### UNIVERSITY OF NAPLES FEDERICO II DEPARTMENT OF AGRICULTURAL SCIENCES



### Ph.D. in Agricultural and Food Sciences XXXI Cycle (2015-2018)

Reduction of Mycotoxins by Biological, Enzymatic & Physical Methods and Technological Performance of UVC and Heat Treated Semolina

> Ph.D. Dissertation By Hina Shanakhat

Supervisor Prof. Silvana Cavella Coordinator **Prof. Guido D'Urso** 

## REDUCTION OF MYCOTOXINS BY BIOLOGICAL, ENZYMATIC & PHYSICAL METHODS AND EVALUATION OF TECHNOLOGICAL PERFORMANCE OF UVC AND HEAT TREATED SEMOLINA

### HINA SHANAKHAT

Thesis

Submitted in fulfilment of the requirements for the degree of doctor

In Agricultural and Food Sciences

(XXXI cycle) at Federico II- University of Naples

## To my parents, Mr & Mrs. Shanakhat Ali, My husband, Mubashir Ali Love of our life, my son Zohaan Mubashir

#### ACKNOWLEDGMENTS

Undertaking this PhD has been a truly life-changing experience for me and it would not have been possible to do without the support and guidance that I received from many people. It is a great pleasure to have the possibility to thank the people, to whom I will be forever grateful.

First of all, I would like to thank very much my supervisor Prof Silvana Cavella for her precious teachings, support and encouragement she gave me. Without her guidance and constant feedback this PhD would not have been achievable.

I gratefully acknowledge Angela Sorrentino, who always supported me with interesting scientific discussions, Moreover, for all the support and encouragement she gave me during my PhD. I am also very grateful to Assunta Riola for her invaluable advice and feedback on my research and for always being so supportive of my work. It has been always challenging, but also enjoyable, working together. I would like to thank Annalisa Romano for giving me the possibility to work on technological characterization of bread baking.

Special thanks go to Matthew Bakker from the United States Department of Agriculture, Who gave me the possibility to join their working group in Peoria, IL, USA. It was a great time there, I learned many things about biological and enzymatic detoxification of mycotoxins, and also I had the possibility to meet wonderful guys like SusanMcCormick, Mark Busman, who I would like to thank for the scientific discussions and collaborations.

I gratefully acknowledge my institution 'University of Naples Federico II' and the finical support of the Italian funding received towards my PhD fellowship.

I would also like to say a heartfelt thank you to my lovely parents, Hannan, Asma, Fazeeha and Ahmed for always believing in me, encouraging me to follow my dreams and for helping me in whatever way they could during this challenging period.

And finally to my husband Mubashir Ali, who has been by my side throughout this PhD, living every single minute of it, and without whom, I would not have had the courage to embark on this journey in the first place. And to love of my life Zohaan Mubashir for being such a good little baby that past two years, and making it possible for me to complete what I started.

#### **TABLE OF CONTENTS**

F	Page
Acknowledgments	iii
Research Aims and outline	11
1. State of Art for Analysis & Reduction of Mycotoxins	14
1.1 Mycotoxins	15
1.2 Analysis of mycotoxins	17
1.2.1 Sampling	17
1.2.2 Extraction Methods	18
1.2.3 Clean-up Methods	19
1.2.4 Quantitative and Qualitative Analysis	21
1.3 Detoxification/Inactivation of Mycotoxins	25
1.3.1 Physical Processing	25
1.3.2 Chemical Treatments	29
1.3.3 Effect of medical plants and food ingredients	30
1.3.4 Enzymatic Detoxification	31
1.3.5 Biological Control Agents	32
1.4 Technological Parameters of Food Processing	36
1.5 References	37
2. Prospecting for Microbes Able to Transform Deoxynivalenol	51

2.1 Introduction	52
2.2 Materials and Methods	54
2.2.1 Materials	54
2.2.2 Enrichment Media	54
2.2.3 Development of Enrichment Cultures for DON Degradation	54
2.2.4 Extraction and Analysis of DON	56
2.2.5 Microbial Population Diversity in Enrichment Cultures	57
2.2.6 Isolation of Individual DON Degrading Microbes	58
2.2.7 Phylogenetic Identification of Isolated Microbes	58
2.3 Results and Discussion	60
2.3.1 Enrichment of DON Degrading Cultures from Soil	60
2.3.2 Analysis of DON Degrading Ability of Enrichment Cultures	61
2.3.3 Microbiome Profiling of Enrichment Cultures	66
2.3.4 Isolation of individual DON Modifying Microbes	68
2.4 Conclusion	71
2.5 References	72
3. Transformation of Deoxynivalenol by Laccas-Mediated System	75
3.1 Introduction	76
3.2 Materials and Methods	78
3.2.1 Materials and Reagents	78
3.2.2 Determination of Optimum Conditions for Laccase Activity	78

3.2.3 Degradation of Deoxynivalenol in Laccase-Mediated System	79
3.2.4 GC-MS Analysis of DON and its Transformation Products	79
3.2.5 LC-MS Analysis of DON and its Metabolities	80
3.2.6 Collection and Characterization of DON transformation Products	80
3.3 Results and Discussion	82
3.3.1 Optimization of Conditions for Enzyme Assays	82
3.3.2 Activity of various laccase-mediator combinations	84
3.3.3 Evaluation of laccase-mediator activity across a pH range	86
3.3.4 Achieving complete transformation of DON	88
3.3.5 Characaterizing DON transformation product	90
3.3.5.1 Gas Chromatography and mass spectroscopy	90
3.3.5.2 Liquid Chromatography and mass Spectroscopy	94
3.3.5.3 DON Precursors for protection of transformed groups	99
3.4 Conclusion	104
3.5 References	105
4. Reduction of Mycotoxins by Heat and UVC Irradiations	109
4.1 Introduction	110
4.2 Materials and Methods	112
4.2.1 Materials	112
4.2.2 Thermal and UVC Treatments	112
4.2.3 Chemicals and Reagents	112

4.2.4 Mycotoxins Extraction and LC/MS analysis	113
4.2.5 Technological Properties of Semolina	114
4.2.5.1 Color	114
4.2.5.2 Total Phenols determination and antioxidant power	114
4.2.5.3 Hydration Indices	114
4.2.5.4 Water Sorption Isotherm	115
4.2.6 Gluten Parameters	116
4.2.7 Farinograph Analysis	117
4.2.8 Differential Scanning Calorimetry Analysis	117
4.2.9 Microstructures	117
4.2.10 Statistical Analysis	118
4.3 Results and Discussion	119
4.3.1 Degradation of Mycotoxins	119
4.3.2 Technological Properties of Semolina	125
4.3.2.1 Color	125
4.3.2.2 Total Phenols determination and antioxidant power	127
4.3.2.3 Hydration and gluten Indices	129
4.3.2.4 Water Sorption Isotherm	132
4.3.3 Farinograph Analysis	134
4.3.4 Differential Scanning Calorimetry Analysis	140
4.3.5 Microstructures	143

4.4 Conclusion	145
4.5 References	146
5. Impact of Heat and UVC Treated Semolina on Pasta and Bread Making	153
5.1 Introduction	154
5.2 Materials and Methods	157
5.2.1 Materials	157
5.2.2 Thermal and UVC Treatments	157
5.2.3 Pasta Processing	158
5.2.4 Pasta Quality	158
5.2.5 Pasta Microstructures	159
5.2.6 Bread Dough Mixing	160
5.2.7 Bread Dough Leavening	160
5.2.8 Bread Quality	161
5.2.8.1 Moisture Content	161
5.2.8.2 Water Activity	162
5.2.8.3 Bubble Structure of the Loaf	162
5.2.8.4 Bread Hardness	162
5.2.9 Data Analysis	163
5.3 Results and Discussion	164
5.3.1 Pasta Quality	164
5.3.2 Pasta Microstructures	167

5.3.3 Bread Dough Mixing	170
5.3.4 Bread Dough Leavening	172
5.3.5 Bread Quality	174
5.4 Conclusion	180
5.5 References	181
Publications	185
Poster Presentation	186

Chapter 1

#### **RESEARCH AIMS AND OUTLINE**

The overall objective of this research is to contribute to the evidences supporting the application of different biological, enzymatic and physical approaches for the reduction of mycotoxins from the cereals and their derivatives.

This general aim has been addressed through the accomplishment of three main research topics such as i) the in vitro studies for the degradation of mycotoxins; ii) the evaluation of the physical treatments (UVC and heat) for reduction of mycotoxins and iii) the investigation of physical treatments on the technological performance of semolina, through pasta production and bread baking, attributed to application on industrial scale.

New approaches are needed to reduce or eliminate the mycotoxin from food and feed chain. These objectives were achieved making use of a research plan structured according to the following thesis outline.

In vitro study of enrichment cultures was conducted for the exploration and characterization of novel bacterial taxa that biotransform DON. Enrichment cultures were developed from the soil samples in minimal media containing chitin and DON  $50\mu$ g/mL and incubated for longer period of time. DON content was analyzed by gas chromatography mass spectrometer (GC/MS) DON. After three weeks complete transformation of DON was observed in cultures. Microbiome profiling was performed to reveal the reduction in microbial complexity from week 1 to 8 and the screening of pure strains from the cultures were carried out to identify the organism responsible for DON conversion. Such organisms may serve as a source of enzymes or genes involved in detoxification of mycotoxins, which could be useful for decontaminating agricultural commodities (Chapter 2)

11

Another in vitro study of laccase mediated reactions was performed for DON degradation activity in different combinations, in order to find the most appropriate combination. Laccase from *Trametes veriscolor* mediated by TEMPO was found most interesting to explore. The reactions were performed with enzyme, mediator and DON in McIlvaine's buffer solution. GC/MS analysis has revealed the complete transformation of DON into two other metabolites, which were isolated and purified by column chromatography. Characterization of DON metabolites was performed by gas chromatography mass spectrometery (GC/MS), and liquid chromatography mass spectrometery (LC/MS) (Chapter 3).

Chapter 4 analyzed some strategies that could be integrated in semolina-based products chains, with the aim of reducing mycotoxin contamination. Thermal treatment and UVC irradiation are selected fotr this research since they are known to be cost-effective techniques, combining a high thermal efficiency with simplicity of application. The effects of heating at 100, 150 and 200°C and UVC irradiation at 254 nm for 15, 30, 60, 120 min applied on semolina for the reduction of mycotoxins and their impact on technological semolina properties was studied. The impact of the most efficient treatments (150°C for 30 min and UVC irradiation for 120 min) were evaluated both on semolina and dough technological properties, such as color, hydration and gluten indices, farinograph and calorimetric parameters and microstructure. Thermal treatment significantly increased hydration of semolina and swelling power; farinograph analyses revealed a marked increase in water absorption and dough development time, and a reduction of dough stability, degree of softening and elasticity compared to the control. Calorimetric analyses showed a significant reduction of both temperature peaks and enthalpy of starch gelatinization, and a decrease of enthalpy for melting of lipids with starch. Scanning electron micrograph

images representing the dough microstructure confirmed the occurrence of these changes; UVCtreated samples showed less pronounced changes comparing to the control.

The impact of heat and UVC treatments applied for the reduction of mycotoxins was evaluated for technological performance by pasta and bread production. Pasta quality parameters and their micrographs by SEM analysis revealed no significant changes after the studied treatments compared to the control, in particular after UV treatment of semolina, so this treatment can be applied for reduction of mycotoxins without affecting the technological performance of the product, while the impact after heating was marked for some parameters. Technological parameters of bread baking were investigated by the dough consistency at the end of mixing, leavening behaviour of dough and the bread loaf characteristics revealing that until 120°C for 30 min no significant changes were observed in bread quality, while at 150°C a marked reduction of bread performance was shown (Chapter 5). Results from this study could be useful to food companies for the realization of safer semolina-based products such as for baby-food where the limit of mycotoxins fixed by European Commission is lower. In particular, the treatments at 120°C for 30 min can be applied without loss of technogical performance, while the treatment at 150°C could be applied for different types of cereal products, where the process of leavening is not required.

## Chapter 1

## State of Art for

# Analysis & Reduction of

# Mycotoxins

### STATE OF ART FOR ANALYSIS AND REDUCTION OF MYCOTOXINS 1.1 MYCOTOXINS

Mycotoxins are basically secondary fungal metabolites, which are extensively produced by many different fungal species that may contaminate a wide range of agricultural food products (Bennett and Klich, 2003). Most countries respond to food safety threat by establishing and enforcing maximum regulatory limits of mycotoxins (van Egmond et al, 2007). Despite the development of several measures to control fungal contamination, toxigenic fungi exist everywhere in the environment and can contaminate a wide range of agricultural products, both in the field before harvest and in warehouses after harvest. High levels of contamination in raw materials are usually more tolerated compared to a finished product intended for direct human consumption.

Thousands of different kinds of mycotoxins exist, but only a few of them have considerable food safety concerns. Among all fungal flora, the most studied fungal genera are *Aspergillus*, *Penicillium* and *Fusarium*, which are very well known for mycotoxin production in food. The most prominent mycotoxins are aflatoxins produced by *Aspergillus niger*, (Iqbal et al, 2015) deoxynivalenol (DON), zearalenone (ZEN) and fumonisins produced by *Fusarium* species, and patulin (PAT), produced by *Penicillium* species (Audenaert et al, 2014).

These compounds have adverse health effects such as carcinogenic, teratogenic, mutagenic and birth defects, which may result in varying levels of symptoms from skin irritation and neurotoxicity to immune suppression and death (Wu et al, 2014). Mycotoxins represent a serious health risk to humans, especially in low-income countries, where prolonged exposure, even in low quantities, may cause immune disorders as well as liver damage and cancer (Prietto et al, 2015). In children, aflatoxin contamination of milk can lead to delay in growth, (Raiola et al,

2015) while Fumonisins have been reported to be responsible for esophageal cancer, and ochratoxin can induce nephropathy. Prolonged DON exposure is reported to cause immunotoxicity (Hassan et al, 2015). Moreover, mycotoxin contamination adversely affects crop and animal production, significantly reducing market value. These issues require the application of control strategies to limit the health risks associated with the production of these contaminants.

Effective measures should be taken to avoid mycotoxin contamination in food. The first and foremost priority is prevention of mycotoxin production in the field before harvest and after harvesting in storage (Schmidt-Heydt et al, 2013). A number of different agricultural practices, e.g. crop rotation, growing resistant varieties, soil tillage, insect control, and biological and chemical control of plant diseases, have been developed to inhibit the growth of mycotoxin-producing fungi in the field, which ultimately lowers their production in foodstuffs (Munkvold, 2014; Alberts et al, 2016). Post-harvest measures are also crucial to avoid mycotoxin production during storage; in particular, humidity and temperature are the two main factors affecting mould growth during storage (Jacobsen, 2014). Several post-harvest methods can be used for the detoxification of food materials during food production (Zhu et al, 2016).

Food processing can also have a valuable impact on mycotoxin level (Santini, 2016) by:

- (i) Physical removal
- (ii) Chemical transformation to less toxic compounds
- (iii) Enzymatic detoxification
- (iv) Microbial degradation by adsorption on their surfaces

The accurate quantitative and qualitative analysis of mycotoxins is crucial for food safety measures. All the analytical procedures being used for estimation and determination of

16

mycotoxins include three basic steps: (i) extraction, (ii) purification and clean-up, and (iii) identification and quantification (Rahmani et al, 2009). In order to detect the lower level of mycotoxins in food commodities, it is necessary to develop sensitive, rapid and accurate assays. Several analytical techniques have been developed for different purposes, having different accuracy and sensitivity.

Most commonly used methods are chromatographic techniques and bioassays. Among the chromatographic methods, high-performance liquid chromatography (HPLC) is the most popular, with two different types of detectors, such as UV or fluorescence detectors. Recently, liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS) have become more feasible for qualitative and quantitative determination of mycotoxins (Sulyok et al, 2010).

