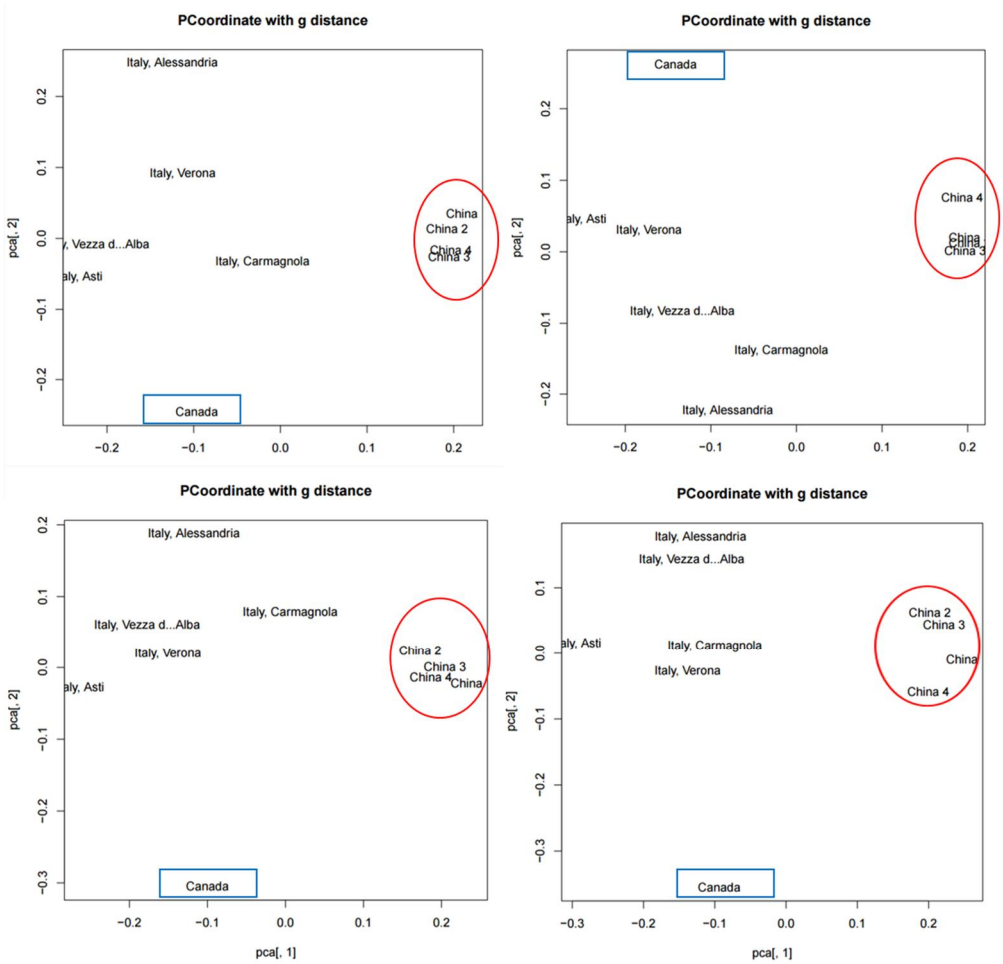

Figure 2. Principal Component Analysis (PCA) plots obtained by using four primers pairs, which allow the clustering of the 4 Chinese ecotypes from the 5 Italian and the 1 Canadian ecotypes. From the top left to the bottom right: A10 and B20; A10 and C4; A10 and F3; A11 and F3.

Mycotoxin content

Regarding the mycotoxin content, none of the Italian samples had quantifiable levels of either aflatoxin B1 or aflatoxin G1 (Table 2). In one sample, the aflatoxin G1 concentration resided below the limits of quantification (LOQ). None of the Italian sample resulted contaminated by ochratoxin A.

Concerning hemp samples from China, low amount of AFB1 and OTA were found in all the considered samples, with the exception of de-hulled seeds. De-hulling, in fact, is widely considered as a mitigation procedure for seeds and grains *(26)*. Mycotoxins, on the other side, were not found in the Canadian hemp sample (Table 2).

Table 2. Aflatoxins and ochratoxin A concentration in hempseed related to the origin.

Origin of the samples	**[OTA] µg/Kg**	**[AFB1+AFG1+AFB2+AFG2] µg/Kg**
Carmagnola, Italy	< LOD	0.06±0.01 (AFG1)
Vezza d'Alba,Italy	< LOD	< LOD
Verona, Italy	< LOD	< LOD
Asti, Italy	< LOD	< LOQ (AFB1)
Alessandria, Italy	< LOD	< LOD
China 1(Dehulled)	< LOD	< LOD
China 2	**0.85±0.01**	**0.74±0.04 (AFB1)**
China 3	**1.42±0.02**	**0.88±0.03 (AFB1)**
China 4	< LOQ	**1.24±0.05 (AFB1)**
Canada	< LOD	< LOD

OTA: Ochratoxin A; LOD (limit of detection): 0.05 µg/Kg; LOQ (limit of quantification): 0.1 µg/Kg

AF: Aflatoxins; LOD (limit of detection): 0.05 µg/Kg for AFB1; LOQ (limit of quantification): 0.1 µg/Kg for AFB1

DISCUSSION

The first step in assuring quality and safety of crops is the correct identification of the origin. In the present paper, 10 ecotypes of *Cannabis sativa* for food use (producing very low quantity of tetrahydrocannabinol, not expressed in the seeds) were analyzed through RAPD technique. To optimize the identification at genotype/ecotype level, indirectly, testing the capacity to identify the geographical origin of hempseed, 20 random decamers were tested. The combined results given by only 6 primers (A10, A11, B20, C4, F3 and J20) showed the best resolution in clustering the ecotypes depending on where they were cultivated. This RAPD approach led us to conclude that the minimum number of RAPD products providing a reproducible clustering was 38, but considering all 48 bands a higher resolution is achieved, as already observed in other studies for *Brassica* and *Boesenbergia* genera *(27, 28)*.

RAPD markers in *Cannabis sativa* has been proven effective for clustering cultivars, as observed in other previously published works *(29, 30)*. Considering primer A10 and A11, other couples of primers where at least one member was either A10 or A11, all the ecotypes analyzed showed distinguishable RAPD patterns. Such a degree of polymorphism in *C. sativa* is explained by its allogamy, and it is comparable to other allogamous species like potato (*Solanum tuberosum) (31)*.

It is interesting to notice that 5 primer pairs are enough to group Chinese ecotypes in an isolated location, indicating that there is a real genetic distance between Chinese cultivars and cultivars cultivated in other countries that goes beyond the geographic distance. This result is in accordance with the hypothesis that the hemp has originated from the Central Asia and has then spread in Europe *(32)*. Also in previous studies based on morphology *(33)* and allozyme data *(34)*, Chinese and European ecotypes resulted separated, and four groups (ecotypes) of hemp were identified: Northern (Northern Russian, Finland), Central (Central

Russian, Ukraine), Southern (Mediterranean region, Balkan, Turkey, Caucasus), and Far Eastern ecotypes (China, Japan and Korea).