#### 1.2 ANALYSIS OF MYCOTOXINS

#### 1.2.1 Sampling

The purpose of sampling is to obtain a portion for the analysis and estimation of attributes of a specific lot. Sampling methods are crucial in obtaining representative samples, owing to the high heterogeneity of contamination by mycotoxins (Wagner, 2015). The small percentages of extremely contaminated portions are randomly distributed in a lot. Thus, due to the high heterogeneity of mycotoxin concentration in samples, traditional methods of sampling are not good enough for mycotoxin estimation in agricultural foodstuff (Johansson et al, 2000).

To follow a correct procedure, the substance to be tested, the analytical method, the numbers of replicates, the numbers of measurements per each replicate and the sampling method must be defined. A good sampling is of primary importance for the management actions to implement

17

with lots that may be contaminated by mycotoxins (Wagner, 2015). A general criterion is that the whole primary sample must be ground and mixed to obtain the same concentration of toxin as the original sample, which is fundamental in raw cereals, since some mycotoxins, such as DON, are mainly present in the pericarp of grain (Vidal et al, 2013).

#### **1.2.2 Extraction Methods**

The physicochemical properties of sample material and type of toxin to analyze determine the method of extraction to be used. Generally, the sample is ground, homogenized in extraction solvent and filtered for the purification step. During the extraction procedure, the analyte will move in the extraction solvent and, in this way, the desired compound from the sample matrix is removed for analysis. The selection of extraction solvent is made wisely by considering that there is no specific solvent which can remove only the desired mycotoxins; thus, solvents are selected which can remove as many mycotoxins as possible (Capriotti et al, 2012).

*Solid–liquid extraction:* Most of the mycotoxins are soluble in polar solvents and insoluble in nonpolar solvents (Ridgway, 2012). The most common solvents that have been used in different studies for the extraction of mycotoxins from cereals are polar or relatively polar solvents, such as water, acetone, acetonitrile, (Raiola et al, 2012; Tolosa et al, 2017) methanol, (Juan et al, 2014) chloroform (Venkataramana et al, 2015) or a mixture of them (Lai et al, 2014; Skendi et al, 2016). *Innovative approaches for extraction:* The classic procedure adopted to favour the extraction of mycotoxins is carried out by using mechanical shaking or ultrasound, but in the last few years several new methods have been developed for extraction from cereals, including supercritical fluid extraction (SFE), accelerated solvent extraction (ASE) and microwave-assisted extraction (MAE), having the advantage of needing lower amounts of extraction solvent to optimize the performance. The SFE method for isolation and clean-up of macrocyclic lactone

mycotoxins, such as ZEN, from maize flour was carried out (Zougagh and Ríos, 2008). Several experimental conditions, e.g.  $CO_2$ , time of extraction, temperature and flow rate, were optimized. The use of methanol as solvent allowed 100% recovery for all mycotoxins. This technique is fast but requires expensive equipment.

The ASE method uses a low amount of solvent and requires extraction under high pressure (1500 psi for ASE 300) and high temperatures (50–200 °C); the sample is kept under these conditions for a short time, using compressed gas to remove the sample extract from the container; thus, fast extraction can be established. The ASE method was used in maize flour for the analysis of ZEN and its derivatives, (Zougagh and Ríos, 2008) and in rice and maize for the analysis of aflatoxins (Li et al, 2014). A MAE technique for OTA extraction in bread was reported, with optimized extraction conditions based on an orthogonal composite design coupled with response surface methodology (Paíga et al, 2012). An innovative method for the extraction of aflatoxins from grains and grain products, based on the combination of MAE and solid phase extraction (SPE) techniques, was also performed (Chen and Zhang, 2013).

#### **1.2.3** Clean-up Methods

Prior to analysis of the target molecule, sample extracts are cleaned up to remove the coextracted materials, which may interfere with the analyte during instrumental analysis.

SPE is generally based on molecularly imprinted polymers, which represent one of the most significant improvements in the purification procedure. There are several column packings commercially available with different sorbents, and the choice is function of matrix, analytes and interferences. The most common materials are silica gel, Florisil and cyano for determination of trichothecenes, while strong anion-exchange is used for fumonisins (Lattanzio et al, 2013). In

SPE, synthetic receptors are adopted for the extraction of the main mycotoxins. This method allows the complete removal of interfering compounds from the sample matrix by reducing the sample preparation time and increasing the sample output, since washing, conditioning and elution can be performed automatically (Stecher et al, 2007). Some authors optimized the purification step by SPE cartridges for the clean-up of 23 mycotoxins from sorghum (Njumbe Ediage et al, 2015).

Immunoaffinity columns (IAC) are often utilized in mycotoxin analysis, for their high specificity, since they contain an activated solid phase support with antibodies that bind a single mycotoxin, removing interfering substances; after that, the mycotoxins are eluted with a solvent or by antibody denaturation. The scientific literature provides examples of the application of IAC columns for the analysis of aflatoxin and ochratoxin in wines, (Di Stefano et al, 2015) animal feedstuff, (Di Stefano et al, 2015) pasta, (Keller Bol, 2016) infant formula and baby foods (Juan et al, 2014).

Another strategy for clean-up is represented by Mycosep and Multisep columns, which are filled by adsorbents such as ion-exchange resins, celite charcoal and other packed materials: the purified extracts seep through the column, while proteins, fats and pigments are held in the solid phase. This method is simple and quick, but the efficiency of purification depends on the matrix. (Montes et al, 2012).

The QuEChERS (quick, easy, cheap, effective, rugged and safe) sample preparation method has been widely described for several analytes and, in last few years, it was also reported for the analysis of mycotoxins. This approach is favourable for its simplicity, low volumes of consumed solvents, good recovery, repeatability, within- and inter-laboratory reproducibility, linearity and

20

low analytical limit of detection (LOD) and limit of quantitation (LOQ), but it cannot be easily automated (Tolosa et al, 2017)

Moreno *et al.* (2016) reported the application of hybrid nanoparticles for the extraction of ZEN and its metabolites from maize samples. This method, based on magnetic separation technology, presents advantages since it simplifies sample treatment; in fact, the sorbent does not need to be packed into the cartridge, the separation can be carried out by an external magnetic field, and nanomaterials could be used six times with sensitivity and recovery. Moreover, the method could be improved by combining the on-line use of magnetic nanoparticles with more sensitive detectors, such as tandem mass spectrometry (MS/MS).

#### **1.2.4** Quantitative and Qualitative Analysis

Mycotoxin analysis methods should be accurate, rapid, simple, robust and selective to enable determination. Different methods for quantitative and qualitative analysis of mycotoxins have eased the surveillance of foods for contamination of mycotoxins. The analytical method should be selected according to the purpose of analysis, and sensitive methods are required for low tolerance levels in food commodities. The determination of mycotoxins is usually achieved by different chromatographic techniques or immunochemical methods, as shown in Table 1.

Method	Advantages	Disadvantages	Reference
HPLC	high sensitivity good selectivity precise identification short analysis time automated (autosampler) selective and sensitive detection ability to generate structural information of analyte	expensive equipment specialist expertise required derivatisations may be required very expensive equipment	Kong et, al., 2014 Juan et, al., 2016 Zhang et, al., 2016 Spanjer et al., 2008
LC/MS	low detection limits simultaneous analysis of multiple mycotoxins minimum requirements for sample preparation convenient and sensitive	specialist expertise required sensitivity depends on ionization techniques matrix interference	Silva et al., 2009 Leon et al., 2016 Tolosa et al., 2017
ELISA	detection ease of operation rapid means of screening the sample limited use of organic solvents	problems cross reactivity with related mycotoxins semi- quantitative narrow operating range	Krska & Molinelli, 2007 Wang et al., 2014 Urusov et al., 2015
Spectral analysis technology	rapid screening of large number of samples qualitative and quantitative information about the structure of mycotoxins	complicated interpretation of spectral data spectra overlapping	Lee et al., 2014 Aiko et al., 2015

**Table 1**: Comparison of different qualitative and quantitative techniques for mycotoxins analysis

Among chromatographic techniques of analysis, HPLC, coupled with fluorescence derivatization (FLD) and MS/MS, is the most popular method for the detection of mycotoxins in cereals (Kong et al, 2014; Juan et al, 2016). The analytical tool LC/MS/MS has many advantages over other chromatographic techniques, such as selective and sensitive detection, ability to generate the structural information of the analyte, low detection limits, minimum requirements for sample preparation, the possibility to identify the wide range of analytes at different polarities and the use of general detectors, which are not dependent on chemical characteristics (Spanjer et al, 2008). Mass spectrometry is capable of separating mass fragments to the fourth or fifth decimal place (exact mass), where previous instrumentation was limited to single-digit mass units (integer mass).

The introduction of high-resolution mass spectrometry (HRMS) offers the possibility to analyse multiple contaminants with a single extraction, including ergot alkaloids, pesticides and veterinary drugs (León et al, 2016). High-resolution LC/Orbitrap mass spectrometry allowed the detection of masked mycotoxins derived from type A trichothecenes in corn, identified as neosolaniol–glucoside (NESGlc) and diacetoxyscirpenol–glucoside (DASGlc) on the basis of accurate mass measurements of characteristic ions and fragmentation (Nakagawa et al, 2012). LC Coupled to quadrupole Orbitrap MS was also adopted for the analyses of 17mycotoxins in 27 samples of durum wheat pasta and two samples of baby food (Tolosa et al, 2017).

Some researchers showed the use of GC technique for the analysis of mycotoxins such as ZEN, Fusarenon X, DON and derivatives, in breakfast cereals and flour (maize, wheat and cassava) (Cunha and Fernandes, 2010). This technique is convenient since it combines superior separation on the capillary columns with a variety of general or specific detectors such as MS. A twodimensional separation using Deans switch heart-cutting or comprehensive systems have also been carried out, improving separation power. Briefly, in heart-cutting a part of the sample from the first column is transferred to a second column by an interface such as Deans switch (Seeley, 2012). This approach requires a derivatization phase, such as silylation and acylation, since most mycotoxins are characterized by low volatility.

In general, the physicochemical detection methods of mycotoxins require a lengthy preparation of the sample, as well as an unstable chromatographic trend of mycotoxins. Therefore, bioassays have become widespread for mycotoxin detection. In these methods, mycotoxin specific antibody (at fixed concentration) is mixed with a sample containing a known amount of mycotoxin. A complex is formed, and responses are generated over a range of standard mycotoxin concentrations, which are used to generate a calibration curve and table and, finally, unknown samples are determined by referring to the calibration curve. Advances in research have made it possible to develop highly specific antibody-based tests; several kits are commercially available, which can be used to identify and measure the mycotoxins in food commodities, sometimes in less than 10min. Bioassays are generally based on the affinities of monoclonal and polyclonal antibodies, and three different types of immunochemical methods are distinguished: (i) radioimmunoassay (RIA); (ii) enzyme-linked immunosorbent assay (ELISA); and (iii) immuno-affinity column assay (ICA). By the late 1960s, RIA was introduced to the diagnostic market and subsequently ELISA began to replace RIA. A solid-phase RIA for detecting AFB1 in corn and wheat was developed (Jacobs et al, 1972). RIA was also adopted for detecting nivalenol in barley (Teshima et al, 1990).

ELISA is the most widespread immunoassay technique used in OTA analysis for its simplicity and capability for parallel analysis of multiple samples. Bioassay methods have several advantages over other methods, such as rapid means of sample screening, convenient and sensitive detection, ease of operation, high output, and quick and reliable results with limited sensitivity. However, like other methods, it also has some demerits, such as cross-reactivity and matrix dependence (Krska and Molinelli, 2007).

Finally, the introduction of spectral analysis technology for mycotoxin detection can ensure rapid screening of many samples. These techniques include near-infrared reflectance (NIR), Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy, which can provide qualitative and quantitative information about the structure of mycotoxins (Hernandez-Hierro et al, 2008). Some examples of Raman spectroscopy application have been reported for qualitative and quantitative analysis of aflatoxins in ground maize (Lee et al, 2014). On the other side, spectroscopic technology has been limited owing to the difficult interpretation of spectral data and overlapping of spectra.

#### 1.3 DETOXIFICATION/INACTIVATION OF MYCOTOXINS

#### **1.3.1** Physical Processing

*Sorting:* Mycotoxin contamination is higher in broken and damaged kernels; unprocessed cereals often contain admixtures and dust particles (Johansson et al, 2006; Juan et al, 2012). Sorting, dehulling or washing is usually applied before processing methods or after harvesting, which removes admixtures from the cereals (Table 2). Sorting is generally a technique to separate substandard particles from the food to maintain quality. In the case of cereal grains, it can be carried out based on different physical properties like shape, colour, size and density, as well as the identification of broken grains with fungal growth. Mycotoxin contamination is heterogeneously distributed among the grains; therefore, separating the damaged grains can effectively reduce the contamination level. Grain sorting for aflatoxin reduction using UV light is

also common. Ergot alkaloids are even more heterogeneously distributed than the aflatoxins; sclerotia loaded with alkaloids can be efficiently removed from the rye by opto-electronic sorting (Miedaner and Geiger, 2015). In spite of all this, sorting is basically an inefficient, laborious and impractical method.

Detoxification			Reduction	
method	Product	Mycotoxin	(%)	Reference
	pistachio nut	Aflatoxins	95	Shakerardekani et al., 2012
Sorting	rye	Ergot alkaloid	70-80	Miedaner and Geiger, 2015
Dehulling	Maize	Aflatoxins	46.6	Kilonzo et al., 2014
Milling	Wheat Maize	Aflatoxins DON ZEN	40-50	Schwake-Anduschus et al., 2015 Tibola et al., 2015
-	Cereal	Aflatoxins	50-80	Oliveira et al., 2013
Extrusion	Maize	Fumonisins	34-95	Hahn et al., 2015
	Maize	Aflatoxins	95	Markov et al., 2015
Irradiations	red chili	Aflatoxins	85	Iqbal et al., 2013
	cereal	Aflatoxins	75	Herzallah et al., 2008
	cereal	DON	40	Vearasilp et al., 2015
~	Nuts	Aflatoxins	50	Basaran et al., 2008
Cold Plasma	palm fruits	Aflatoxins	All spores	<i>Ouf et al., 2015</i>

**Table 2**: Physical approaches for detoxification of mycotoxins during food processing

An innovative application based on image processing is represented by near-infrared spectroscopy (NIRS), which is very effective for screening of fungal growth on a large scale from cereal grains and other agricultural products (Udomkun et al, 2017). Hyperspectral imaging (HSI) is another innovative technique to produce the complete NIR spectrum and localize contamination in food samples, especially in grains. In a recent study on maize kernels inoculated with *Aspergillus flavus* spores, the contamination level of aflatoxins was estimated using hyperspectral imaging (Wang et al, 2015).

**Processing:** Removal of the outer layers of the grains is known as dehulling, a basic processing step prior to the grinding of grains. The level of mycotoxin contamination can be decreased by dehulling, since fungal colonization accumulates on the surface of grains, and so this method has been reported to reduce the fungal load from the bulk (Vučković et al, 2013). A study carried out on maize in Kenya has shown the reduction of aflatoxins by dehulling, during the preparation of muthokoi, which is a traditional dehulled dish of maize. Aflatoxin content was significantly reduced, leading to lower exposure in muthokoi as compared to entire maize kernels (Kilonzo et al, 2014). Spatial distribution of mycotoxins was studied in wheat milling fractions, after the milling process of cereals. Finished flour was found to be less contaminated, while high mycotoxin levels were found in bran (Tibola et al, 2015). Temperature and time are important parameters for industrial food processing, which can affect the mycotoxin content in the finished product. Although mycotoxins are thermally very stable compounds, some conventional food preparation methods, or temperatures above 100 °C, can affect some mycotoxins. Hightemperature methods such as toasting, roasting, frying and extrusion can reduce mycotoxin contamination (Oliveira et al, 2013).