The Canadian sample showed genetic association with the Italian genotypes, suggesting a common genetic origin. Main hemp cultivars cultivated in Canada are Finola (originating from Finland), Crag (an indigenous ecotype from Canada), and USO 14 (from Ukraine) *(35)*. To allow a better separation between European and Canadian ecotypes, a higher number of primers is suggested. This could permit also the discrimination among the Italian ecotypes according to the geographic area of growth, which is not feasible using only a set of six primers. The correlation of genomic clustering with geographical origin can only be established when ecotypes are associated to a specific known area of cultivation: DNA analysis can provide the origin at "ecotype" level, but if the same ecotype is grown in different geographical areas the traceability cannot be reliable. For this reason, we suggest this DNA-based method to be used as first screening test to identify the hemp's germoplasm; more advanced approaches (particularly Isotopic Ratio Mass Analysis, IRMS) should be a complementary analysis to assess geographical origin, even if a lot of work needs to be done in order to create a solid database. This approach has already been used to distinguish marijuana's geographic origin exploiting carbon and nitrogen stable isotopes *(36)*.

Mycotoxin contamination on hemp samples from different geographic origin

As reported, some samples of hempseeds analysed in this study showed the presence of mycotoxins. No specific correlation with original Country of production can be made, in consideration of the small set of samples considered for each origin. None of the contaminated samples exceeded the legal limit fixed in Europe for flours intended as food (Commission Regulation (EC) No 1881/2006) or feed (Directive 2002/32/EC) *(37)*. Moreover, the conditions/parameters of preservation (and particularly the time that elapses between the harvest and the drying of the seeds) are critical for the quality and the

safety of the seeds. In Italy, until today, no industrial advanced protocols of drying are used for hemp, often produced in small lots by small farmers. This step represents perhaps the first criticism for the fungal growth and the mycotoxin production. The second criticism is surely represented by the transport of the seeds as well as the preservation before the processing (cold pressing and production of flours). On the other side, the low contamination found in Chinese seeds may suggest that hemp from China should be carefully monitored. As already reported for other commodities, this higher incidence of mycotoxins could be due to multiple factors, such as the environment and the agronomic parameters, or the storage and transport conditions. In addition, the lack in harmonised control policies may allow contaminated batches to enter the market.

This fact must to be investigated more deeply, also considering the derived products (hemp oil and hemp flours), increasing the size of the sampling in order to obtain robust data.

CONCLUSIONS

The RAPD technique resulted an inexpensive and easy approach to identify and cluster hemp ecotypes of different geographical origins. Currently, the cultivation of more that 40 hemp cultivars is allowed in the EU, and the RAPD approach may help in the control of the commodities at the reception, to direct the crops to suitable management and utilization. More specific DNA markers, such as sequence characterized amplified regions (SCAR), can be developed in further studies, as an alternative tool to monitor the origin of hempseeds. The percentage of mycotoxin contamination of the samples considered for this work was low; apparently, no correlation between geographical origin and mycotoxin concentration can be made.

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Chapter 6: Unpublished results.

Report of the activities carried out during the internship at RIKILT (Wageningen University and Research, Wageningen, The Netherlands)

*****_This work is currently on going, and it will be completed during the next months, following the publication and discussion of this PhD Thesis. The data presented here are not definitive and have to be completed soon with the complete analytical validation of the best performing method, also comparing it with the ELISA detection._

Rapids methods for the detection of Staphylococcal Enterotoxin A: SPR-based Biacore versus Luminex xMAP approach

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ABSTRACT

Staphylococcal enterotoxin A (SEA) is the most commonly recovered staphylococcal enterotoxin in food poisoning outbreaks. Our research objective was to select different immunoassays, using the high throughput Luminex xMAP technology and a surface plasmon resonance (SPR-Biacore) biosensor, to detect SEA in milk, with the final aim to set up a rapid and sensitive method for the quantification of the toxin. Several antibodies, one polyclonal, one antiserum and five monoclonal, were tested in several formats (direct, competitive and sandwich).

Preliminary results confirmed that the SPR technology (Biacore) displays low sensitivity for all the tested antibodies in the different assays compared to traditional Luminex xMAP, while the Luminex xMAP sandwich assays in which a polyclonal antibody was used to trap the toxin result the most promising method, either using the same polyclonal antibody or using one of the monoclonal SETJ21310 and SETJ643 for the detection.

INTRODUCTION

Staphylococcal food poisoning is the first leading cause of bacterial food poisoning in Europe. In 2014, staphylococcal toxins caused 393 foodborne outbreaks, 7.5% of all foodborne outbreaks reported in Europe, with a small increase compared to 2013 *(1)*. Staphylococcal Enterotoxins (SEs) are short, extracellular and very stable proteins, resistant to heat as well as gut proteases. SEs constitute a family of different serological types of water soluble and single chain globular proteins, with molecular weight between 27 and 34 kDa *(2)*. To date, 23 SEs have been identified, and staphylococcal enterotoxin A (SEA), either alone or together with other SEs, is the most common SE encountered in food poisoning outbreaks *(3 - 4)*.

Staphylococcus aureus is a common commensal of the skin and the mucosal membranes of humans, and the major source of food contamination is due to the

improper handling of cooked or processed food, followed by storage under conditions that allow bacterial replication and enterotoxins synthesis. A further source of *S. aureus* contamination, typical of animal-derived foods and especially milk, is its presence in cattle, sheep and goats affected by subclinical mastitis *(5)*. A large variety of foods, such as meat and meat products, egg products, salads, bakery products, milk and dairy products including cheeses can be contaminated. The ingestion of less than 1 μg of the preformed toxin causes emesis, diarrhea, prostration, severe dehydration, dizziness and abdominal pain, which are the main symptoms of food poisoning and toxic shock syndrome *(6)*. In an outbreak due to Staphylococcal Enterotoxin A (SEA) contaminating chocolate milk, the amount of toxin infecting the children was reported to be only 200 ng *(7)*. Elderly and young children are more susceptible, but the disease is usually self-resolving and rarely lethal.

The European Commission has set the maximum tolerated level of coagulase-positive staphylococci in cheeses and milk-derived products *(8)*, therefore the presence of staphylococcal enterotoxins is currently searched only in dairy products contaminated by *S. aureus* at higher levels. In these foods enterotoxins must be absent in 25 g (8), while foods presenting a level of contamination lower than the threshold are not even tested. If the toxin is preformed and is present in the food prior to consumption, a sensitive detection of SE in food is crucial, given the consequences of staphylococcal foodborne poisoning. European legislation (Commission Regulation 1441/2007) stipulates the reference procedure for SE analysis in milk and dairy products, which is based on extraction, concentration through dialysis and immunochemical detection using one of two approved assays (VIDAS® SET2, Ridascreen® SET Total) *(9)*. While Ridascreen® SET Total is a sandwich Enzyme-Linked Immunosorbent Assays (ELISA), VIDAS® SET2 is an Enzyme Linked Fluorescent Assay (ELFA). Both of the assays are based on the capture of the toxin in the samples by antibodies adsorbed onto a solid phase

before the detection by an enzyme-labelled antibody, which generate a colorimetric signal in the first case or a fluorescent signal in the latter case. Both these methods have been considered the most practical and powerful method for the analysis of SEs in foods because of their sensitivity and reliability.