**Radiation:** Irradiation such as ionizing (gamma) and non-ionizing (UV, solar, microwave) can partly remove mycotoxins from foods, by eliminating or reducing pathogenic microorganisms. Hence irradiation may be an industrial approach to remove contamination from foods by providing energy to both food constituents and contaminants. Gamma radiation generates veryhigh-energy photons, which directly damage the DNA of microorganisms in the cell (Da Silva Aquino, 2012). Several studies have reported the efficacy of the application of gamma radiation for the reduction of aflatoxins. In a study on naturally infected maize, AFB1 was found to be reduced by 95% when exposed to a dose of 10 kGy gamma radiation (Markov et al, 2015). In the literature, the application of UV irradiation was also reported to be effective as a non-thermal and economic strategy for the destruction of mycotoxins. Further alternative methods of irradiation for the detoxification of food commodities are the microwave and dielectric processes of radio frequency (Vearasilp et al, 2015). Microwave heat treatment was found to be less successful for lowering mycotoxin levels in food commodities (Numanoglu et al, 2012). Another innovative and recent application for mycotoxin reduction is pulsed light (PL), which generates short, high-intensity broad-spectrum white light. PL was reported to be efficient in reducing toxicity from Aspergillus flavus in rice (Wang et al, 2016).

*Cold plasma:* The use of cold plasma technique in food processing to eliminate pathogens is reported to be a valid method. A recent review of this technique highlighted the potential of its strong antimicrobial effects, which can be used for the sterilization of temperature-sensitive surfaces, such as food products. (106) Cold plasma is basically created by atmospheric dielectric discharge, with synthetic air as working gas; in a case study, the concentrations of ZEN and DON were found to be reduced from 100  $\mu$ gmL–1 to a few  $\mu$ gmL–1 (ten Bosch et al, 2017).

Food products treated by plasma need to be assessed for the formation of any toxic compound during the process, since no investigations have yet been conducted using this technique.

#### **1.3.2** Chemical Treatments

Chemical processing of food for decontamination or detoxification of mycotoxins is not authorized within the EU for human food commodities; therefore, chemical processing requires regulatory approval. Many studies have been conducted to investigate the suitability of chemical processing to inactivate or destroy mycotoxins. Chemicals may change the structure of mycotoxins into other compounds; therefore, the toxicity level must be assessed after chemical treatment. Furthermore, it is known that chemical processing also impairs the nutritional quality of the food product by affecting texture, taste or flavour. Recently, defined criteria for chemical detoxification of feed may be used as a model for corresponding developments in food regulations (European Commission, 2015).These chemical processing methods involve the use of acids, bases, reducing agents or oxidizing agents. Usually these chemicals are applied to mycotoxin reduction by packing, immersion or fumigation (Bender et al, 2012; Guzmán and Hernández, 2014).

Codex Alimentarius General Standards for Food Additives and general food legislation provide a list of chemicals that can be used. Several studies have demonstrated the effect of different food additives on the detoxification of mycotoxins. For example, glycerol is a general-purpose non-toxic food additive; thus, a powerful detoxification effect was observed when combined with calcium hydroxide (Venter, 2014). In a more recent study, aflatoxins were treated with acetic, lactic and citric acids under conditions simulating cooking, and lactic acid showed the highest efficiency (Aiko et al, 2016). Therefore, they can be used as a preservative in different food commodities. In the oxidation process of aflatoxins, primarily phenol formation occurs, by an

addition reaction towards the double bond of the furan ring, destroying the structure of aflatoxin. It is well understood that compounds which have a terminal double bond, such as aflatoxins G1, B1, and M2, are more susceptible to reaction with oxidizing agents such as ozone, which remove their double bond (Inan et al, 2007).

#### **1.3.3** Effects of Medical Plants and Food Ingredients

To overcome the residual toxicity of synthetic additives and resistance of fungal species against them, there are certain natural plant materials such as some spices, plant-based additives, herbs or other food ingredients that are used during food processing or home cooking for their detoxification ability against mycotoxins. The effect of isothiocyanates was evaluated for reduction of aflatoxins present in oriental mustard flour as an effect of reduced *Aspergillus parasiticus* growth: aflatoxins were reduced from83.1% to 87.2% (Hontanaya et al, 2015). In Asian cooking, ajwain spice (carom) is used in some foods; therefore, the potential effect of reducing aflatoxins after incubation with ajwain extract was studied (Velazhahan et al, 2010). In another study on medicinal plants, an extract of *Ocimum tenuiflorum* was evaluated as an aflatoxin detoxifier, even at room temperature; (Panda and Mehta, 2013) also, aqueous extracts of *Adhatoda vasica*were found to completely degrade AFB1 after 24 h at 37 °C (Vijayanandraj et al, 2014).

Generally, essential oils originating from different aromatic plants have also been used as food preservatives because of their antimicrobial properties. For example, the antifungal activity of eugenol (a compound derived fromessential oils) was determined against aflatoxin production in sorghum grains (Komala et al, 2012). In another similar study, the antimicrobial potential of thyme (essential oil from *Thymus vulgaris*) was reported to inhibit fungal growth (Kohiyama et al, 2015). Detoxification of mycotoxins by herbs and spices has been recently reviewed (Aiko

and Mehta, 2015). In a study of plant material as detoxifier, neem (*Azadirachta indica*) leaves were found to have strong fungicidal and anti-aflatoxigenic properties. The application of 20% neem powder completely inhibited all types of fungal growth when used during the storage of wheat, rice andmaize (Sultana et al, 2015). Among the other food ingredients, reducing sugars have also been found to be detoxifiers; for example, D-fructose and D-glucose reduced the level of fumonisins by blocking the primary amino group in their structure, hence preventing their toxicity in food commodities (Fernandez-Surumay et al, 2005).

#### **1.3.4 Enzymatic Detoxification**

A distinguishing feature of detoxification using enzymes is its specificity to food commodities, among all the potentially suitable detoxification techniques, since enzymatic catalysis has a unique position (Wang et al, 2011). Enzymes have evolved as important catalytic agents used for different industrial processing methods, because of recent developments in protein engineering and recombinant DNA technology (Pfliegler et al, 2015). Some notable exceptions were found during studies of enzymatic detoxification: peroxidases and laccases can also modify the substrate and may destroy valuable food components. It is reported that the laccase from Trametes versicolor can degrade AFB1 (Scarpari et al, 2014). The potential for mycotoxin detoxification by enzymatic activities has been recently reviewed (Vanhoutte et al, 2016). Another distinguishing feature of enzymatic detoxification is that enzymes are basically proteins, which can cause allergies when used during food processing. Thus investigation of the allergenic potential of enzymes is also required for approval as processing aids (European Food Safety Authory, 2009). Enzymes possess an unexplored profile to detoxify contaminants in food, because of their favourable toxicology and specificity. In the EU, so far, no enzyme has been authorized for the removal of mycotoxin contamination from foods.

Enzymes are used in the food industry as processing aids; for example, in the manufacturing of cheese the aspartic protease chymosin is used as an alternative to rennet, and other industrial enzymes are also used in bread making (Whitehurst and van Oort, 2010). Some other food production processes that can also benefit from the use of enzymes in the detoxification of mycotoxins are malting and brewing. Enzymes for the detoxification of DON may also have desirable effects on wheat flour during the baking, and some enzymes can also be added to flour with other commonly used enzymes during the baking process, such as amylases, proteases and xylanases. Unfortunately, for irreversible detoxification of DON, no suitable enzymes are available yet. New detoxification activities for industrial production have been identified, but whether the enzymes responsible for these activities are suitable for food production remains to be evaluated (He et al, 2015). A bacterium belonging to *Sphingomonas* spp. has been characterized as fumonisin detoxifying, since it was able to produce the enzyme for decontamination of fumonisins (Heinl et al, 2010). ZEN degrading ability has been found in the fungus *Clonostachys rosea*, producing some enzymes for decontamination.

In addition, enzymatic detoxifying activities have been found in bacteria (Tan et al, 2014), yeast (Vekiru et al, 2010) and fungi (Popiel et al, 2014). Many peptidases are able to hydrolyse OTA, exerting their detoxifying activity by hydrolyzing the amide (Abrunhosa et al, 2010). Enzymatic detoxification appears to be a conceivable approach for mycotoxin decontamination in food processing.

#### **1.3.5** Biological Control Agents

Different physical and chemical methods have many disadvantages as well as many undesirable effects on the quality of food products. However, biological methods have been reported as the most promising approach to degrade mycotoxins and also to prevent exposure of the human body to them (Table 3) (Ahlberg et al, 2015). Fermentation is a common food process, based on the use of microorganisms widely used in the food industry. Bacteria and fungi are used for the fermentation process; the activities of these microorganisms are responsible for required transformations in food components.

However, many additional metabolic enzymes also work in their cells, which are released into the food matrix after the autolysis or disintegration of cells. Transformation of mycotoxins into non-toxic compounds can occur as a consequence of these activities of microbes (Saladino et al, 2016). For targeting mycotoxin decontamination, so far no strain of any microorganism has been authorized. Brewing and malting are major examples of fermentation processing which can significantly benefit from such technologies to remove mycotoxins (Moss and Long, 2002). Fermentation with lactic acid bacteria (LAB) is also used in the manufacture of many dairy products. Detoxification by LAB has been studied for a long time against aflatoxin M1, the major aflatoxin in milk. LAB known as *Lactobacillus, Propionibacterium, Lactococcus* and *Bifidobacterium* have been reported as very good binders of aflatoxins, which can ultimately degrade aflatoxins from the food product (Popiel et al, 2014). More recently, in another study the effect of LAB against aflatoxin development was investigated during bread processing, which resulted in reduction of aflatoxins (Saladino et al, 2016).

Other microorganisms have also been reported as mycotoxin binders or detoxifiers, such as cultures of *Saccharomyces cerevisiae*, a yeast used in the brewery and in sourdough production, able to detoxify OTA (Petruzzi et al, 2014) and aflatoxins, (Topcu et al, 2010) thus reducing the contamination level in fermented food products. In the 1960s, the US Department of Agriculture carried out the first screening for mycotoxin-degrading microbes, especially targeting the aflatoxins (Ciegler et al, 1966). Many screening studies were reported but rarely confirmed that

degradation was a result of enzymatic activity rather than physical adsorption of microorganisms. Numerous bacterial and fungal species were screened for detoxification purposes, but progress in elucidating the mechanism of action was very slow. After almost three decades of research it was found that the detoxification of aflatoxins by Flavobacterium aurantiacum involved enzymatic activities (Smiley and Draughon, 2000). The fungal species Rhodococcuserythropolis was also able to detoxify aflatoxins, (Alberts et al, 2006) and the degradation of thesemycotoxins by Rhizopus oryzae and Tricoderma reesei was recently studied (Hackbart et al, 2014). In some molecular-level studies, for the detoxification of mycotoxins, it has been revealed that these activities are not feasible for food processing, particularly for the degradation of AFB1 by Actinomycetes spp (Alberts et al, 2006). White-rot fungus (Phanerochaete sordid) was reported to produce peroxidase, responsible for degradation of aflatoxins (Wang et al, 2011). However, in most of cases the mechanism of action remained undefined. The number of microorganisms reported for detoxification of mycotoxins from food commodities is high (He et al, 2015; He et al, 2016) and their use for decontamination of food commodities during food process ing requires regulatory approval.

Biological agent	Product	Mycotoxin	Reference
Lactic acid bacteria	Milk	Aflatoxin M1	Ahlberg et al., 2015
Saccharomyces cerevisiae	fermented food	OTA, Aflatoxin	Petruzzi et al., 2014
Lactobacillus spp.	Bread	Aflatoxins	Saladino et al., 2016
Rhodosporidium paludigenum	Fruits	PAT	Zhu et al., 2015
Bacillus strain	animal feed	Trichothecene	Zhou et al., 2014
Rhizopus oryzae	food processing	Aflatoxins	Hackbart et al., 2014

#### Table 3: Application of biological agents for detoxification in food commodities

#### 1.4 TECHNOLOGICAL PARAMETERS OF FOOD PROCESSING

Many physical approaches such as, dehulling, milling, extrusion, irradiation and cold plasma are being applied during food processing. Temperature and time are important parameters for industrial food processing, which can affect the food quality by damaging the nutritional value. For example, during bread baking, starch molecules play a significant role. Starch granules have the ability of gelatinization and to trap the air bubbles, facilitating the process of fermentation by gas retention (Hug-Iten et al, 2001).

During processing, the foods undergo many physical, physiochemical and sensory changes (Robertson, 1993), Such as heating at a very high temperature can affect the gluten content present in cereal's derivatives. Gluten is a complex of protein, composed of two main groups: gliadins, which is responsible for the viscosity of the dough, and glutenins, ensures the dough elasticity (Becker et al, 2007). However, dough produced in the absence of gluten presents poor rheological properties, affecting the final quality.

These complex physical and chemical processes can significantly affect the bread technological parameters during baking such as dough volume during bread leavening, crumb firmness and gas bubble area fraction in bread loaf, a consequence of the starch granules gelatinized during baking. Different technologies are being used to access the food quality parameters such as differential scanning calorimetry (DSC) to evaluate the impact of treatments on starch gelatinization, while a Brabender Farinograph is used to test the behaviors of doughs (Hager et al, 2012; Therdthai et al, 2016).
#### **1.5 REFERENCES**

- Abrunhosa L, Paterson RRM and Venâncio A, Biodegradation of ochratoxin a for food and feed decontamination. *Toxins* 2:1078-1099 (2010).
- 2. Ahlberg SH, JoutsjokiV and Korhonen HJ, Potential of lactic acid bacteria in afaltoxin risk mitigation. *Int J Food Microbiol* **207**:87-102 (2015).
- Aiko V and Mehta A, Occurrence, detection and detoxification of mycotoxins. *J Bio sci* 40:943-954 (2015).
- Aiko V, Edamana P and Mehta A, Decomposition and detoxification of aflatoxin B1 by lactic acid. *J Sci Food Agric* 96:1959-1966 (2016).
- Alberts JF, Engelbrecht Y, Steyn PS, Holzapfel W and van Zyl W, Biological degradation of aflatoxin B1 by *Rhodococcus erythropolis* cultures. *Int J Food Microbiol* 109:121-126 (2006).
- Alberts JF, van Zyl WH and Gelderblom WCA, Biologically based methods for control of fumonisin producing *Fusarium* species and reduction of the fumonisins. *Front Microbiol* 7:548 DOI: 10.3389/fmicb.2016.00548 (2016).
- 7. Audenaert K, Vanheule A, Höfte M and Haesaert G, Deoxynivalenol: a major player in the multifaceted response of fusarium to its environment. *Toxins* **6**:1-19 (2014).
- Becker D, Folck A, Knies P, Lörz H, and Wieser H. Silencing the α-gliadins inhexaploid bread wheat. In: G. L. Lookhart & P. K. W. Ng (Ed,.), *Gluten proteins* (pp. 86-89) Campinas: St Paul (2007).
- Bender RJ, Farias NPCE, Nunes DSSJ and Pranke PHL, Use of nucleophilic chalcogen or amino acid compounds as reagents for reducing or eliminating patulin from fruit-based beverages and processed foodstuffs. WO Patent Application Publication No. 2012106794 A1 (2012).

- 10. Bennett JW and Klich M, Mycotoxins. Clin Microbiol Rev 16:497-516 (2003).
- 11. Capriotti AL, Cavaliere C, Piovesana S, Samperi R, Laganà A, Multiclass screening method based on solvent extraction and liquid chromatography–tandem mass spectrometry for the determination of antimicrobials and mycotoxins in egg. *J Chromat A* **1268**:84–90 (2012).
- Chen S and Zhang H, Development of a microwave-assisted-extraction-based method for the determination of aflatoxins B1, G1, B2, and G2 in grains and grain product. *Anal Bioanal Chem* 405:1623–1630 (2013).
- Ciegler A, Lillehoj EB, Peterson RE and Hall HH, Microbial detoxification of aflatoxin.
  *Appl Microbiol* 14:934-939 (1966).
- 14. Cunha SC and Fernandes JO, Development and validation of a method based on a QuEChERS procedure and heart-cutting GC-MS for determination of five mycotoxins in cereal products. J Sep Sci 33:600–609 (2010).
- 15. Da Silva Aquino KA, Sterilization by gamma irradiation, in *Gamma radiation*, ed. by Adrovic F. In Tech, Vienna, Austria, pp. 171-206 (2012).
- 16. Di Stefano V, Avellone G, Pitonzo R, Capocchiano VG, Mazza A, Cicero N and Dugo G, Natural co-occurrence of ochratoxin A, ochratoxin B and aflatoxins in Sicilian red wines. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **32**:1343-1351 (2015).
- 17. Di Stefano V, Pitonzo R, Cicero N and D'Oca MC, Mycotoxin contamination of animal feedingstuff: detoxification by gamma-irradiation and reduction of aflatoxins and ochratoxin A concentrations. *Food Addit Contam Part A* **31**:2034-2039 (2014).