During the last 20 years, several methods have been developed for the identification of SEA, including mass spectrometry, PCR based methods *(10, 11)*, biosensor-based techniques, reversed passive latex agglutination (RPLA) *(12)*, and immunoblotting. Recently, a mouse polyclonal antibody-based sandwich ELISA has been reported to detect SEA in milk and cheese at concentrations as low as 0.064 ng/mL *(13)*, whereas a monoclonal antibody-based sandwich ELISA has reached the LOD of 0.0282 ng/mL *(14)*.

This work aims to set up a rapid and sensitive method for the detection of SEA in milk and milk derived products, exploiting xMAP and SPR based technologies. The aim of this work is to choose the best promising xMAP and Biacore assay to detect SEA in spiked samples of milk, also comparing the efficiency with an ELISA method, after complete analytical validation.

MATERIAL AND METHODS

Chemicals and Materials

SEA produced by *Staphylococcus aureus* (1 mg/ml) and anti-SEA defatted whole antiserum (S7656) (1 mg/ml) were purchased from Sigma. The polyclonal anti-SEA IgG (LAI101) (1 mg/ml) was purchased from Toxin Technology. Three peptides contained in SEA, namely peptide 1, peptide 2 and peptide 3, and three monoclonal anti-SEA antibodies (anti-SEA14, anti-SEA15 and anti-SEA19) were kindly provided by the Russian group, while two monoclonal antibodies, named SETJ643 (1 mg/ml) and SETJ21310 (1 mg/ml), were obtained by the Chinese group of Professor Chuanlai Xu. RPE-goat anti-rabbit (1 mg/ml) and RPE-goat anti-mouse (1 mg/ml) were purchased by Prozyme.

Solutions

The following solutions were used in this study: phosphate-buffered saline (PBS, pH 7.4), phosphate-buffered with Tween 20 (PBS containing 0.05% (v/v) Tween 20). PBS pH 6, 7, 8 and 9 were tested to optimize the reaction between SEA and anti-SEA defatted whole antiserum, while PBS pH 6 and 8.5 were tested to improve the sensitivity of the reaction between monoclonal antibodies provided by the Russian group and SEA.

Luminex xMAP assays

Two variant of competitive assays were tested by Luminex xMAP technology: the competition between SEA coupled to colour-coded paramagnetic beads and the free SEA in samples, and the competition between 3 different peptides contained in SEA and free SEA in samples were evaluated (*https://www.luminexcorp.com/*). One set of paramagnetic beads was prepared through carbodiimide coupling to SEA, and three sets were prepared through neutravidin coupling to peptide 1, peptide 2 and peptide 3 respectively.

Different concentration of anti-SEA antibody were tested before finding the optimal concentration to use as capture antibodies in the competitive assay (data not shown): for the defatted whole antiserum (Sigma) 8.33 ng/ml, for the polyclonal antibody (Toxin Technology) 83.3 ng/ml, for the monoclonal antibodies SETJ643 and SETJ21310 8.33 µg/ml and for the monoclonal anti-SEA19 8 µg/ml.

The estimation of the SEA concentration in an unknown sample depends upon the creation of a standard curve, prepared by making serial dilutions of SEA in the range 0.0001 µg/ml to 100 µg/ml. The ELISA plates with 10 µl per well of anti-SEA antibody diluted in PBS, 10 µL per well of paramagnetic beads coupled suspension (1000 beads/well) in PBS and 40 µl per well of SEA dilution were incubated on a plate shaker at room temperature for 1 hour. During incubation,

the free SEA in the sample compete with the mycotoxin-reporter conjugates for antibody binding. For each concentration of the toxin, the reaction was evaluated in duplicate or in triplicate. After incubation, the paramagnetic beads were trapped by a magnet and the plates were washed with PBS. Eighty microliters of RPE-labelled goat anti-rabbit immunoglobulin previously diluted in PBST (2 µg/ml) were added in each well and then the plate was incubated on a plate shaker at room temperature for 30 minutes. For the detection of monoclonal antibodies, the secondary antibody was replaced by RPE-labelled protein A/G (3 ng/ml). After washing, the paramagnetic beads were suspended in 100 µL of PBST and the plate was read on a planar bead array analyser (MAGPIX), running on XPONENT software (all from Luminex, Austin, USA). In the planar array analyser, the beads are magnetically trapped in a flow chamber to create a monolayer array. After excitation by red light, red and infra-red images are recorded by the CCD camera. Next, a reporter image is recorded upon green light excitation. A calibration curve for each reaction was drawn.

The competition between each of the 3 peptides contained in SEA and free SEA in samples were tested also using the monoclonal antibodies anti-SEA14, anti-SEA15 and anti-SEA19. The monoclonal antibodies SETJ643 and SETJ21310 were not tested.

To improve the sensitivity, the sandwich format was also tested. To capture the free SEA present in the samples, a set of coloured paramagnetic beads was coupled to the purified polyclonal antibody through carbodiimide coupling reaction. This set of beads was used to trap free SEA spiked in different concentration. The trapped peptide was subsequently bound by either free biotinylated polyclonal antibody (4 mg/ml) or by monoclonal anti-SEA antibodies SETJ643 or SETJ21310 (4 mg/ml). The binding of the free antibody was highlighted by fluorescence, due to the recognition of the detecting antibody by

either Streptavidin – Phycoerithrin (SAPE) (4 µg/ml), or RPE-labelled goat anti-mouse secondary antibody, respectively.

In a 96-wells microplates 10 µl of bead suspension (1000 beads/well) and 40 µl of a serially 10-fold diluted SEA, ranging from 0.0001 µg/ml to 100 µg/ml, were added to each well. After incubation on a plate shaker at room temperature in the dark for 30 minutes, the wells were washed. The addition of the detecting antibodies was followed by incubation on a plate shaker at room temperature in the dark for 30 minutes. After another washing step, SAPE or RPE- labelled goat anti mouse, depending on the detecting antibody used, was added to each well, and then the plates were incubated for 30 minutes. After the plate was washed, 100 µL of PBST were added to each well, and the emission was measured. All measurements were performed at least in duplicate.

Biacore assays

Experiments were performed using Biacore 3000 equipped with research-grade CM5 sensor chips (Biacore AB, Uppsala, Sweden) (https://www.biacore.com/lifesciences/index.html). The instrument temperature was set at 20 °C for all analytical steps. Sensor chips and amine-coupling reagents (N-ethyl-N-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), ethanolamine HCl and buffer HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20) were purchased from Biacore AB.

Different variants of direct and competitive assays were carried out using a chip on which the SEA was bound, while for the sandwich format the chip was coated with the purified polyclonal antibody or the monoclonal SETJ21310. The coating of the carboxymethylated dextran layer on the sensor surface of the CM5 chip was performed using the amine coupling kit running the surface preparation wizard procedure in the Biacore 3000 control software. In short, the biosensor surface was activated by injecting a mixture of EDC and NHS (1:1, v/v) into a

flow channel (Fc) at a flow of 5L min−1. The protein to be immobilized was diluted to 50 µg/mL in coupling buffer (10 mM sodium acetate; pH 4.5) in a final volume of 500 µl, which were injected over the activated surface until a total immobilization level of 10000 response units (RU) was reached. After coupling, active groups were blocked by injecting 100 µl of ethanolamine (10 M).