- 18. EFSA, European Food Safety Authory, Guidance on the submission of a Dossier on food enzymes for safety evaluation by the scientific panel of food contact material, enzymes, flavourings and processing aids. *EFSA J* 1305:1-26 (2009).
- European Commission (EC) Regulation n. 2015/786 defining acceptability criteria for detoxification processes applied to products intended for animal feed. *Off J Eur Union* 125:10-14 (2015).
- European Commission (EC) Regulation n. 401/2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Off J Eur Comm*, L70:12-34 (2006).
- 21. Fernandez-Surumay G, Osweiler GD, Yaeger MJ, Rottinghaus GE, Hendrich S, Buckley LK and Murphy PA, Fumonisin B glucose reaction products are less toxic when fed to swine. *J Agric Food Chem* 53:4264-4271 (2005).
- 22. Guzmán ODA and Hernández VLJ, Methods for neutralizing aflatoxins by potassium salt of naphtaleneacetic acid. WO Patent Application Publication No. 2014114982 A1 (2014).
- Hackbart HCS, Machado AR, Christ-Ribeiro A, Prietto L and Badiale-Furlong E, Reduction of aflatoxins by *Rhizopus oryzae* and *Trichoderma Reesei*. *Mycotoxin Res* 30:141-149 (2014).
- 24. Hager AS, Wolter A, Jacob F, Zannini E, and Arendt EK, Nutritional properties and ultra-structure of commercial gluten free flours from different botanical sources compared to wheat flours. *J Cereal Sci***56**: 239-247 (2012).
- 25. Hassan YI, Watts C, Li XZ and Zhou T, A novel peptide-binding motifs inference approach to understand deoxynivalenol molecular toxicity. *Toxins* **7**:1989-2005 (2015).

- 26. He JW, Bondy GS and Zhou T, Toxicology of 3-epideoxynivalenol, a deoxynivalenoltransformation product by *Devosia mutans* 17-2-E-8. *Food Chem Toxicol* 84:250-259 (2015).
- 27. He JW, Hassan YI, Perilla N, Li XZ, Boland GJ and Zhou T, Bacterial epimerization as a route for deoxynivalenol detoxification: the influence of growth and environmental conditions. *Front Microbiol* **7**:572 (2016).
- 28. Heinl S, Hartinger D, Thamhesl M, Vekiru E, Krska R, Schatzmayr G, Moll WD and Grabherr R, Degradation of fumonisin B1 by the consecutive action of two bacterial enzymes. *J Biotechnol* **145**:120-129 (2010).
- 29. Hernandez-Hierro JM, Garcia-Villanova RJ and Gonzalez-Martin I, Potential of near infrared spectroscopy for the analysis of mycotoxins applied to naturally contaminated red paprika found in the Spanish market. *Anal Chim Acta* **622**:189-194 (2008).
- 30. Hontanaya C, Meca G, Luciano FB, Manes J and Font G, Inhibition of aflatoxin B1, B2, G1, and G2 production by *Aspergillus parasiticus* in nuts using yellow and oriental mustard flours. *Food Control* 47:154-160 (2015).
- Hug-Iten S, Echer F, and Conde-Petit B, Structural properties of starch in bread and bread model systems:influence of an antistaling α-amylose. *Cereal Chem*78: 421-428 (2001).
- 32. Inan F, Pala M and Doymaz I, Use of ozone in detoxification of aflatoxin B1 in red pepper. *J Stored Prod Res* **43**:425-429 (2007).
- 33. Iqbal SZ, Jinap S, Pirouz AA and Ahmad Faizal AR, Aflatoxin M1 in milk and dairy products, occurrence and recent challenges: A review. *Trends in Food Sci Technol* 46:110-119 (2015).

- Jacobs LS, Mariz IK and Daughaday WK, A Mixed Heterologous Radioimmunoassay for Human Prolactin. *The J Clin End & Metab* 34:484–490 (1972).
- 35. Jacobsen BJ, Good agricultural and harvest practices to reduce mycotoxin contamination in wheat in temperate countries, in *Mycotoxin reduction in grain chains*, ed. by Leslie JF, Logrieco AF. Wiley Blackwell, New Delhi, pp. 209–219 (2014).
- 36. Johansson AS, Whitaker TB, Hagler Jr, WM, Giesbrecht FG and Young JH, Testing shelled corn for aflatoxin, Part III: Evaluating the performance of aflatoxin sampling plans. *J Assoc Off Anal Chem Int* **83**:1279-1284 (2000).
- 37. Johansson AS, Whitaker TB, Hagler WM, Bowman DT, Slate AB and Payne G, Predicting aflatoxin and fumonisin in shelled corn lots using poor quality grade components. *J AOAC Int* **89**:433-440 (2006).
- 38. Juan C, Covarelli L, Beccari G, Colasante V and Mañes J, Simultaneous analysis of twenty-six mycotoxins in durum wheat grain from Italy. *Food Contr* **62**:322-329 (2016).
- 39. Juan C, Raiola A, Mañes J and Ritieni A, Presence of mycotoxin in commercial infant formulas and baby foods from Italian market. *Food Control* **39**:227-236 (2014).
- 40. Juan C, Ritieni A and Mañes J, Determination of trichothecenes and zearalenones in grain cereal, flour and bread by liquid chromatography tandem mass spectrometry. *Food Chem* **134**:2389-2397 (2012).
- 41. Keller Bol E, Araujo L, Fonseca Veras F and Welke JE, Estimated exposure to zearalenone, ochratoxin A and aflatoxin B1 through the consume of bakery products and pasta considering effects of food processing. *Food Chem Toxicol* **89**:85-91 (2016).

- 42. Kilonzo RM, Imungi JK, Muiru WM, Lamuka PO and Njage PM, Household dietary exposure to aflatoxins from maize and maize products in Kenya. *Food Addit Contam* 31:2055–2062 (2014).
- 43. Kohiyama CY, Ribeiro MMY, Mossini SAG, Bando E, Bomfim NS and Nerilo SB, Antifungal properties and inhibitory effects upon aflatoxin production of *Thymus vulgaris* L. by *Aspergillus flavus* Link. *Food Chem* 173:1006-1010 (2015).
- 44. Komala VV, Ratnavathi CV, Vijay Kumar BS and Das IK, Inhibition of aflatoxin B1 production by an antifungal component, eugenol in stored sorghum grains. *Food Control* 26:139-146 (2012).
- 45. Kong W, Wei R, Logrieco AF, Wei J, Wen J, Xiao X and Yang M, Occurrence of toxigenic fungi and determination of mycotoxins by HPLC-FLD in functional foods and spices in China markets. *Food Chem* **146**:320-326 (2014).
- 46. Krska R and Molinelli A, Mycotoxin analysis: state-of-the-art and future trends. Anal Bioanal Chem 387:145-148 (2007).
- 47. Lai XW, Sun DL, Ruan CQ, Zhang H and Liau CL, Rapid analysis of aflatoxins B1, B2, and ochratoxin A in rice samples using dispersive liquid-liquid microextraction combined with HPLC. *J Sep Sci* **37**:92–98 (2014).
- 48. Lattanzio VMT, Ciasca B, Haidukowki M, Infantino A, Visconti A and Pascale M, Mycotoxin profile of *Fusarium langsethiae* isolated from wheat in Italy: production of type-A trichothecenes and relevant glucosyl derivatives. *J Mass Spectrom* 48:1291–1298 (2013).

- 49. Lee K, Herrman TJ and Yun U, Application of Raman spectroscopy for qualitative and quantitative analysis of aflatoxins in ground maize samples. J Cereal Sci 59:70-78 (2014).
- 50. León N, Pastor A and Yusà V, Target analysis and retrospective screening of veterinary drugs, ergot alkaloids, plant toxins and other undesirable substances in feed using liquid chromatography–high resolution mass spectrometry. *Talanta* **149**:43-52 (2016).
- 51. Li C, Xie G, Lu A, Ping H, Ma Z, Luan Y and Wang J, Determination of aflatoxins in rice and maize by Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry with accelerated solvent extraction and solid-phase extraction. *Anal Lett* 47:1485–1499 (2014).
- 52. Markov K, Mihaljevi CB, Domijan AM, Pleadin J, Dela, SF and Frece J, Inactivation of aflatoxigenic fungi and the reduction of aflatoxin B1 in vitro and in situ using gamma radiation. *Food Control* **54**:79-85 (2015).
- 53. Miedaner T and Geiger HH, Biology, genetics, and management of ergot (*Claviceps* spp.) in rye, sorghum, and pearl millet. *Toxins* **7**:659-678 (2015).
- 54. Montes R, Segarra R and Castillo MA, Trichothecenes in breakfast cereals from the Spanish retail market. *J Food Comp Anal* **27**:38-44 (2012).
- 55. Moreno V, Zougagh M and Ríos Á, Hybrid nanoparticles based on magnetic multiwalled carbon nanotube-nanoC18SiO2 composites for solid phase extraction of mycotoxins prior to their determination by LC-MS. *Microchim Acta* 183:871–880 (2016).
- 56. Moss MO and Long MT, Fate of patulin in the presence of the yeast *Saccharomyces cerevisiae*. *Food Add Contam* **19**:387-399 (2002).

- 57. Munkvold GP, Crop management practices to minimize the risk of mycotoxins contamination in temperate zone maize, in *Mycotoxin reduction in grain chains*, ed. by Leslie JF, Logrieco AF. Wiley Blackwell, New Delhi, pp. 59–77 (2014).
- 58. Nakagawa H, Sakamoto S, Sago Y, Kushiro M and Nagashima H, The use of LC-Orbitrap MS for the detection of *Fusarium* masked mycotoxins: the case of type A trichothecenes. *World Myc Journ* 5:271-280 (2012).
- 59. Njumbe Ediage E, Poucke CV and De Saeger S, A multi-analyte LC–MS/MS method for the analysis of 23 mycotoxins in different sorghum varieties: The forgotten sample matrix. *Food Chem* **177**:397-404 (2015).
- 60. Numanoglu E, Gökmen V, Uygun U and Koksel H, Thermal degradation of deoxynivalenol during maize bread baking. *Food Add Contam: Part A* **29** (2012).
- 61. Oliveira G, da Silva DM, Pereira RGFA, Paiva LC, Prado G and Batista LR, Effect of different roasting levels and particle sizes on ochratoxin a concentration in coffee beans. *Food Control* 34:651-656 (2013).
- 62. Paíga P, Morais S, Oliva-Teles T, Correia M, Delerue-Matos C, Duarte SC, Pena A and Lino CM, Extraction of ochratoxin A in bread samples by the QuEChERS methodology. *Food Chem* 135:2522-2528 (2012).
- Panda P and Mehta A, Aflatoxin detoxification potential of *Ocimum tenuiflorum*. J Food Safety 33:265-272 (2013).
- 64. Petruzzi L, Bevilacqua A, Baiano A, Beneduce L, Corbo MR and Sinigaglia M, In vitro removal of ochratoxin a by two strains of *Saccharomyces cerevisiae* and their performances under fermentative and stressing conditions. *J Appl Microbiol* **116**:60-70 (2014).

- 65. Pfliegler WP, Pusztahelyi T and Pocsi I, Mycotoxins-prevention and decontamination by yeasts. *J Basic Microbiol* **55**:805-813 (2015).
- 66. Popiel D, Koczyk G, Dawidziuk A, Gromadzka K, Blaszczyk L and Chelkowski J, Zearalenone lactone hydrolase activity in *Hypocreales* and its evolutionary relationships within the epoxide hydrolase subset of a/b-hydrolases. *BMC Microbiol* 14:82 DOI: doi:10.1186/1471-2180-14-82 (2014).
- 67. Prietto L, Moraes PS, Kraus RB, Meneghetti V, Fagundes CAA and Furlong EB, Postharvest operations and aflatoxin levels in rice (*Oryza sativa*). *Crop Protection* 78:172-177 (2015).
- 68. Rahmani A, Jinap S and Soleimany F, Qualitative and quantitative analysis of mycotoxins. *Compr Rev Food Sci Food Saf* **8**:202-251 (2009).
- 69. Raiola A, Meca G, Mañes J and Ritieni A, Bioaccessibility of Deoxynivalenol and its natural co-occurrence with Ochratoxin A and Aflatoxin B1 in Italian commercial pasta. *Food Chem Toxicol* 50:280 (2012).
- 70. Raiola A, Tenore GC, Manyes L, Meca G and Ritieni A, Risk analysis of main mycotoxins occurring in food for children: An overview. *Food Chem Toxicol* 84:169-180 (2015).
- 71. Ridgway K, Sample preparation for food contaminant analysis. *LC/GC Europe* 25:1-8 (2012).
- 72. Robertson GL, Food packaging Principles and practice. New York, USA:Marcel Dekker, Inc. (1993).

- 73. Saladino F, Luz C, Manyes L, Fernandez-Franz M and Meca G, In vitro antifungal activity of lactic acid bacteria against mycotoxigenic fungi and their application in loaf bread shelf life improvement. *Food Control* **67**:273-277 (2016).
- 74. Santini A, Mycotoxins: An Open Challenge for Food Safety. Toxicol open access 2:103 (2016).
- 75. Scarpari M, Bello C, Pietricola C, Zaccaria M, Bertocchi L, Angelucci A, Ricciardi MR, Scala V, Parroni A, Fabbri AA, Reverberi M, Zjalic S and Fanelli C, Aflatoxin control in maize by *Trametes versicolor*. *Toxins* 6:3426-3437 (2014).
- 76. Schmidt-Heydt M, Stoll D and Geisen R, Fungicides effectively used for growth inhibition of several fungi could induce mycotoxin biosynthesis in toxigenic species. *Int J Food Microbiol* 166:407-412 (2013).
- 77. Seeley JV, Recent advances in flow-controlled multidimensional gas chromatography. *J Chromatogr A* **1255**:24-37 (2012).
- 78. Skendi A, Irakli MN, and Papageorgiou MD, Optimized and validated high-performance liquid chromatography method for the determination of deoxynivalenol and aflatoxins in cereals. *J Sep Sci* **39**:1425–1432 (2016).
- 79. Smiley RD and Draughon FA, Preliminary evidence that degradation of aflatoxin B1 by *Flavobacterium aurantiacum* is enzymatic. *J Food Prot* **63**:415-418 (2000).
- 80. Spanjer MC, Rensen PM and Scholten JM, LC-MS/MS multi-method for mycotoxins after single extraction, with validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs. *Food Addit Contam Part A* **25**:472-89 (2008).