The interaction between SEA and both monoclonal and polyclonal antibodies in different variants (toxin or antibody on the chip), was tested to decide over the volume and the concentration of antibodies and SEA to use.

Competitive assays were carried out by mixing 50 µl of sample (blank or serial dilutions of SEA) to 50 µl of antibody (when polyclonal in concentration of 4 µg/ml, monoclonal anti-SEA19 5 µg/ml, monoclonal SETJ643 and SETJ21310 50 µg/ml) in an empty tube. Seventy-five microliters of this suspension are injected in the chip. After the reaction, the chip is regenerated by injecting HCl 0.1 M.

For direct assays 50 µl of sample (blank or SEA dilutions) are injected in the chip. Regeneration is achieved by injecting HCl 0.01 M.

For the sandwich assay the same protocol is used, except for the additional injection of 20 µl of the polyclonal antibody (5µg/ml) or of 50 µl of the monoclonal antibody (100µg/ml).

RESULT AND DISCUSSION

Luminex xMAP assays

This preliminary part of the work focused on the optimization of different Luminex xMAP assays, in order to select some as promising way to quantify the SEA in the model food milk spiked with different concentration of the toxin. Parameters like the concentration of each antibody, the incubation time, the composition and the pH of buffers used were optimized before performing the

preliminary experiments. Several antibodies, including some monoclonal described as very sensitive in ELISA *(14)*, were tested. The main parameters considered in this study were the linearity of the signal and the sensitivity, while other parameters will be considered only for the validation of the most suitable method for SEA quantification in milk. The linearity of the signal across the range of the assay assures that the analyte is detectable within the chosen range, while the R^2 value, determined by linear regression analysis, confirms that the quantification is reliable.
The key factor to determine the sensitivity of an assay lies in having an antibody with high specificity and affinity. The IC50 value, the concentration of standard solution producing 50% of inhibition, is estimated by using the equation of the inhibition standard curve.

Among the Luminex xMAP competitive assays tested, the highest sensitivity was achieved when SEA was coupled to paramagnetic beads and the whole antiserum was used for the capture (Fig. 1). After having set the best conditions (pH of the buffer, time of incubation, concentration of the antibody), the ratio between the fluorescence measured for each sample and the blank (B/B_0) was linear in the range from 0.01 µg/ml to 100 µg/ml. Although in the same competitive assay performed using the purified polyclonal antibody the fluorescent signal was as high as in the assay performed using the whole antiserum, the linearity was observed only in the range from 0.1 µg/ml to 10 µg/ml. Further experiment should be performed, changing the conditions and the buffers with the aim of improving the sensitivity. Both of variants will be considered to test for the detection in SEA spiked milk. The use of purified polyclonal antibody might in fact assure higher specificity than the use of the antiserum.

Three more monoclonal antibodies (anti-SEA19, SETJ643 and SETJ21310) were tested in the competitive assays. None of them provided a satisfying sensitivity, so further experiments considering lower concentrations of each monoclonal

antibody should be performed, in order to verify whether the low sensitivity could be due to the presence of an excess of antibody, causing the saturation of all the epitopes of the SEA.

As for the reaction between SEA peptides coupled to the beads and the whole antiserum, the signal and the sensitivity registered were low for the whole range of concentrations tested, making the beads coupled to the peptides not suitable for further tests of competition between them and the SEA in foods. Moreover, the monoclonal antibodies anti-SEA14, anti-SEA15 and anti-SEA19 did not display reactivity against the three peptides contained in SEA tested; therefore the reaction between the peptides and the monoclonal antibodies was not tested in further Luminex xMAP or SPR assays. The reactions between the beads coupled to each peptide and the purified polyclonal antibody were not tested, considering that the expected signal is lower than the signal obtained having the whole SEA coupled to the beads.

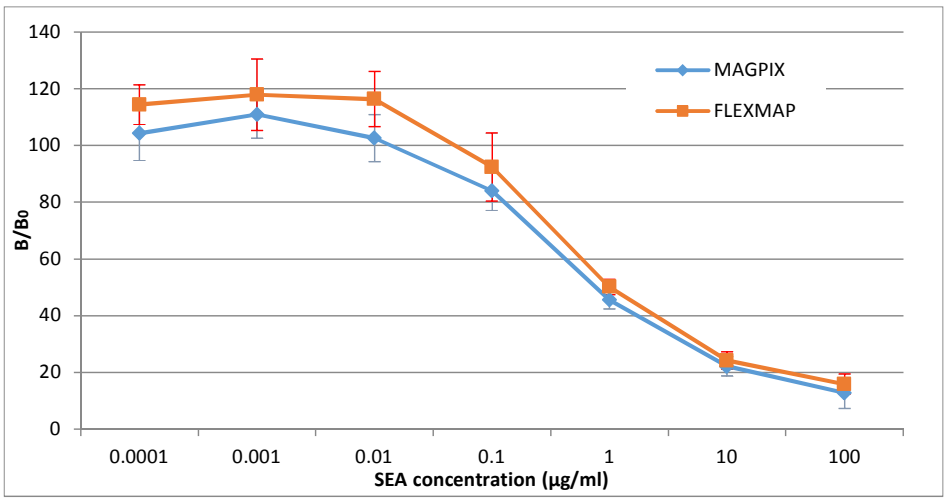

Figure 1. Dose-response curve of the competitive assay in which SEA is coupled to the beads and anti-SEA defatted whole antiserum is used. Points represent the mean value of the triplicate of the ratio between the fluorescence measured for each sample and the blank (B/B_0), measured by MAGPIX (blue line) and by FLEXMAP (orange line) instruments. Standard deviation is shown per each concentration.

The xMAP sandwich assays performed using beads coupled to the purified polyclonal antibody showed the highest sensitivity when the purified polyclonal antibody was also used for the detection. The calibration curve appeared linear between the concentration 0.01 µg/ml and 10 µg/ml, but at the highest SEA concentrations tested the standard deviation was too high, and the quantification could not be considered as reliable. The same sandwich format was not considered for the whole antiserum, since the presence of other proteins than the antibodies can cause unspecific reactions.

The xMAP sandwich assay where the detection was performed by the monoclonal antibodies SETJ643 and SETJ21310 showed promising sensitivity, especially when SETJ21310 was used (Fig. 2). Even if the slope of the calibration curve presented an R^2 of 0.96 (further experiments are needed to adjust the sensitivity), this format of assay where the purified polyclonal antibody is coated on the beads and the best performing monoclonal antibody is used for the detection is definitely the most promising one. In fact, the use of the polyclonal antibody as a capture antibody allows to bind higher amount of toxin, and the use of a monoclonal antibody as detection antibody should improve the specificity, because the various staphylococcal enterotoxins share a certain degree of homology. The specificity of the reaction still needs to be assessed, by measuring the degree of cross-reactivity among different enterotoxins, including all other staphylococcal enterotoxins.

Few works were led to find a sensitive assay for SEs detection thatexploits the xMAP Luminex technology. Simonova and colleagues determined the LOD of SEA by xMAP detection in a sandwich assay to be 10 pg/ml *(15)*, while in the same format Garber and colleagues obtained a LOD of 0.5-2 ng/ml for different SEs *(16)*. Both studies reached high sensitivity, mainly due to the affinity with the target of the antibodies used in each study. The monoclonal SETJ21310 and SETJ643 antibodies used in the present studies showed promising LOD (0.0282

ng/mL for SETJ21310) when used in an ELISA sandwich assay *(14)*. These values indicate that the method is sufficiently sensitive to detect SEA concentrations lower than 0.5 ng/mL, which is still a toxic level in milk *(17)*.