- 81. Stecher G, Jarukamjorn K, Zaborski P, Bakry R, Huck CW and Bonn GK, Evaluation of extraction methods for the simultaneous analysis of simple and macrocyclic trichothecenes. *Talanta* 73:251-257 (2007).
- 82. Sultana B, Naseer R and Nigam P, Utilization of agro-wastes to inhibit aflatoxins synthesis by *Aspergillus parasiticus*: A biotreatment of three cereals for safe long-term storage. *Biores Technol* 197:443-450 (2015).
- 83. Sulyok M, Krska R and Schuhmacher R, Application of an LC–MS/MS based multimycotoxin method for the semi-quantitative determination of mycotoxins occurring in different types of food infected by moulds. *Food Chem* **119**:408-416 (2010).
- 84. Tan H, Hu Y, He J,Wu L, Liao F, Luo B, He Y, Zuo Z, Ren Z, Zhong Z, Peng G and Deng J, Zearalenone degradation by two *Pseudomonas* strains from soil. *Mycotoxin Res* 30:191-196 (2014).
- 85. ten Bosch L, Pfohl K, Avramidis G, Wieneke S, Viöl W and Karlovsky P, Plasma-Based Degradation of Mycotoxins Produced by Fusarium, Aspergillusand Alternaria Species. *Toxins* 9:97 DOI: 10.3390/toxins9030097 (2017).
- 86. Teshima R, Hirai K, Sato M, Ikebuchi H, Ichinoe M, Terao T, Radioimmunoassay of nivalenol in barley. *Appl Environ Microbiol* 56:764-768 (1990).
- 87. Therdthai N, Tanvarakom T, Ritthiruangdej P, and Zhou W, Effect of microwave assisted baking on quality of rice flour bread. *J Food Quality***39**: 245-254 (2016).
- 88. Tibola CS, Cunha Fernandes JM, Guarienti EM and Nicolau M, Distribution of *Fusarium* mycotoxins in wheat milling process. *Food Control* **53**:91-95 (2015).

- 89. Tolosa J, Graziani G, Gaspari A, Chianese D, Ferrer E, Mañes J and Ritieni A, Multi-Mycotoxin Analysis in Durum Wheat Pasta by Liquid Chromatography Coupled to Quadrupole Orbitrap Mass Spectrometry. *Toxins* **9**:59 (2017).
- 90. Topcu A, Bulat T, Wishah R and Boyacı IH, Detoxification of aflatoxin B1 and patulin by *Enterococcus faecium* strains. *Int J Food Microbiol* **139**:202-205 (2010).
- 91. Udomkun P, Wiredu AN, Nagle M, Müller J,Vanlauwe B and Bandyopadhyay R, Innovative technologies to manage aflatoxins in foods and feeds and the profitability of application -A review. *Food Control* **76**:127-138 (2017).
- 92. van Egmond HP, Schothorst RC and Jonker MA, Regulations relating to mycotoxins in food: perspectives in a global and European context. *Anal Bioanal Chem* 389:147–157 (2007).
- 93. Vanhoutte I, Audenaert K and De Gelder L, Biodegradation of mycotoxins: tales from known and unexplored worlds. *Front Microbiol* **7**:561 DOI: 10.3389/fmicb.2016.00561 (2016).
- 94. Vearasilp S, Thobunluepop P, Thanapornpoonpong S, Pawelzik E and von Hörsten D, Radio frequency heating on lipid peroxidation, decreasing oxidative stress and aflatoxin B1 reduction in *Perilla frutescens* L. highland oil seed. *Agric Agricu Sci Proc* 5:177-183(2015).
- 95. Vekiru E, Hametner C, Mitterbauer R, Rechthaler J, Adam G, Schatzmayr G, Krska R and Schuhmacher R, Cleavage of zearalenone by *Trichosporon mycotoxinivorans* to a novel nonestrogenic metabolite. *Appl Environ Microbiol* **76**:2353-2359 (2010).
- 96. Velazhahan R, Vijayanandraj S and Vijayasamundeeswari A, Detoxification of aflatoxins by seed extracts of the medicinal plant, *Trachyspermum ammi* (L.) Sprague ex Turrill-

structural analysis and biological toxicity of degradation product of aflatoxin G1. *Food Control* **21**:719-725 (2010).

- 97. Venkataramana M, Rashmi R,Uppalapati SR, Chandranayaka S,Balakrishna K, Radhika M, Gupta VK, and Batra HV, Development of sandwich dot-ELISA for specific detection of Ochratoxin A and its application on to contaminated cereal grains originating from India. *Front Microbiol* 6:511 (2015).
- Venter AC, Glycerol compositions and solutions. US Patent Application Publication No. 20140106008 A1 (2014).
- 99. Vidal A, Marín S, Ramos AJ, Cano-Sancho G and Sanchis V, Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market. *Food Chem Toxicol* **53**:133-138 (2013).
- 100. Vijayanandraj S, Brinda R, Kannan K, Adhithya R, Vinothini S, Senthil K, Chinta RR, Paranidharan V and Velazhahan R, Detoxification of aflatoxin B1 by an aqueous extract from leaves of *Adhatoda vasica* Nees. *Microbiol Res* 169:294-300 (2014).
- 101. Vučković J, Bodroža-Solarov M, Đura Vujić Đ, Bočarov-Stančić A and Bagi F, Protective effect of hulls on the occurrence of *Alternaria* mycotoxins in spelt wheat. J Science Food Agric **93**:1996-2001 (2013).
- 102. Wagner C, Critical Practicalities in Sampling for Mycotoxins in Feed. *J AOAC Int***98**:301-308 (2015).
- 103. Wang B, Mahoney NE, Pan Z, Khir R, Wu B and Ma H, Effectiveness of pulsed light treatment for degradation and detoxification of aflatoxin B1 and B2 in rough rice and rice bran. *Food Control* **59**:461-467 (2016).

- 104. Wang J, Ogata M, Hirai H and Kawagishi H, Detoxification of aflatoxin B1 by manganese peroxidase from the white-rot fungus *Phanerochaete sordida* YK-624. *FEMS Microbiol Lett* **314**:164-169 (2011).
- 105. Wang W, Lawrence KC, Ni X, Yoon SC, Heitschmidt GW and Feldner P, Near-infrared hyperspectral imaging for detecting Aflatoxin B1 of maize kernels. *Food Control* 51:347-355 (2015).
- 106. Whitehurst RJ and van Oort M, Enzymes in food technology. Wiley-Blackwell, Singapore (2010).
- 107. Wu F, Groopman JD and Pestka JJ, Public health impacts of foodborne mycotoxins. *Ann Rev Food Sci Technol* **5**:351-372 (2014).
- 108. Zhu Y, Hassan YI, Watts C and Zhou T, Innovative technologies for the mitigation of mycotoxins in animal feed and ingredients - A review of recent patents. *Animal Feed Sci Technol* 216:19-29 (2016).
- 109. Zougagh M and Ríos A, Supercritical fluid extraction of macrocyclic lactone mycotoxins in maize fluor samples for rapid amperometric screening and alternative liquid chromatographic method for confirmation. *J Chomatogr A* **177**:50-57 (2008)

## Chapter 2

# Prospecting for Microbes Able to Transform Deoxynivalenol

#### PROSPECTING FOR MICROBES ABLE TO TRANSFORM DEOXYNIVALENOL

#### 2.1 INTRODUCTION

The plant pathogens *Fusarium graminearum* and several other *Fusarium* species infect many crops and cause the destructive disease *Fusarium* Head Blight (FHB) in wheat, barley, and other small grain cereals (Goswami et al., 2006; Yoshida & Nakajima, 2010). These pathogens can contaminate grains with trichothecene mycotoxins such as deoxynivalenol (DON), 15-acetyl deoxynivalenol (15-ADON), 3-acetyl deoxynivalenol (3-ADON), and nivalenol (NIV), causing significant reduction in grain yield and quality (Ito, et al, 2012).

Biological detoxification is a promising approach to mitigate mycotoxin contamination, which involves the use of microorganisms for the biotransformation of mycotoxins into less toxic compounds (Saladino et al, 2016). Mycotoxin degrading microorganisms are promising alternatives to chemical or physical strategies for mitigating mycotoxin contamination (Karlovsky, 2011).

Several efforts have been reported for the isolation of bacterial strains with the ability to degrade or transform DON, and resulting by-products have been sometimes been identified and characterized (Zhou et al, 2008; He et al, 2010). Bacterial detoxification of DON has been reported by two main transformations: 1) de-epoxidation of DON to form the de-epoxy-DON(3epi-DOM-1), generally accomplished by anaerobic bacteria isolated from the digestive tract of various animals (He et al., 2010; Karlovsky, 2011); and 2) oxidation of DON to form 3-keto DON, which sometimes progresses to isomerization forming 3-epi DON ( $3-\beta$ -hydroxy), transformations that have been reported by aerobic bacteria in the genera *Nocardioides* and

52

*Devosia* (Ikunaga et al. 2011; Sato et al. 2012). Deoxynivalenol is 55 times more toxic than the de-epoxy form, DOM-1 (Eriksen et al. 2004).

In some cases, transformation of DON has been described by consortia of microbes, with no clear linkage of the activity to a single community member. For instance, a study under aerobic conditions showed the conversion of DON to DOM-1, by a mixed community of Enterobacteria, including *Serratia*, *Clostridium*, *Citrobacter*, and *Enterococcus*, originating in an agricultural soil. There is a great interest in cloning de-epoxidase genes from these microorganisms to engineer trichothecene detoxification into cereal plants (Islam et al, 2012).

The exploration and characterization of novel bacterial taxa that biotransform DON will lead to a better understanding of the pathways involved in detoxification and the diversity of organisms with the ability to transform DON. These efforts may provide us with more specific choices of the bacterial isolates and the methods to be used in screening for the biotransformation of DON. However, these microbial sources might be limited in their utility for detoxifying contaminated cereals.

The objective of this part of the thesis was to isolate microbes from soil that are capable of biotransforming DON to less-toxic products. Such organisms may serve as a source of enzymes or genes involved in detoxification of mycotoxins, which could be useful for decontaminating agricultural commodities..

53

#### 2.2 MATERIALS AND METHODS

#### 2.2.1 Materials

We used long-term incubations in a minimal medium containing DON and chitin, in order to enrich for taxa capable of metabolizing DON.

#### 2.2.2 Enrichment Media

The minimal medium (MM) used for the enrichment procedure of microbes with slight modifications is that of Kirimura et al. (1999). The medium consisted of (per liter) 1.6 g of Na<sub>2</sub>HPO<sub>4</sub>, 1 g of KH<sub>2</sub>PO<sub>4</sub>, 0.5 g of NaNO<sub>3</sub>, 0.5 g of MgSO<sub>4</sub>·7H2O, 0.5 g of (NH)<sub>2</sub>SO<sub>4</sub>, 0.025 g of CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 mL of trace metals solution (TekNova; per L: 1.5 g of FeCl<sub>2</sub>·4H<sub>2</sub>O, 0.190 g of CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.1 g of MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.07 g of ZnCl<sub>2</sub>, 0.062 g of H<sub>3</sub>BO<sub>3</sub>, 0.036 g of Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.024 g of NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.017 g of CuCl<sub>2</sub>·2H<sub>2</sub>O, and 0.01 g of AlK(SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O), 1 mL of vitamin solution (ATCC; per L: 0.02 g of biotin, 0.02 g of folic acid, 0.05 g of thiamine-HCl, 0.05 g of riboflavin, 0.010 mg of pyridoxine-HCl, 0.050 g of cyanocobalamin, 0.05 g of niacin, 0.05 g of Ca-pantothenate, 0.05 g of p-aminobenzoate, and 0.05 g of thiolactic acid), and supplemented with 1% (w/v) chitin flakes. After autoclaving, DON (50mg/L) was added into the media.

#### 2.2.3 Development of Enrichment Cultures for DON Degradation

The source of microorganisms for initiating enrichment cultures was a soil sample collected from an experimental wheat field at the National Center for Agricultural Utilization Research in Peoria, Illinois, USA. A 100-fold dilution was prepared by suspending 20 g of soil in 180 mL of water and then further diluting 1 mL of this suspension in 9 mL of water.

Chapter 2

Enrichment cultures were established in two different culture vessels and with an initial selection by one of two different antibiotics. Two cultures were established in flat bottomed spinner flasks (ChemGlass Life Sciences; 100 mL volume) and were incubated on stir plates at room temperature (fluctuating between 20-23 °C) (fig. 1a). Two additional cultures were established in baffled culture flasks (50 mL culture in 125 mL capacity flasks) and incubated on a shaker table (200 RPM, 28 °C) (fig. 1b). Spinner flask cultures were seeded with 1 mL of soil dilution and initial selection was imposed with 0.5 mL of a 1% solution of either aztreonam (activity against Gram-negative bacteria) or vancomycin (activity against Gram-positive bacteria). Baffled flask cultures received equivalent doses, adjusted for culture size (i.e., 0.5 mL soil dilution and 0.25 mL antibiotic solution).

Enrichment cultures were sampled every 7 days for analysis of DON content and for microbiome profiling. At each sampling, duplicate 1.5 mL subsamples were collected to microcentrifuge tubes and cells plus chitin flakes were pelleted. Supernatant (1.4 mL) was withdrawn for DON analysis, while the pellets were frozen for microbiome analysis. Additional aliquots were collected to cryovials containing glycerol, and were stored at -80 °C. Fresh media (MM + chitin + DON; no additional antibiotics) was added to replace the volume withdrawn during sampling. Immediately after mixing of cultures with replacement media, duplicate 1.4 mL aliquots (as above) were collected, representing the reference concentration against which reduction in DON content over the next 7 day incubation period was assessed. This process of sampling, incubation and analysis was carried out for eight weeks.



a) Baffled flask



b) Spin flask

### Fig. 1: Enrichment cultures established in a) baffled flasks (50 mL culture in 125 mL capacity flasks) and b) flat bottomed spinner flasks (100 mL volume)

#### 2.2.4 Extraction and Analysis of DON

Deoxynivalenol (DON) was extracted from 1.4 mL aliquots of culture supernatant with 8.6 mL of acetonitrile and mixed on vortexer for 5 s. The samples were centrifuged at 4000 rpm for 5 min and 2 mL of the resultant extract was transferred to 1 dram vial to dry down completely under a stream of nitrogen on a heating block (55 °C). Trimethylsilyl (TMS) derivitization was carried out by preparing TMS reagent N-trimethylsilylimadazole and trimethylchorosilane (100:1; Sigma-Aldrich) and 100  $\mu$ L of it was added in each 1-dram vial containing the sample. The samples were vortexed to mix and allowed to incubate at room temperature for 30 min. 900  $\mu$ L of isooctane was added in each sample, followed by 1 mL of distilled water. Vials were gently vortexed until the top, organic layer became clear. The organic layer was transferred into GC vials and TMS-derivatized DON was analyzed by gas chromatography-mass spectrometry (GC-MS).

The gas chromatograph (Agilent 7890) was fitted with a HP-5MS column (30 m length x 0.25 mm internal diameter x 0.25  $\mu$ m film thickness) and was connected to quadrupole mass spectrometer (Agilent 5977). The column was held at 250 °C at injection and the column flow rate was 1mL/min. The initial temperature of 150 °C was held for 1 min and then increased to 280 °C at a rate of 30 °C/min and held for 3.5 min. Selective ion monitoring (SIM) was applied to detect the characteristic ions of tri TMS-DON with fragments ion (m/z value) of 235.1 as the target ion and 259.1, 295.1, 392.2, 422.2, and 512.2 as reference ions. The detection limit with this method was 0.025  $\mu$ g DON.

#### 2.2.5 Microbial population diversity in enrichment cultures

DNA was extracted from enrichment culture cell pellets using the Quick-DNA Fungal/Bacterial 96 Kit (Zymo Research). To profile the community composition of enrichment cultures, amplicon libraries were prepared for sequencing. The V4 region of the bacterial 16S-rRNA gene was amplified using primers 515F and 806R (Caporaso et al. 2011). The second internal transcribed spacer (ITS2) region of fungal rRNA genes was amplified using primers ITS3\_KYO2 and ITS4\_KYO3 (Toju et al. 2012). Primers were modified with 5' overhangs for the compatibility with the MiSeq workflow, and to create a frameshifted mixture of oligos to provide signal diversity within each cycle of sequencing through the primer regions.

The reaction mixture for amplicon generation consisted of 0.5 U Phusion High Fidelity DNA polymerase with associated Phusion Green HF reaction buffer (Thermo Fisher), dNTPs at 200  $\mu$ M final concentration, 0.5  $\mu$ M of each forward and reverse primers, 2.5  $\mu$ L DNA template and nuclease free water to a total volume of 25  $\mu$ L per reaction. Thermocycling consisted of 98 °C for 30 s, 25 cycles of (98 °C for 10 s, 57 °C for 30 s, 72 °C for 15 sec), final extension at 72 °C for 5 min. PCR products were cleaned using the SequalPrep Normalization Plate Kit (Thermo

Fisher) and amplicons were sent for sequencing. Amplicon sequences and resulting taxon abundance tables were processes with Mothur v.1.40.5, and R v. 3.4.

#### 2.2.6 Isolation of Individual DON Degrading Microbes

1 mL of growing enrichment culture (8<sup>th</sup> week of incubation) was taken and transferred into a flask containing 100 mL of fresh minimal media containing DON, chitin, and serial dilutions of enrichment cultures were prepared and incubated under same conditions to produce secondary enrichment cultures. After 14 days of incubation, the cultures were assessed for reduction in DON content. The most dilute culture showing complete loss of DON was selected. The same dilution procedure was repeated, in an attempt to reduce the microbial diversity, resulting in tertiary enrichment cultures. The most dilute tertiary enrichment culture showing loss of DON was selected for isolations. One hundred  $\mu$ L of culture broth was spread on TGY (tryptone-glucose-yeast extract) agar plates, and incubated for two days. Morphologically different colonies were picked and re-inoculated on TGYA media. Purified single colonies were tested for DON (28°C, 14 days). All pure cultures that demonstrated a reduction in concentration of DON were selected for identification and stored as glycerol stocks at -80°C.

#### 2.2.7 Phylogenetic Identification of Isolated Microbes

Individual microbes that demonstrated decreased levels of DON were selected and genomic DNA was extracted using the DNEasy UltraClean Microbial kit (Qiagen). To obtain 16S-rRNA marker gene sequences, universal bacterial primers 27f (5'-GAGTTTGATCCTGGCTCAG) and 1492r (5'-AGAAAGGAGGTGATCCAGCC) were used. Amplification was carried out in a Gene-Amp PCR System (Applied Biosystems). PCR was performed in a total reaction volume of

25 µl, which contained 2 µl of each reverse and forward primer (10 µM stock concentration), 12.5 µl of GoTaq Master Mix (2x) (Promega) and 2 µl of DNA template. The PCR program included the following steps: 94°C (5 min); 30 cycles of 94°C (30 s), 56°C (1 min), 72°C (1 min 30 s); and 72°C (5 min) extension step. After purification of the PCR products using ExoSAP-IT (Affymetrix) PCR purification kit, the purified PCR product was subjected to sequencing reaction using each primer and BDV 3.1 dye terminator kit (ThermoFisher Scientific). The nucleotide sequences were determined with a sequencer (Applied Biosystems 3730 DNA Analyzer). The nucleotide sequence similarities of the amplified region were performed with BLAST program available at the National Centre for Biotechnology Information website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Chapter 2

#### 2.3 RESULTS AND DISCUSSION

#### 2.3.1 Enrichment of DON Degrading Cultures from Soil

The application of different enrichment strategies is being adopted due to the complexity of microbial communities in the environmental samples (Zhu et al, 2017). Enrichment strategies differ based on mycotoxins to be studied, possible transformation pathway and microbiota source. The aim of our enrichment cultures was to create conditions in which organisms able to metabolize DON could increase in abundance, which assists in achieving further isolations (He et al, 2016). Successful transformation of DON by our enrichment cultures demonstrates that the ability to metabolize DON exists within the soil microbial community that was used to inoculate our cultures.

Given the complexity of soil microbial communities, however, the next challenge is to identify the particular community member(s) that carry the ability to metabolize DON. Exposure to combinations of different or individual antibiotics has been reported in many studies as a useful approach for screening of functional microorganisms based on different antimicrobial spectra (Benedetti et al, 2006; Islaam et al, 2012). In one study of DON biotransformation by a bacterial consortium, selection was imposed through many combinations of ten different antibiotics at varying concentrations. This study demonstrated the combination of three antibiotics (salinomycin, virginiamycin, and lincomycin) were able to significantly reduce microbial community complexity without a loss of the DON degradation activity (He et al, 2010). Similarly, our study also suggests the utility of applying different antibiotics for screening of potentially functional microbiota from the soil.

60

#### 2.3.2 Analysis of DON Degradation Ability of Enrichment Cultures

GC-MS analysis of enrichment culture broth revealed complete transformation of DON after three weeks of incubation in either a baffled flask (fig. 2) or a spinner flask (fig. 3), for cultures that had been treated with the antibiotic aztreonam. However, DON degradation activity was not observed in samples that had been exposed to vancomycin (data not shown). These results demonstrated the possibility of utilizing antibiotics for screening of potentially functional bacterial strains involved in biotransformation of DON into new metabolites.

Enrichment culture broths were analyzed weekly for DON content and the presence of potential metabolites of DON. Each week, a portion of the culture media was consistently replaced by fresh minimal media containing 50  $\mu$ g/mL of DON. Weekly GC-MS analysis demonstrated a consistent transformation of DON under both conditions, baffled flask (fig. 4a) and spinner flask (fig. 4b).

DON is a chemically very stable compound, yet it does not show much accumulation in agricultural soils, suggesting that DON is being degraded by soil microorganisms (Karlovsky, 2011). Strategies are needed to eliminate or detoxify DON in animal feed and the human food chain. Several reports have been made of DON modification by anaerobic organisms (He et al., 2010; Karlovsky, 2011). However, anaerobic activity restricts the variety of potential uses of these microbes for industrial applications. Discovery of aerobic detoxification reactions would be useful for a different set of applications. Various studies have been done to isolate microorganisms from the soil that possess DON degradation activity. For instance, conversion of DON to de-epoxy DON has been investigated (He et al, 2016). In this study, we have extended the previous studies by developing enrichment cultures for DON biotransformation and characterizing the microbes involved in DON degradation.

We also characterize the DON degradation activity of Gram-positive and Gram-negative bacteria; it is interesting that the Gram-negative bacteria exhibited no DON degradation activity. We assume that the Gram-negative bacteria are not responsible for the DON degradation activity, suggesting Gram-positive bacteria may have some regulatory system for DON degradation. All previously studied anaerobic DON degrading bacteria belong to Gram-positive genera (*Bacillus, Eubacteria, Anaerofilum, Cllinsella*) (Yu et al, 2010).

GC-MS profiling suggested that different pathways may be involved in the degradation of DON under different incubation conditions. GC-MS scans from the baffled flask enrichment culture showed the appearance of one peak with a similar mass/charge ratio as DON, but a different retention time (6.451 vs. 6.294 for DON) (fig. 2), suggesting some modification to the structure of the molecule that altered its behavior within the GC column. On the other hand, GC-MS profiles of the spinner flask enrichment culture demonstrated two peaks, one with a retention time of 6.451 and another peak at 6.305 (fig.3). The formation of an additional transformation product by the spinner flaks enrichment culture, which was not observed in the baffled flask enrichment culture, suggests either a change in gene expression by the community member(s) responsible for DON transformation, or a different community structure under the two incubation conditions. In a previous study, it has been reported that 3-keto-DON may be further reduced to 3-epi-DON (Karlovsky, 2011). Our work extends the previous studies that have highlighted the changes in microbial assemblages before, during and after DON degradation in enrichment cultures.



**Fig. 2**: GC-MS chromatograms (week 1-8) of extracts from culture broth for an enrichment culture growing in a baffled flask. DON (week 1-2, retention time of 6.294 min) was biotransformed to a DON derivative (week 3-8, retention time of 6.451 min).

Chapter 2

Prospecting For Microbes Able To Transform Deoxynivalenol



**Fig. 3**: GC-MS chromatograms (week 1-8) of extracts from culture broth for an enrichment culture growing in a spinner flask, showing a different mechanism of biotransformation. DON (week 1-2, retention time of 6.294 min) was biotransformed to two different DON derivatives (week 3-4, retention time of 6.451 min; and week 5-8, retention time of 6.305 min).



Fig. 4: Transformation of DON by enrichment cultures; a) baffled flask, b) spinner flask.

DON transformation was observed by the second week of incubation and complete transformation was observed after three weeks of incubation. Transformation activity persisted through week 8. The graphical representation of GC-MS data illustrates that enrichment cultures remained consistent for their biotransformation activity.

#### 2.3.3 Microbiome Profiling of Enrichment Cultures

Results from microbiome profiling of enrichment cultures that consistently modified DON indicated a progressive simplification of the microbial communities over time (week 1 to 8). Microbiome profile from week one cultures (fig. 5a), indicated the presence of mostly members of *Chitinophaga*, followed by *Reyranella*, Rhizobiaceae, *Bosea*, and Pseudomonadaceae. Bacterial communities could be clearly differentiated by time of incubation, as by week 8 *Chitinophaga* (99%) were found to be most abundant member of the bacterial community, nearly eliminating all other community members which were initially present in enrichment cultures (fig. 5b).

To our knowledge, *Chitinophaga* have not been reported previously for DON degradation in cultures. Strains of *Pseudomonas* have been reported for modification of different mycotoxins such as aflatoxin degradation by *Pseudomonas aeruginosa* (Sangare et al, 2014) and biotransformation of DON to de-epoxy-DON (He et al, 2016). However, there is also the possibility that co-metabolism by several microorganisms may be required for the conversion of DON into other metabolites.



Fig. 5: Taxonomic summary of bacteria in enrichment cultures a) after one week of incubation, and b) after 8 weeks of incubation. Different colors represent different taxa, with divisions towards the perimeter reflecting more narrowly defined taxonomic ranks.

#### 2.3.4 Isolation of Individual DON Modifying DON Modifying Microbes

Once an enrichment culture containing the DON degrading microflora was obtained, further screening to isolate single pure bacterial colonies was performed. Microbiome profiling of the enrichment cultures had indicated the presence of *Chitinophaga* (99%), followed by Proteobacteria. We were able to isolate the members of the *Chitinophaga* (*Chitinophaga rhizosphaerae*, *Chitinophaga eiseniae*), Proteobacteria (*Acidovorax* sp, *Stenotrophomonas maltophilia*), Dyadobacter (*Dyadobacter fermentans*), Bacillaceae (*Bacillus oceanisediminis*, *Bacillus firmus*) and Actinobacteria (*Arthrobacter* sp, *Microbacterium* sp, and *Rothia terrae*).

We evaluated the DON transformation activity of isolated pure cultures in minimal media containing the 50  $\mu$ g/mL of DON (28°C, 14 days). Among all the tested pure strains, we were unable to isolate a pure culture that transformed the DON completely in culture media. However, five isolated pure strains have shown their activity for DON degradation. *Chitinophaga rhizosphaerae* and*Arthrobacter aurescens* have indicated the highest degradation of DON (shown by reduction in DON peak area) as compared to other three strains (*Arthrobacter* sp, *Microbacterium* sp, and Acidovorax sp) (fig. 6).

In this study, the enrichment cultures were successfully developed from the soil samples for the complete DON transformation. From the initial screening of these enrichment cultures, we were able to isolate few bacterial strains, which were consistent in reducing the DON from the culture media, but none of them was found to show complete conversion. Völkl et al, (2004) were also unable to isolate a pure strain from the enrichment cultures that was consistently involved in transformation of DON.

Screening of DON modifying microbes is very critical; as multiple microorganisms could be responsible for the transformation of DON, or due to growth restrictions since some microbes could be inhibited by others (Yu et al, 2010).

Isolation of a pure strain from the enrichment cultures always remains challenging. Additional work needs to be done on the physiological characteristics of the culture media to improve the culturability of DON modifying strains (Ikunaga et al, 2011; Ito et al, 2012). Efforts such as choosing media with different carbon sources, extending the incubation time for slow growing bacteria and changing the media-solidifying agent, as reported, that rather than agar, gellan gum is better for soil based bacteria (Janssen et al, 2002). Additional testing and analysis by adopting different strategies of screening can increase the probability of identifying the pure strain that was responsible for DON transformation, or the possibility of co-metabolism in the enrichment cultures.



**Fig.6:** Reduction in DON peak area by individual bacterial colonies isolated from the enrichment cultures (8<sup>th</sup> week), measured by GC/MS after 14 days of incubation in culture media containing

DON.

#### 2.4 CONCLUSION

Microbial detoxification is an important strategy to eliminate the mycotoxins from the food and feed chain. Microoragasims that can biotransform mycotoxins to less toxic metabolities serve as biological control agents in agricultural commodities. Enrichment cultures from the soil samples were successfully developed for DON transformation activity. Due to the complexity of microbial communities in the environmental samples, we adopted the application of different enrichment strategies such as the treatment with different antibiotics. GC-MS scans from the soil samples after the  $3^{rd}$  week of inoculation.

The enrichment cultures were incubated for longer period of time (8<sup>th</sup> week) and analysed for their consistent DON transforming activity. As expected, the reduction in microbial complexity was observed from 1<sup>st</sup> to 8<sup>th</sup> week of incubation, revealed by microbiome profiling. Screening of single pure isolate from the enrichment culture is always challenging, we were unable to isolate a pure strain responsible for the complete conversion of DON in culture media. However, some of the isolated pure strains have shown their partial activity for DON degradation.

Our work extends previous studies that have demonstrated the potential to use mixed cultures to detoxify the mycotoxins. However, additional work needs to be done for the screening of specific microoragasims that are unable to grow under examined conditions. As future perspective; it could be interesting to apply different stratagies to increase the probability of isolating an organism that is responsible for DON transformation. Finally, a better understanding of co-metabolism of different microbes in the enrichment culture could be fascinating to explore.

#### 2.5 **REFERENCES**

- 1. Benedetti R, Nazzi F, Locci R, and Firrao G, Degradation of fumonisin B1 by a bacterial strain isolated from soil. *Biodegradation*, **17**: 31-38 (2006).
- De Bellis P, Tristezza M, Haidukowski M, Fanelli F, Sisto A, Mulè G, and Grieco F, Biodegradation of Ochratoxin A by Bacterial Strains Isolated from Vineyard Soils. *Toxins*, 7: 5079-5093 (2015).
- Eriksen GS, Pettersson H, and Lundh T, Comparative cytotoxicity of deoxynivalenol, nivalenol, their acetylated derivatives and de-epoxy metabolites. *Food Chem Toxicol*, 42: 619-624 (2004).
- Goswami RS, Xu JR, Trail F, Hilburn K, and Kistler HC, Genomic analysis of hostpathogen interaction between Fusarium graminearum and wheat during early stages of disease development. *Microbiology*, 152: 1877-1890 (2006).
- He J, and Zhou T, Patented techniques for detoxification of mycotoxins in feeds and food matrices. *Recent Pat Food Nutr Agric*, 2: 96-104 (2010).
- He WJ, Yuan QS, Zhang YB, Guo MW, Gong AD, Zhang JB, Wu AB, Huang T, Qu B, and Li HP, Aerobic De-Epoxydation of Trichothecene Mycotoxins by a Soil Bacterial Consortium Isolated Using In Situ Soil Enrichment. *Toxins*, 8: E277 (2016).
- Ikunaga Y, Sato I, Grond S, Numaziri N, Yoshida S, Yamaya H, Hiradate S, Hasegawa M, Toshima H, Koitabashi M, Nocardioides sp. strain WSN05-2, isolated from a wheat field, degrades deoxynivalenol, producing the novel intermediate 3-epi-deoxynivalenol. *Appl Microbiol Biotechnol* 89: 419-427 (2011).
- Islam R, Zhou T, Young JC, Goodwin PH, and Pauls KP, Aerobic and anaerobic deepoxydation of mycotoxin deoxynivalenol by bacteria originating from agricultural soil. *World J Microbiol Biotechnol*, 28: 7-13 (2012).
- Ito, M.; Sato, I.; Koitabashi, M.; Yoshida, S.; Imai, M.; Tsushima, S. A novel actinomycete derived from wheat heads degrades deoxynivalenol in the grain of wheat and barley affected by Fusarium head blight. Appl. Microbiol. Biotechnol. 2012, 96, 1059–1070.
- Janssen, P.H.; Yates, P.S.; Grinton, B.E.; Taylor, P.M.; Sait, M. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. Appl. Environ. Microbiol. 2002, 68, 2391–2396.
- Karlovsky P, Biological detoxification of the mycotoxin deoxynivalenol and its use in genetically engineered crops and feed additives. *Appl Microbiol Biotechnol*, **91**: 491-504 (2011).
- 12. Kirimura K, Nakagawa H, Tsuji K, Matsuda K, Kurane R, and Usami S, Selective and continuous degradation of carbazole contained in petroleum oil by resting cells of Sphingomonas sp. CDH-7. *Biosci Biotechnol Biochem*, **63**: 1563-1568 (1999).
- 13. Saladino F, Luz C, Manyes L, Fernandez-Franz M and Meca G, In vitro antifungal activity of lactic acid bacteria against mycotoxigenic fungi and their application in loaf bread shelf life improvement. *Food Control* **67**:273-277 (2016).
- 14. Sangare L, Zhao Y, Folly YME, Chang J, Li J, Selvaraj JN, Xing F, Zhou L, Wang Y, and Liu Y, Aflatoxin B1 degradation by a Pseudomonas strain. *Toxins*, 6: 3028-3040 (2014).