However, three sandwich formats of xMAP immunoassay, in which either the purified polyclonal antibody or the monoclonal SETJ21310 or SETJ643 serves as detector, should be tested on spiked milk samples, to demonstrate the applicability of this format.

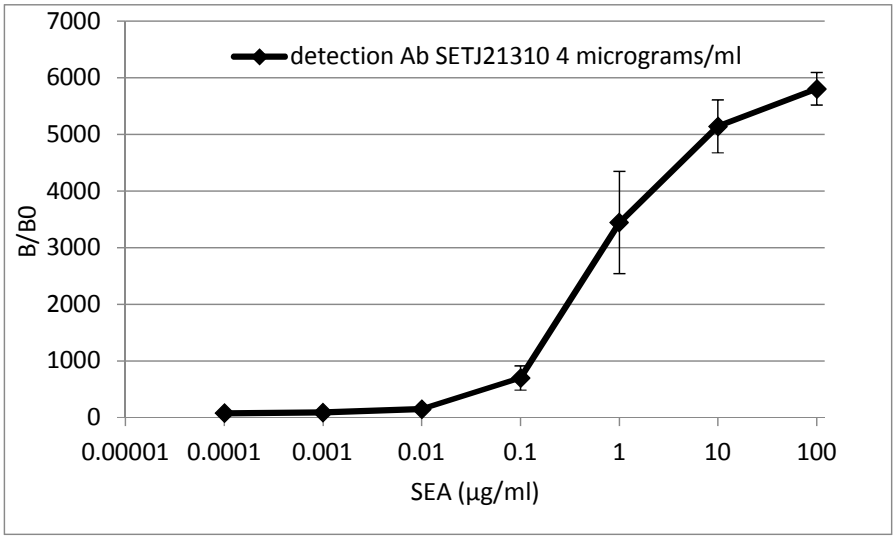

Figure 2. Dose-response curve of xMAP sandwich assay when SETJ21310 is used as detection antibody and anti-SEA polyclonal antibody is coupled to beads for the SEA capture. Points represent the mean values of triplicate ratio B/B_0. Standard deviation is shown for each concentration.

SPR Biacore assays

Three different formats, direct, competitive and sandwich, were assayed to determine which one could assure higher sensitivity using the antibodies at our disposal. The competition assays in which the purified polyclonal or the antiserum are used showed best sensitivity among the experiment performed in this format,

as expected. In general, the format where the highest sensitivity was registered was the sandwich assay, in which the signal (in Response Unit, RU) was linear in a wider range. Among the SPR Biacore sandwich assays, the highest sensitivity was obtained when the purified polyclonal antibody was bound to the chip surface and the same antibody (Fig. 3), or the monoclonal antibody SETJ21310 (Fig. 4), were employed as detectors. Sandwich assays are expected to be more sensitive than direct and competitive ones, since in SPR the detector response is proportional to the mass of the analyte that binds to the ligand and, therefore, the responses resulting from the binding of small molecules are low. For this reason the use of a further antibody to detect the toxin bound to the capture antibody allows the amplification of the signal, especially when the polyclonal antibody, which recognizes a higher number of epitopes, is used.
The direct assay, where no amplification of the signal occurs, gave lower signal respect to the other two formats, even if our target has the correct size to be tested in this format, as showed by Naimushin and colleagues, who performed the direct detection of Staphylococcal enterotoxin B (SEB) by a SPR sensor system, based on a prototype two-channel system. That proves that SEs are big enough to produce significant responses even at low molar concentrations. In this study the SEB, with molecular weight 28.400 Da, was detected at concentrations above 0.5 nM *(18)*.

However, the sensitivity obtained in our study using each assay of SPR was anyway lower than the sensitivity showed by both the Luminex xMAP assay as well as less performing that the European reference methods Ridascreen (R-Biopharm) or Vidas, (Biomerieux) both based on ELISA format.

The SPR Biacore immunoassay often shows less sensitivity when compared to ELISA or to other traditional bioassays. Hsieh and collaborators (1998) *(19)* showed that the SPR Biacore immunoassays applied to detection of β-toxin of *Clostridium perfringens*, display less sensitivity that the same format in ELISA.

However, in this study the SPR assay lasted only 20 minutes, while the ELISA one ran for six hours. Low sensitivity is reported also by Liu and colleagues (2004) *(20)* for the tetanus toxin. Better sensitivity than what described by Hsieh *(19)* and Liu *(20)* was obtained by Rasooly (2001) *(21)* in an SPR sandwich biosensor assay, where SEB was detected at ~10 ng/ml rapidly in milk and meat, and by Homola and colleagues (2002) *(22),* who reported the lowest detection limit to be 0.5 ng/ml in sandwich detection mode for SEB detection in buffer and milk samples. Finally, Medina (2006) *(23)* detected SEA using Biacore from 1 to 40 ng/mL in spiked liquid egg.

Since one of the most important characteristic of Biacore is the regeneration of the chip and its reuse, a mild regeneration of the biosensor surface is essential for sustainable and optimal use of the biosensor with large series of samples. Among the antibodies that we have bound to the chip surface, the binding of the polyclonal antibody was relatively strong and needed relatively harsher conditions for the complete recovery of the chip surface. Regeneration conditions affected the performance, and thus the lifetime, of the biosensor chip. For this reason, the flow cell in which the purified polyclonal antibody was bound underwent to washes with a volume of HCl 0.01 M higher than the usual (15 µl instead of 5 µl). On the other hand, the binding of the monoclonal antibody on the chip is weaker than the binding between the chip and the polyclonal antibody, and the regeneration, even if performed with 5 µl of HCl 0.01 M, still represents an issue, since the maximum signal decreases after every use.

Considering the wideness of the range in which the signal is linear, the IC50 and the issue related to the regeneration, both the best performing sandwich assay were chosen for the test of the detection of SEA in spiked milk. Performance parameters like specificity, accuracy, precision, robustness, LOD and LOQ still need to be calculated.

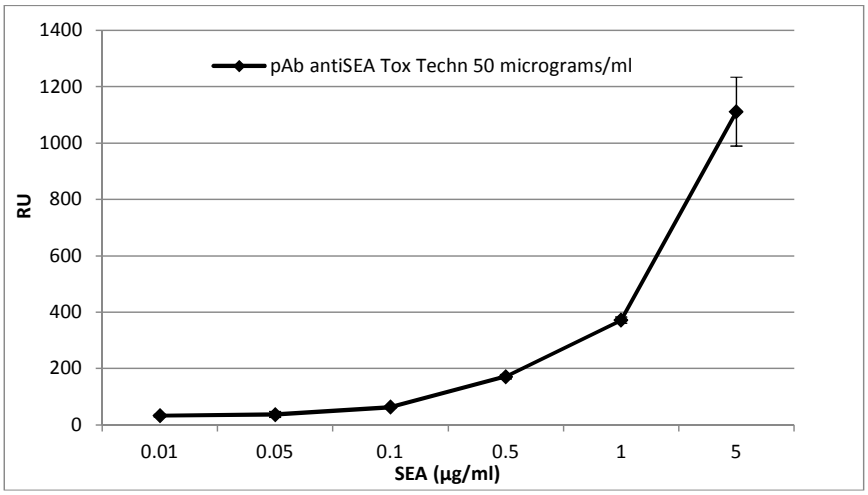

Figure 3. Dose-response curve for the determination of SEA when polyclonal antibody is used in a SPR Biacore sandwich assay both as capture antibody on the chip and as detection antibody. Points represent mean values of duplicates. Standard deviation is shown per each concentration.

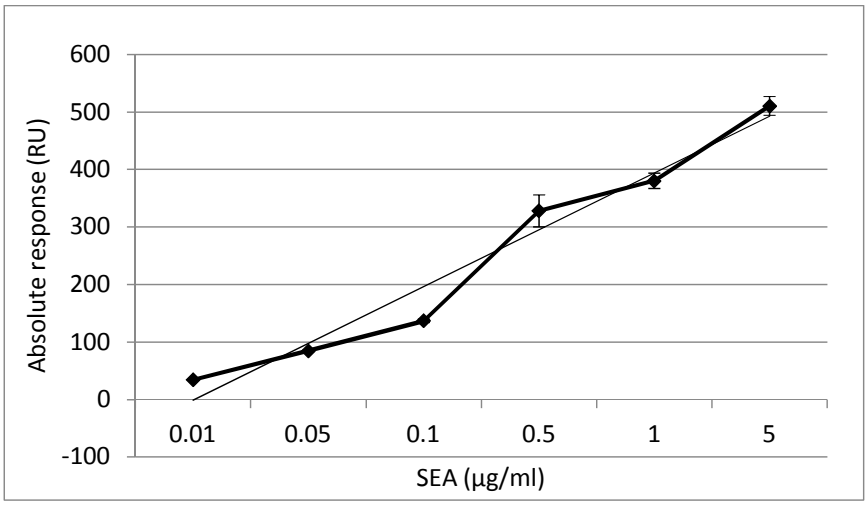

Figure 4. Dose-response curve for the SPR Biacore sandwich assay when the purified polyclonal antibody is used as detection antibody and the monoclonal SETJ21310 is bound on the chip. Each points represent mean values of duplicates of measurements of Response Unit (RU). Standard deviation is shown per each concentration.

CONCLUSION

We have shown in this preliminary study that the developed xMAP assays are good candidates for a sensitive method to detect SEA. The time required for quantifying SEA using this method matches that required by traditional ELISA, but it requires much less reagents consumption as well as volume of the analyzed sample. Moreover, the assay has the possibility to be multiplexed in future.
Among the Luminex xMAP assays tested, two competitive ones, performed using either the antiserum or the purified polyclonal antibody, were considered suitable for testing the presence of SEA in spiked milk. Regarding the Luminex xMAP sandwich assays, those employing either the purified polyclonal antibody or the monoclonal antibodies SETJ643 or SETJ21310 as detectors, will be selected for testing SEA-spiked milk samples.

Moreover, two sandwich SPR Biacore assays will be tested for SEA detection in spiked milk. Even if their performance seem less promising, the lack of need for molecular labelling, the low sample consumption and speed of detection, makes this technology suitable for fast screening.

Following the setting up in the model food and the selection of the best performing method, and following a complete analytical validation in order to assess sensitivity, robustness and repeatability, this protocol must to be compared to a common ELISA kits, particularly the kits considered “reference standard” in Europe.

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Chapter 7. Discussion

Chapter 7: Discussion

This PhD Project, carried out in collaboration with Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna (Pavia Unit), RIKILT Department of Wageningen University and Research (WUR, Wageningen, The Netherlands) and University of Parma, aimed to evaluate rapid and sensitive approaches to guarantee food free from microbiological risks due to the presence of pathogens and/or their toxic metabolites.

Although generally the safety of the commonly consumed foods has improved over the last decades, foodborne illnesses continue to be a serious public health challenge. Even if Companies processing food operate HACCP protocols, certain risks cannot be totally eliminated, like those involving the steps of preparation or storage. In order to assure safe food for consumers, national food control systems control all food produced, processed and marketed within the country, including imported food. Sampling procedures are ruled by specific EU Regulations, but not all contaminants are comprised in the controls: microbial pathogens and mycotoxins are included in this list. The laboratory analyses employed to detect these contaminants must be rapid, reliable and sensitive. Currently many limitations regarding the sensitivity, the cost of analysis and the representativeness of the sample of the methods used still exist. Moreover, some hazards that used to be major concerns have declined (thanks to controls or changing situations), while others are emerging or increasing, like for example multi-drug resistant strains of monophasic variant of *Salmonella* ser. Typhimurium 4,[5],12:i:-. Recent advances on the development of more rapid and sensitive analytical tests have helped to identify hazards that were detected in the last century without being clearly recognized as a health risk, like mycotoxins.

During the first PhD year, the isolates from human and salami obtained in the framework of three foodborne salmonellosis outbreaks caused by *Salmonella* ser.

Typhimurium and monophasic variant of *Salmonella* ser. Typhimurium, were evaluated through serotyping, phage-typing, antimicrobial susceptibility testing, PFGE and MLVA, in order to investigate their origin. The same approach was exploited for *Salmonella* ser. Typhimurium strains isolated from 360 salami produced in Pavia Province in the three years following the outbreaks.

This investigation allowed to estimate the prevalence of *Salmonella* ser. Typhimurium and its monophasic variant in salamis during the outbreaks and for the years 2011-2013 in the area of Pavia Province, and to highlight that the monophasic variant has replaced *Salmonella* ser. Typhimurium as the major risk in the environments of the salami producers monitored in this study. These data confirm the dominance of this emergent serovar along the swine supply chain, as reported also by EFSA. Moreover, this work allowed to detect the ASSuT (Ampicillin, Streptomycin, Sulphonamides and Tetracycline) antimicrobial resistance pattern in 47% of isolates of the monophasic variant of *Salmonella* ser. Typhimurium, while no resistant *Salmonella* ser. Typhimurium were identified.

The use of PFGE and MLVA techniques for genotyping the isolates allowed to verify the correlation between the isolates from humans and the isolates from salamis manufactured by three different producers, during a short timeframe. The salamis were identified as the food source of the first outbreak. In fact, *Salmonella* ser. Typhymurium isolates that showed the same PFGE-type and shared the same rare MLVA profile (3-18-9-NA-211) were recovered from salami and patients with symptoms of gastroenteritis. The fact that this strain with a very rare MLVA profile has been found only among these isolates out of three thousand isolates examined by MLVA in the IZSLER database (and it has never been reported in the literature worldwide so far) reinforced the evidence of the presence of a link between this food and the human cases. The probable common source of infection of the salamis was identified in the slaughterhouse from which all the three producers had purchased the meat.

The salamis were identified as the speculative source also of the second outbreak, since the same PFGE, the rare MLVA profile and the phenotypic profiles were also found in the *Salmonella* ser. Typhimurium isolate recovered from a sample related to outbreak II. For this isolate, the confirmation of the link was impossible due to the lack of subtyping data from the available human isolate associated with this outbreak.