- 15. Sato I, Ito M, Ishizaka M, Ikunaga Y, Sato Y, and Yoshida S, Thirteen novel deoxynivalenol-degrading bacteria are classified within two genera with distinct degradation mechanisms. *FEMS Microbiol Lett*, **327**: 110-117 (2012).
- Völkl A, Vogle B, Schollenberger M, and Karlovsky P, Microbial detoxification of mycotoxin deoxynivalenol. *J Basic Microbiol*, 44: 147-156 (2004).
- Yoshida M, and Nakajima T, Deoxynivalenol and nivalenol accumulation in wheat infected with Fusarium graminearum during grain development.*Phytopathology*, **100**: 763-773 (2010).
- 18. Yu H, Zhou T, Gong J, Young C, Su X, Li X, Zhu H, Tsao R, and Yang R, Isolation of deoxynivalenol-transforming bacteria from the chicken intestines using the approach of PCR-DGGE guided microbial selection. *BMC Microbiol*, **10**: 182 (2010).
- Zhou T, He J, and Gong J, Microbial transformation of trichothecene mycotoxins. *World Mycotoxin J*, 1: 23-30 (2008).
- 20. Zhu Y, Hassan YI, Lepp D, Shao S, and Zhou T, Strategies and Methodologies for Developing Microbial Detoxification Systems to Mitigate Mycotoxins. *Toxins*, **9**: 130 (2017).

# Chapter 3

# Transformation of Deoxynivalenol by Laccase-Mediated System

Chapter 3

#### TRANSFORMATION OF DEOXYNIVALENOL BY LACCASE-MEDIATED SYSTEM

### 3.1 INTRODUCTION

Enzymatic degradation is potentially suitable for individual mycotoxins, due to expected catalytic specificity of enzymes. However, some enzymes such as laccases (LCs) have broader spectrum activities that may be attributable to the formation of reactive intermediates with strong oxidoreductive properties. Such enzymes may be useful for the simultaneous degradation of multiple mycotoxins. Enzymatic bioremediation of mycotoxins has been recently reviewed (Loi et al 2017).

Laccases (LCs) are a family of multicopper oxidases that typically contain four cupric ions, and are classified into three distinct spectroscopic types (T1, T2, and T3) (Pardo and Camarero, 2015; Mate and Alcalde, 2016). Laccases have been shown to catalyze aromatic amines, oxidation of phenols, and other non-phenolic compounds by means of reduction of molecular oxygen into water. The activity of laccases in reacting with a range of different substrates can be extended by forming a Laccase Mediator System (LMS) in which the enzyme is combined with a redox mediator (Zucca, 2015). In LMS, several compounds have been widely used as redox mediators in many biocatalytic processes. These include 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), 1-hydroxybenzotriazole (HBT), and 2,2-azino-bis-[3-ethylbenzo-thiazolin-sulfonate] (ABTS) (Camarero et al, 2005; Moldes et al, 2008).

LCs are being used in food industries (i.e., baking, fermentation, dairy processing) for multiple purposes, such as improvement in the techno-functional properties during food processing, modification of food sensory parameters and to improve product shelf-life (Pezzella, 2015). Moreover, laccases have also been applied in industries for purposes of bioremediation, chemical synthesis, pharmaceutical and cosmetic manufacturing (Zucca P, 2015). The degradation of some mycotoxins, such as aflatoxins (AFM1, AFB1), and zearalenone (ZEN), by laccases has been reported (Banu et al, 2013; Loi et al, 2016, 2017).

Trichothecenes are a group of sesquiterpenoid mycotoxins, produced by several different fungi belonging to the genus *Fusarium* (Scott, PM 1990). One of the most economically devastating trichothecenes in wheat, barley and maize production is deoxynivalenol (DON) (McCormick, 2011). DON, also known as vomitoxin, causes diarrhea, abdominal pain, vomiting, skin irritation (Pestka, 2010) and immunosuppressive effects (Sobrova, 2010), depending on the time and dose of exposure. DON is generally characterized by a keto functional group on C-8 (Kimura M, 2007; McCormick et al., 2011; Alizadeh, 2016), and is commonly produced by *Fusarium graminearum* and *Fusarium culmorum* (Desjardins AE, 2006). These fungal pathogens are the casual agents of the destructive disease Fusarium head blight (FHB) (McMullen et al., 2012), which leads to tremendous loss in yield and quality of cereal grains and ultimately poses a great threat to food safety and public health (Wagacha and Muthomi, 2008). DON contamination is a crucial issue in major cereal crops, so proper control strategies are required to mitigate economic losses and to ensure food safety.

In this part of the thesis, the application of a commercially available laccase for the chemical modification of deoxynivalenol (DON) is studied, by testing tested a range of enzymes and mediators to develop a Laccase Mediated System (LMS). Laccase from *Trametes versicolor*, coupled with 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) as a mediator, achieves complete transformation of DON. Two transformation products were generated by this LMS, and were assessed by gas chromatography mass spectrometry (GC/MS) and liquid chromatography mass spectrometry (LC/MS).

# 3.2 MATERIALS AND METHODS

#### **3.2.1** Chemicals and Reagents

TEMPO free radical (TEMPO) and phenolphthalein (PPt) were obtained from Aldrich (Steinheim, Germany), syringaldazine (SYRaz) and 3-hydroxyanthranilic acid (HAA) were obtained from SAFC (St. Louis, MO), 2,2-azino-bis (3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt (ABTS) and laccase from *Trametes versicolor* 53739 were obtained from Fluka (Switzerland). Column chromatography used Silica gel 70-230 mesh, 60 Å for column chromatography (Sigma-Aldrich, St. Louis, MO), methylene chloride (Fischer, Fair Lawn, NJ), and methanol (Millipore, Billerica MA).

#### 3.2.2 Determination of Optimum Conditions for Laccase Activity

The optimum pH for laccase preparation was measured by using a Sigma enzymatic assay for Laccase procedure EC 1.10.3.2 with some modifications. Assays were performed in triplicate 100  $\mu$ L volumes in polystyrene assay plates (96-well). Assays consisted of an appropriate amount of enzyme, 10 % (v/v) methanol, McIlvaine buffer (sodium phosphate dibasic 0.2 M and citric acid monohydrate 0.1 M) constituted to yield different pH values, and 0.02 mM syringaldazine. Laccase activity was assessed as oxidation of syringaldazine, and measured using a plate reader (Molecular Devices, Sunnyvale, CA) at 530 nm for 5 minutes. Oxidation of syringaldazine was evident as an increase in absorbance.

Laccase activity was also evaluated using ABTS, with the reaction mixture prepared in McIlvaine buffer at the optimum pH, with 180  $\mu$ L of ABTS (1 mM) and 20  $\mu$ L of enzyme solution. Oxidation of ABTS was assessed as an increase in absorbance at 420 nm. The optimum

temperature for enzyme activity was assessed using 1 mL of ABTS (1 mM) and McIlvaine buffer under a temperature range of 25-80 °C (Larson et al, 2012).

#### 3.2.3 Degradation of Deoxynivalenol in the Laccase Mediator System

Assays for laccase-mediated transformation of deoxynivalenol (DON) were performed in triplicate in 1.5 mL microfuge tubes. Reaction mixtures consisted of 10 mg/mL laccase, 6.5 mM TEMPO dissolved in McIlvaine buffer at pH 5 (optimum pH), and 5 mg/mL DON. Reactions were set up by adding 30  $\mu$ l of DON (5 mg/ml) into tubes followed by the addition of 150  $\mu$ l of TEMPO (6.5 mM) and 20  $\mu$ l of laccase (10 mg/ml) and incubated at 28 °C, 200 rpm for 72 h. Control samples containing DON but no laccase or no mediator were incubated under the same conditions. Reactions were stopped by the addition of an equal volume (200  $\mu$ L) of acetonitrile, and were transferred to 1 dram vials. The reactions were dried under nitrogen gas (N<sub>2</sub>) at 55 °C. Samples were analyzed by GC/MS after 24, 48, and 72 h of incubation. In some cases, 15-acetyl-deoxynivalenol (15-ADON) or 3-acetyl-deoxynivalenol (3-ADON) was substituted for DON.

## 3.2.4 GC/MS Analysis of Deoxynivalenol and its Transformation Products

Deoxynivalenol and its transformation products were recovered by adding 500  $\mu$ L of methanol to each vial, mixing vigorously and transferring into GC vials. GC-MS analyses were performed with a gas chromatograph (Hewlett Packard 6890) fitted with a HP-5MS column (30 m length × 0.25 mm internal diameter × 0.25  $\mu$ m film thickness) and a mass detector (HP 5973). The carrier gas was helium with 20:1 split ratio and a flow rate of 20 mL/min. Samples were injected at 120 °C, the temperature was held for 1 min and then the column was heated to 260 °C at a rate of 20 °C/min and held for 15 min. The presence of DON and its transformation products was examined by their individual peaks on chromatograms, with the corresponding mass spectra.

#### 3.2.5 LC/MS Analysis of Deoxynivalenol

Deoxynivalenol and its metabolities were recovered by adding 500  $\mu$ L of methanol-water (86:14, mixed vigorously and transferred into LC vials for analysis. Analyses were performed with a LC-MS instrument (Dionex UltiMate 3000 UPLC) attached to a Thermo QExactive mass spectrometer. The MS was operated utilizing electrospray ionization and set to detect ions in full scan high resolution mode (resolution = 70,000) over the mass range 100-1000. For each sample two different LC-MS experiments were performed. Both methods involved separation of compounds on a Phenomenex Kinetex F5 column (150 mm L x 2.1 mm dia, 1.7 uM particle size, 100A pore dia.) with gradient elution using water / methanol (0.2 mL/min, 15-95% methanol over 15 min) containing 5 mM ammonium acetate. The first method was done with the MS set to detect negativelycharged ions, while the second method was done with the MS set to detect positively charged ions.

#### 3.2.6 Collection and Characterization of DON Transformation Products

To isolate sufficient quantities of the DON transformation products for further characterization, we scaled up the enzymatic reactions under the same conditions. Specifically, 90  $\mu$ L of DON (5 mg/ml) was added into microfuge tubes, followed by the addition of 450  $\mu$ L of TEMPO (6.5 mM) and 60  $\mu$ L of laccase (10 mg/ml) in McIlvaine buffer at pH 5. Reactions were incubated at 28°C, 200 rpm for 72 h. Reactions were stopped by the addition of an equal volume (600  $\mu$ L) of acetonitrile. Individual reactions were combined and concentrated under nitrogen gas (N<sub>2</sub>). The dried product was dissolved in methanol and collected into new vials, leaving behind insoluble components. Constituents were separated on a silica gel column (2.54 cm diameter; 22 cm long) and eluted with methanol-dichloromethane (5:95). Fractions (15 mL) were collected and monitored by GC-MS. Fractions 4-6 contained (metabolite 1) and fractions 7-9 contained

(metabolite 2). Fractions carrying the same compound were combined and further purified on a Sephadex LH-2 (Sigma, St. Louis MO) column (2.54 cm diameter, 22 cm long) and eluted with methanol.

Chapter 3

# 3.3 RESULTS AND DISCUSSION

#### **3.3.1** Optimization of Conditions for Enzyme Assays

The optimum conditions for enzyme activity were examined at a varying range of pH (pH 2.5-8.0) and temperature (25-80 °C). The optimum pH of the enzymes was determined by the oxidation of syringaldazine in McIlvaine's buffer. All of the enzymes tested were found to be consistent in the pH range of 4.5 to 5.5 (Table 1).

The optimum temperature for enzyme activity was assessed using ABTS. The initial activity of all the tested enzymes was retained over a wide range of temperatures, as reported by another study (Larson et al, 2012). Among the tested enzymes, ATCC 11235 and ATCC 20869 had an optimum temperature at 55°C, while the remaining enzymes were still active at 60°C. Enzyme activity was found to be decreased upon a longer time of incubation at higher temperature. Decreasing the reaction temperature from 60°C to 30°C extended the duration of enzyme activity, while a further decrease to 25°C resulted in a slightly lower initial reaction rate.

Generally, we found that Mcllvaine's buffer was suitable for activity of all the enzymes tested. Therefore, we selected Mcllvaine's buffer as a solvent for the reaction mixture, with pH 5.0 at 28°C as the standard conditions for subsequent enzymatic reactions. 

 Table 1: Optimum pH and temperature (°C) activity assays for enzymes from fungal cell extracts and commercial sources.

Enzyme	Source Organism	Preparation	pH <sub>opt</sub>	T <sub>opt</sub> (°C)
ATCC 11235	Trametes versicolor	Cell extract	4.5	55
	ATCC 11235			
ATCC 200478	Pycnoporus cinnabarinus	Cell extract	4.5	60
	ATCC 200478			
ATCC 20869	Trametes versicolor	Cell extract	4.5	55
	ATCC 20869			
Fluka 38429	Trametes versicolor	Commercial	5.5	60
Fluka 53739	Trametes versicolor	Commercial	4.6	60
ASA LacC	Trametes sp.	Commercial	5.4	60

#### 3.3.2 Activity of Various Laccase-Mediator Combinations

Laccase-mediated reactions were performed for the degradation of deoxynivalenol (DON), in a combinatorial fashion with 4 known mediators to evaluate the activity of 6 laccase enzymes, including three fungal crude extracts and three commercial laccases (Table 1). Enzymes were also tested for DON degradation ability without adding any mediator in the reaction. The choice of the mediator is very critical for the success of reaction; all of the tested chemicals have been previously reported as the mediators in many enzyme-mediated reactions (Riva et al, 2006; Larson et al, 2012).

Among reactions without a mediator, only enzymes Fluka 38429 and ASA Laccase C modified appreciable amounts of DON (Fig. 1). Among the tested mediators, TEMPO was found to be the most effective mediator, increasing the activity of all of the tested enzymes. The largest transformation rates (up to 70% reduction in DON peak area as compared to control) were observed in the combinations of Fluka 38429 + TEMPO and ASA Laccase C + TEMPO. PPt did not show considerable activity with any of the tested enzymes, while HAA and ABTS were found to be considerably effective with only one enzyme (Fig. 1). TEMPO and its derivatives have been studied in a series of reactions as a mediator in many biotransformation reactions by laccases from *Trametes versicolor* (IWCE Arends et al, 2006; Kobakhidze et al, 2018), and turned out to be the most effective mediator for our laccase assays.

Although each of the four mediators have been previously reported as laccase-mediators, it is necessary to investigate a variety of laccase-mediated combinations for the optimization of required results. We successfully screened our one of the top reactions with TEMPO as a mediator for further studies with the aim of achieving 100% biotransformation of deoxynivalenol.



**Fig. 1**: Evaluation of DON degradation activity of different laccase mediated assays from commercial sources (Fluka 38429, Fluka 53739, and ASA LacC) and fungal cell extracts (ATCC 11235, ATCC 200478, and ATCC 20869) coupled with four different mediators (ABTS, HAA, PPt and TEMPO).