The responsible for the third outbreak was the monophasic variant of *Salmonella* ser. Typhimurium. The strain isolated from all human samples related to this outbreak was not found in any salami analysed in the same period, but the same phenotypic and genetic profile combination was detected in a salami manufactured two years after outbreak III. This suggests that salami or pork meat could have been the source of the three human outbreak III cases.

This work allowed also to prove that the extended sampling method adopted is suitable for the monitoring of pathogens in foods, enabling the detection of *Salmonella enterica* subtypes in salami, and preventing the onset of food-borne outbreaks through hygienic measures adopted to manage the risk. Although the data presented in this thesis confirm the clear role of the pig as an important source of *Salmonella*, a control program for *Salmonella* in the swine meat chain has not been implemented yet in Italy. As suggested by the evidence collected in this work and by the results of other studies conducted in Italy, the application of control plans, as previously done for the poultry sector, is needed to reduce the prevalence of salmonellosis at the National level.

Another major safety concern considered during this PhD course refers to the presence of mycotoxins in food, especially aflatoxins and ochratoxin A (OTA), which are recognized as carcinogenic compounds. During the first year of PhD I focused my attention on the selection of DNA markers useful for the identification of foods and raw materials known to be contaminated by mycotoxins. The use of

Random Amplified Polymorphic DNA (RAPD) has been exploited in this project in order to identify and cluster some Italian ecotypes of hemp (*Cannabis sativa* L.) from hemps imported from foreign countries, considering also the rate of occurrence of aflatoxins and ochratoxin A, evaluated by HPLC-MS in collaboration with the University of Parma. The cultivation of hemp for drug unrelated purposes has been recently reintroduced in Italy, with the purpose of making oil and flour rich in proteins. The Italian hemp production is however still insufficient to cover the commercial requests, and then hemp is imported from China and Canada, among the world-leading hemp producers. Since some hempseeds are grown in countries known for their humid climate, which facilitates the growth of toxigenic filamentous fungi with consequent production and accumulation of mycotoxins, and since the transport and the preservation of the seed can potentially increase the contamination rate, a molecular approach for the separation of hempseeds based on their cultivar identity is proposed in this Thesis.

A set of 20 RAPD primers were tested, and the combination of six RAPD markers resulted sufficient for the geographical clustering of hempseeds: Italian cultivars clustered separately, and could be discriminated from those grown abroad. The RAPD technique resulted an inexpensive and easy approach that could be exploited in rapid screening to verify the correctness of the information written on the label, or for the control of the imported commodities at the reception, to direct the crops to suitable management and utilization. Nevertheless, further investigations that will consider a higher number of ecotypes are needed to validate this approach and verify its utility. More specific DNA markers, such as microsatellite (SSR) or the sequence characterized amplified regions (SCAR), can be developed in further studies, as an alternative tool to monitor the origin of hempseeds. More different analytical approaches will be useful to confirm the data recovered by DNA analysis, particularly the application of IRMS and NMR,

both useful to assess (and confirm) the geographical origin, beside the molecular identification of the ecotype.

The 75% of Chinese and the 20% of Italian samples of hempseeds analysed in this study showed the presence of at least one class of mycotoxins. No specific correlation with original Country of production can be made, in consideration of the small set of samples considered for each origin. None of the contaminated samples exceeded the legal limit fixed in Europe for flours intended as food (Commission Regulation (EC) No 1881/2006) or feed (Directive 2002/32/EC).

During the second and the third year of my PhD studies, the work has been addressed to the monitoring of ochratoxin and ochratoxin-producing fungi presence in traditional Italian cured salami, as well as on the setting up of a new Real Time PCR protocol for the detection of OTA-producing fungi. The Italian cured "salami" is a model of great interest for the study of mycotoxins production: molds play a major role in the correct maturation of artisanal salamis and the lack of the use of fungal starters during the fermentation/maturing process allows the surface colonization by environmental fungi present in the ripening room. One hundred and thirty-three samples of salami produced in Lombardia and Emilia Romagna were investigated. Ochratoxin A was searched in salami through LC-MS/MS (in collaboration with IZSLER, Brescia Unit). For the searching of ochratoxin-producing strains, fungal isolates from salamis were identified at species level through the observation of macro and microscopic morphologic characteristics. Then, the total genomic DNA from the isolates was subjected to the amplification of a target DNA sequence (*otanpsPN*) know to be common to all OTA-producing fungi. Regarding *Penicillium* isolates, also the *otapksPN* gene target to recognise the ochratoxigenic strains of *P. nordicum*, the species identified as the major OTA producer in meat, was considered. The same Real Time PCR reactions were carried out also from 19 salamis casings, including 12 casing belonging to salamis positive to OTA.

This investigation led to new knowledge about the frequency of the occurrence of OTA in this food: 9.8% of samples showed the presence of OTA beyond the limit permitted by Italian regulation, ranging from 1.14 to 691 µg/kg of meat. Among these salamis, three were contaminated by both OTA and an OTA-producing fungus (*Aspergillus westerdijkiae*), two of which were manufactured by the same salamis factory in two different periods. Our monitoring study highlighted a huge problem of contamination in this traditional factory. In fact, in a subsequent sampling of the air and surfaces of this small working plant, *A. westerdijkiae* was found as the predominant fungal species in the air, workspaces and on tools dedicated to the processing and ripening of salami, as well as in the external environments (these data have not been discussed during this thesis report). Since fungal spores in the air are able to produce OTA, a plan for the sanitization of the plant has been proposed.

This monitoring has also allowed highlighting the limit of the prediction method based on the morphologic species identification followed by the analysis of specific genetic characteristic of the isolates. In fact, OTA was detected in 13 out of 133 salamis, but only three salamis carried an isolable OTA-producing species, *A. westerdijkiae*. None of the DNA purified from the isolates, including the three *A. westerdijkiae*, showed to possess either the *otanpsPN* gene target, common to all the strains able to synthesize OTA, or the target for the ochratoxigenic strains of *P. nordicum*, and the inability to produce OTA was confirmed by growing the isolates under conditions favourable to OTA biosynthesis. Conversely, the *otanps* target gene was amplified from the DNA purified from the three salami casings harbouring both *A. westerdijkiae* and OTA, but also from other 11 salami casings where *A. westerdijkiae* was not isolated, suggesting that OTA-producing strains were no longer viable and isolable at the end of maturation. The molecular approach has also permitted to foresee the production of OTA also in those salamis where, despite the colonization by ochratoxigenic fungi, the conditions of

temperature, water activity, pH and nutrients suitable for the mycotoxin production have not occurred. In fact, among the seven salami casings negative to OTA presence, four resulted positives to the amplification of *otanpsPN* gene.

Moreover, in our study, no strain of *P. nordicum* was isolated, but 11 salami casings were positive to the amplification of the *otapksPN* fragment specific for this species. Since *P. nordicum* prefers high water activity, it belongs to the flora of early maturation stages and probably it is not viable and isolable in late maturation stages. Both the microbiological species identification and the molecular search for OTA-producing strains from DNA purified from the isolates did not result a good indicator for the ochratoxin production. Our data show that it is complicated to isolate the strains responsible for the toxin production within a multitude of different fungal strains that colonize the salami at the end of its maturation. This difficulty can be explained considering the different mycofloras present at different stages of maturation, or postulating the presence of different strains belonging to the same species that share the same morphology but not the same genotype. In any case, the PCR-based approach, performed for the *otanpsPN* gene target, and the *otapksPN* fragment specific for OTA-producing *P. nordicum*, failed to recognize two salamis positive to OTA. The origin of the OTA detected in these two salamis could most probably be the meat used for their preparation, since OTA resulting from the contaminated feeding can be carried-over in the swine tissues. Finally, the amplification of sequences specific for OTA-producing strains performed on DNA extracted directly from the washing of the salami casing resulted a better approach to evaluate the risk of OTA presence than amplifying the same target from DNA purified from the salami isolates.

In the same Project, two novel primer pairs were designed on *beta tubulin* region of fungal genomic DNA, and tested in real time PCR in order to identify the species *A. westerdijkiae*, the only OTA-producing species isolated in this study.

The best performing primer pair could be used to monitor the working plants in the salami factory, which was massively colonized by this fungus. Both the designed primer pairs failed the specificity tests, since they showed to amplify also the DNA of *A. ochraceus* and *P. nordicum* (also after the modulation of PCR parameters). Therefore, other DNA regions different from *beta tubulin* should be considered in the next future to design species-specific primers. Recently (just before the completing of this PhD Thesis), *A. westerdijkiae* whole genome was sequenced and published: its comparison with known sequences of *A. ochraceus* or the whole genome of *P. nordicum* can allow the design of more specific primers.

Among the major toxins recovered from foods, staphylococcal enterotoxins were considered a secondary target of this Thesis. Particularly, we aimed to select the most promising Luminex xMAP and SPR assays to detect low levels of the Staphylococcal Enterotoxin A (SEA) in spiked samples of milk. This preliminary study (part of a larger Project and performed in collaboration with WUR) aimed to set up a rapid and sensitive method for the detection of SEA in milk and milk-derived products. The choice of the technologies was made considering quicker and cheap alternatives to ELISA, which can also be exploited for the simultaneous detection of multiple analytes with minimal sample pre-treatment. Different antibodies, one polyclonal, one antiserum and five monoclonal, were tested in several formats of immunoassay (direct, competitive and sandwich), either by using Luminex xMAP or SPR Biacore technologies.

Preliminary results confirmed that the SPR technology (Biacore) displays low sensitivity for all the tested antibodies in the different assays. The real advantage of this biosensor-like approach is that the immunochemical interactions between the toxin and the antibodies are performed in real time and in a completely automated way, since the instrument pre-programmed by the operator performs by itself the dilutions, the admixtures and the analysis. Considering these

advantages, the sandwich format in which the polyclonal antibody is used for the SEA capture and either the same polyclonal or the best performing monoclonal SETJ21310 are used as detection antibody, were selected to test the detection of the toxin in spiked foods.

Among the Luminex xMAP competitive assays tested, both the antiserum and the polyclonal antibody are suitable for testing the presence of SEA in spiked milk. Even if the highest sensitivity is achieved in the first case, we still need to verify in the next future whether different conditions of incubation and the use of alterative buffers can improve the sensitivity of the competitive assay performed with the purified polyclonal antibody.

Regarding the Luminex xMAP sandwich assays, three format display satisfying sensitivity and will be tested for the detection of SEA in spiked milk. The best sensitivity was obtained when the purified polyclonal antibody is used for both the capture and the detection or when the monoclonal antibody SETJ643 or SETJ21310 is used as detector. The use of monoclonal antibody can also improve the specificity, which was not considered in the experiment performed so far. In fact, several enterotoxins share similarities in structure, and especially the lack of cross-reactions between the antibodies at our disposal and staphylococcal enterotoxin E should be verified. Following the setting up and the selection of the best performing methods, this protocol will be compared to a common ELISA kit, in order to assess sensitivity, robustness and repeatability (analytical validation). Our results are preliminary, and only the testing of the two SPR Biacore and the five Luminex xMAP selected formats to detect SEA in spiked foods could give a real indication of which method is the most suitable.

Chapter 8: List of publications

Products of the Research

Posters presented in National/International Congresses:

- "Specie fungine produttrici di ocratossina A nei salami lombardi prodotti negli anni 2011-2014". **Merla C**., Andreoli G., Vicari N., Dalla Valle C., Cavanna C., Guglielminetti M.L., Biancardi A., Fabbi M. 17th Congress of "Società Italiana Tossicologia", 18-20 March 2015, Milan.

- "Hemp seeds traceability and safety: RAPD genotyping and mycotoxin contamination of samples from different geographical origin". **Merla**, **C**., Garino C., Travaglia F., Coisson J.D., Locatelli M., Dall'Asta C., Galaverna G., Arlorio M. Food Integrity. 26-27 March 2015, Bilbao (Spain).

- "Ochratoxigenic molds in the air of salami ripening rooms in Pavia Oltrepò", **Merla C.**, Andreoli G., Guglielminetti M.L., Rovida E., Pozzi C., Fabbi M. 2nd International Plant Science Conference, 14-18 September 2015, Pavia.

- "Indagine sull'origine di una colonizzazione da parte di muffa ocratossinogena in un salumificio dell'Oltrepò Pavese". **Merla C**., Andreoli G., Guglielminetti M. L., Biancardi A., Rovida E., Pozzi C., Gennari L., Scotto di Fasano P., Fabbi M. 16th National Congress of "Società Italiana Diagnostica di Laboratorio Veterinario" (S.I.Di.L.V.), 30 September- 2 October 2015, Montesilvano (PE).

- Chemical and microbiological analysis of cosmetics for hair dyeing and body painting. Rubini S., Bonati A., Andreoli G., **Merla C.**, Caprai E., Baldisserotto A., Vertuani S., Manfredini S. Abstracts of the 52nd Congress of the European Societies of Toxicology (EUROTOX) Fibes Congress Center Seville, Spain, 04th-07th September 2016. Published in: Abstracts / Toxicology Letters 258S:S187. http://dx.doi.org/10.1016/j.toxlet.2016.06.1689

Papers published:

Andreoli G., **Merla C**., Dalla Valle C., Corpus F., Morganti M., D'Incau M., Colmegna S., Marone P., Fabbi M., Barco L., Carra E.(*). Foodborne salmonellosis in Italy. Characterization of Salmonella enterica Serovar Typhimurium and Monophasic Variant 4,[5],12:i- isolated from salami and human Patients. Journal of Food Protection. (Accepted 5 December 2016; doi:10.4315/0362-028X.JFP-16-331).

I have actively participated to the writing of the paper and my contribution to the work was the isolation and identification through microbiological and molecular method of the *Salmonella* strains. I have also contributed to the serotyping of some strains through agglutination and MLVA technique, in collaboraton with other colleagues.

Papers submitted:

Merla C., Andreoli G., Garino C., Vicari N., Guglielminetti M.L., Biancardi A, Arlorio M, Fabbi M. Ochratoxin A and ochratoxin-producing fungi in traditional salami manufactured in Northern Italy.

This paper is the result of the project PRC2013021.

Merla C., Garino C., Rinaldi M., Dall'Asta C., Arlorio M. Hemp traceability and safety: RAPD (Random Amplified Polymorphic DNA) and mycotoxin detection as integrated tools of control.

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