# 3.3.3 Evaluation of Laccase-Mediator Activity Across a pH Range

We have examined the effect of pH in the reaction mixture to proceed for a more comprehensive study on the complete biotransformation of DON. Specifically, we evaluated the DON-transforming activity of all six enzymes, in combination with TEMPO, at a varying range of pH 4.5, 5.0 and 6.0. We found that across all laccases, DON transformation was most effective at pH 5.0, whereas the reactions at pH 4.5 and 6.0 displayed a slightly lower activity (Fig. 2). Therefore, we selected pH 5.0 for subsequent studies of DON biotransformation.



**Fig. 2**: Evaluation of DON degradation activity of different laccase mediated reactions coupled with TEMPO at varying range of pH 4.5, 5.0 and 6.0

# 3.3.4 Achieving complete transformation of DON

Although we had seen up to 80 % reduction in DON peak area, none of the conditions tested so far had provided 100 % biotransformation of DON. Initial studies were carried out in enzymemediated assays containing 10 mg/mL of DON, which may have exceeded the available enzyme capacity. Thus, we evaluated the DON degradation activity of all the laccase-mediator combinations with different concentrations of DON (1 mg/mL, 5 mg/mL and 10 mg/mL) in the reaction mixture.

Three of the combinations (TEMPO+200478, TEMPO+38429 and TEMPO+53739) demonstrated consistent DON transformation across initial DON concentrations (Fig. 3). We had expected that lowering the DON concentration in the reaction would facilitate the complete biotransformation of deoxynivalenol; hence, 100% biotransformation with 1 mg/mL and 5 mg/mL of DON was observed in these assays (Fig. 3).



**Fig. 3**: Evaluation of DON degradation activity of different laccase mediated reactions coupled with TEMPO against a varying concentration of DON, 1 mg/mL, 5 mg/mL and 10 mg/mL.

# 3.3.5 Characterizing DON Transformation Products

With a laccase-mediator system providing complete transformation of DON, we were ready to identify the transformation products. GC/MS analysis revealed the combination of laccase 53739 with TEMPO as the most interesting combination to examine further because of its metabolities. Thus, a stepwise procedure was investigated with TEMPO+53739 to explore the products of biotransformation.

# 3.3.5.1 Gas Chromatography and Mass Spectroscopy

GC/MS analysis of the laccase-mediator reaction with DON indicated the loss of DON peak completely and the appearance of 2 new peaks (Fig. 4)

The obtained GC/MS profiles revealed clear and different retention times of the newly formed two different metabolites. The typical peak corresponding to DON (retention time 6.29 min) disappeared, and was replaced by peaks at 6.92 min (metabolite 1) and at 5.91 min (metabolite 2). A shift in retention time results from interactions with the packing material in the GC column, and indicates that the parent compound has undergone a chemical change.



**Fig. 4**: Gas chromatograms showing A) DON and B) the replacement of DON by 2 new compounds following incubation with laccase ATCC 53739 + TEMPO.

Mass spectra for these two metabolites were consistent with the oxidation of a hydroxyl group to a keto group (forming metabolite 1), followed by a second oxidation of another hydroxyl group to a keto group (forming metabolite 2); the molecular ion decreased by 2 atomic mass units at each step. DON has a molecular ion at 296, whereas the two new peaks had molecular ions at 294 (metabolite 1) and 292 (metabolite 2).

DON has available hydroxyl groups at positions C3, C7, and C15. Mass spectra indicated a very good match for both metabolite 1 and metabolite 2 to a prior report of chemically-derived DON transformation products. Specifically, ions at m/z 55, 77, 108, 124, 136, 175, 204 and 231 observed for metabolite 1 match ions reported for 3-keto DON, while ions at m/z 123, 124, 175, 204, 221, 231, 245, 263, and 292 for metabolite 2 match ions reported for 3,15-diketo DON (Fig. 5; Savard et al. 1987).

Thus, an initial identification by mass spectroscopy suggests the transformation of DON into 3keto DON and 3,15-diketo DON. Further analyses were performed on the LC/MS for better understanding of the transformation products.



**Fig. 5**: GC/MS chromatographic analysis of DON metabolities. Mass spectra for A) metabolite 1 (3-keto DON) and B) metabolite 2 (3,15-diketo DON)

# 3.3.5.2 Liquid Chromatography and Mass Spectroscopy

The strategy for the identification of unknown biotransformation products is always challenging due to the unknown properties of the products. Thus, the sample needs to be subjected to different conditions for better understanding of the transformation products.

Consistent with the indication by GC/MS of the formation of two transformation products of DON by the activity of laccase 53739 + TEMPO, LC/MS profiling also indicated two transformation products with altered retention times relative to DON (Fig. 6; major peak at 15.8 min and minor peak at 17.26 min).

We viewed these two transformation products with a mass detector, in both negative and positive mode. The molecular ions for the two transformation products differed by two atomic mass units (in positive mode, m/z for M+H = 450 for the peak at 15.89 min, and m/z for M+H = 448 for the peak at 17.26 min), which is consistent with the difference between metabolite 1 and metabolite 2 as indicated by GC/MS.

When viewed in negative mode, the main product (retention time 15.89 min) yielded a mass spectrum with dominant ions at 508 and 308 m/z. Tandem mass spectroscopy demonstrated that the 308 ion was a fragmentation product of the 508 ion ESI-MS/MS; data not shown). Interpretation of this observed molecular mass is not entirely certain, but it appears likely that we were observing a conjugate of 3-keto DON (m.w. = 294) with TEMPO (m.w. = 156) plus an additional acetyl group (m.w. = 59). It was unexpected that TEMPO might continue to be associated with DON even after purification of the reaction products. The specific interaction among the putative DON, TEMPO, and acetyl moieties is unclear. However, when a DON standard was run under these LC/MS conditions, we also observed DON at a larger than

expected mass (m/z = 355), which when fragmented produced an ion with m/z = 59, which is consistent with liberation of an acetyl group (data not shown).



**Fig. 6**: LC/MS chromatogram showing the presence of two transformation products after incubating DON with laccase 53739 + TEMPO: metabolite 1 as a major peak at a retention time of 15.89 min, and metabolite 2 as a minor peak at a retention time of 17.26 min.

When viewed in positive mode, the main product (retention time 15.89 min) yielded a molecular ion at m/z = 450, which is consistent with a 3-keto DON-TEMPO conjugate (294 + 156). The minor product (retention time 17.26 min) yielded a molecular ion at m/z = 448, which is consistent with a 3,15-diketo DON-TEMPO conjugate (292 + 156; Fig. 7).

Fragmentation of both metabolites by the electrospray ionization mass spectrometry (ESI-MS/MS) was performed for the better understanding of the transformation products (Fig 7). The MS/MS spectra for the transformation products has some fragment masses that are similar to those seen in the DON MS/MS spectra (Fig. 8), indicating a shared core structure between the parent compound (DON) and the transformation products.



**Fig. 7**: LC/MS chromatographic analyses of A) DON metabolite 1 and B) DON metabolite 2 are shown. Electrospray ionization mass spectrometry (ESI-MS/MS) of the DON metabolities are illustrated as a small chart within the chromatogra



**Fig. 8**: LC/MS chromatographic analysis of DON is shown. Electrospray ionization mass spectrometry (ESI-MS/MS) of the DON is illustrated as a small chart within the chromatograph.

# **3.3.5.3.** DON Precursors for Protection of Transformed Groups

To further validate the structure of the observed DON transformation products, we repeated the enzyme assays using two trichothecene variants (15-ADON and 3-ADON), each of which are differently substituted (acetyl group in place of hydroxyl group) at one of the positions that was modified by laccase 53739 + TEMPO.

- the presence of the acetyl group at C15 in 15-ADON limited transformation products to just the 3-keto form (Fig. 9)
- the presence of the acetyl group at C3 in 3-ADON limited transformation products to just the 15-keto form (Fig. 10)

Conversion of 3-ADON to 15-keto-3-ADON was incomplete (Fig. 10); this is consistent with the incomplete conversion of DON at the C15 position (i.e., the formation of 2 products rather than going all the way to a single product). It appears that the C15 position is more difficult to transform, compared to the C3 position, perhaps due to steric hindrance.



**Fig. 9**: Gas chromatograms showing A) 15-ADON and B) the formation of 1 new compound following incubation of 15-ADON with laccase 53739 + TEMPO.



**Fig. 10**: Gas chromatograms showing A) 3-ADON and B) the formation of 1 new compound following incubation of 3-ADON with laccase 53739 + TEMPO.



**Fig. 11:** Representation of observed DON transformation products. The inset figure shows the conventional numbering of atom positions for trichothecenes such as DON.

Taken together, the results of GC/MS and LC/MS analyses indicate that our laccase-mediator system completely transformed DON into two metabolites. One of them is 3-keto DON, which constituted the majority of the product (75%) and the other is 3,15-diketo DON, produced in a smaller quantity (25%).

The microbial detoxification of DON by various species involving different enzymes has been widely reviewed. Previous studies on the mechanism of DON transformation have focused on complete mineralization of DON or transformation at the C-3 position (Karlovsky, 2015; Zhu et al, 2016). *Devosia* sp has been reported to epimerize the DON into 3-epi DON (He et al, 2015-16). Epimerization at the C-3 position of DON is a two step process, involving two different enzymes: the first step is the oxidation of DON into 3-keto DON, while in a second step 3-keto DON is reduced to 3-epi DON (Hassan et al, 2017; Carere et al, 2018). The oxidation of DON into 3-keto DON reduces the toxicity (He et al, 2015).

In our study, we have reported a new mechanism of DON transformation in laccase-mediated system involving the transformation of DON into two metabolites, 3-keto DON (75%) which has been reported previously as a part of microbial transformations of DON (He et al, 2016) and another 3,15-diketo DON (25%). To our knowledge, 3,15-diketo DON has not been reported before as a product of enzymatic transformation. Only in one study, 3,15-diketo DON was analyzed as a part of a set of synthetic trichothecenes generated through chemical reactions (Savard et al, 1987). Further studies are needed to explore the toxicity of this product.

# 3.4 CONCLUSION

The enzymatic detoxification of mycotoxins represents a promising approach for remediating contamination of cereals by mycotoxins. In this work, a laccase-mediator system was successfully developed for the enzymatic transformation of deoxynivalenol. Different combinations of laccase enzymes with chemical mediators were evaluated for DON transformation; the most interesting combination of laccase from *Trametes versicolor* 53739 with TEMPO as a mediator was investigated in detail. GC-MS and LC-MS analyses were performed for the identification of DON metabolities and mechanism involved in this system.

The GC/MS and LC/MS profiling has revealed the complete transformation of DON into two metabolites, 3-keto DON (75%) and 3,15-diketo DON (25%). We have explored a new mechanism of DON transformation in laccase-mediated system involving 3-keto DON and 3,15-diketo DON. Among these two metabolites, 3-keto DON has been reported before as the intermediate of epimerization of DON at the C-3 position by biological transformation, whereas, 3,15-diketo DON has not been reported before as a product of enzymatic transformation. However, additional analysis on nuclear magnetic resonance (NMR) needs to be done for the better understanding of mechanism involved and to explore the toxicity of 3,15-diketo DON.

The identification of an enzyme that can oxidize DON to 3-keto DON is a significant step towards the detoxification of DON. The capability to transform the DON to less toxic 3-keto DON has important industrial applications, including fuel ethanol production, livestock production by incorporating the enzymes in their feed, and detoxification of contaminated seeds.

# **3.5 REFERENCES**

- 1. Alizadeh A, Braber S, Akbari P, Garssen J, and Fink-Gremmels J. Deoxynivalenol impairs weight gain and affects markers of gut health after low-dose, short-term exposure of growing pigs. *Toxins*, **7**: 2071-2095 (2015).
- 2. Arends IWCE, Li YX and Sheldon RA, Stabilities and rates in the laccase/TEMPOcatalyzed oxidation of alcohols. *Biocatal Biotransformation*, 24: 6 (2006).
- Banu A, Lupu I, and Aprodu I, Degradation of Zearalenone by laccase enzyme. Biotechnol, 14: 79-84 (2013).
- Camarero S1, Ibarra D, Martínez MJ, Martínez AT.Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Appl Environ Microbiol*, **71**: 1775-84 (2005).
- Carere J, Hassan YI, Lepp D, and Zhou T, The enzymatic detoxification of the mycotoxin deoxynivalenol: identification of DepA from the DON epimerization pathway. *Microb biotechnology*, 11: 1106-1111 (2018).
- Desjardins AE, Fusarium Mycotoxins. Chemistry, Genetics, and Biology, American Phytopathological Society Press, St. Paul, MN (2006).
- Hassan YI, He JW, Perilla N, Tang K, Karlovsky P, and Zhou T, The enzymatic epimerization of deoxynivalenol by *Devosia* mutans proceeds through the formation of 3keto-DON intermediate. *Sci Rep* 7: 6929 (2017).
- 8. He JW, Hassan YI, Perilla N, Li XZ, Boland GJ, and Zhou T, Bacterial epimerization as a route for deoxynivalenol detoxification: the influence of growth and environmental conditions. *Front Microbiol*, **7:** 572 (2016).

- He JW, Yang R, Zhou T, Boland GJ, Scott PM, and Bondy GS, An epimer of deoxynivalenol: purification and structure identification of 3-epi-deoxynivalenol. *Food Addit Contam Part A*, 32: 1523-1530 (2015).
- Kimura M, Tokai T, Takahashi-Ando N, Ohsato S, and Fujimura M, Molecular and genetic studies of *fusarium* trichothecene biosynthesis: pathways, genes, and evolution. *Biosci Biotechnol Bioche*, **71**: 2105-23 (2007).
- Kobakhidze A, Elisashvili V, Corvini PFX, and Čvančarová M, Biotransformation of ritalinic acid by laccase in the presence of mediator TEMPO. *New Biotechnol*,43: 44-52 (2018).
- 12. Larson TM, Anderson AM, and Rich JO, Combinatorial evaluation of laccase-mediator system in the oxidation of veratryl alcohol. *Biotechnol Lett*, **35**: 225-231(2013).
- Loi M, Fanelli F, Zucca P, Liuzzi VC, Quintieri L, and Cimmarusti MT, Aflatoxin B1 and M1 degradation by Lac2 from Pleurotuspulmonariusand redox mediators. *Toxins*,8: 245-261(2016).
- 14. Loi M, Fanelli F, Liuzzi VC, Logrieco AF, and Mulè G, Mycotoxin biotransformation by native and commercial Enzymes: Present and future perspectives. *Toxins*, 9: 111-142 (2017).
- 15. Mate DM and Alcalde M, Laccase: a multi-purpose biocatalyst at the forefront of biotechnology *Microbiol Biotechnol*, **10**: 1457-1467 (2016).
- 16. McCormick SP, Stanley AM, Stover NA, and Alexander NJ. Trichothecenes: from simple to complex mycotoxins. *Toxins*, **3**: 802-14 (2011).

- Mcmullen M, Bergstrom G, De Wolf E, Dill Macky R, Hershman D, Shaner G, and Van Sanford D, A unified effort to fight an enemy of wheat and barley: Fusarium head blight. *Plant Dis*96:1712-1728 (2012).
- Moldes CA, Medici LO, Abrahao OS, TsaiSI SM and Azevedo RA, Biochemical responses of glyphosate resistant and susceptible soybean plants exposed to glyphosate. *Acta Physiologiae Plantarum*, 30: 469-479 (2008).
- Pardo I and Camarero S, Laccase engineering by rational and evolutionary design. *Cell Mol Life Sci*, **72**: 897-910 (2015).
- 20. Pestka JJ, Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. *Arch Toxicol*,**84**: 663-79 (2010).
- Pezzella C1, Guarino L, and Piscitelli A. How to enjoy laccases. *Cell Mol Life Sci*,**72**: 923-40 (2015).
- 22. Riva S, Laccases: blue enzymes for green chemistry. *Trends Biotechnol*,**5**: 219-226 (2006).
- 23. Savard ME, Blackwell BA, and Grenhalgh R, An 'H nuclear magnetic resonance study of derivatives of 3-hydroxy-12, 13-epoxytrichothec-9-enesl. *J Chem*,**65**: 2254 (1987).
- 24. Scott PM, Trichothecenes in grains. Cereal Foods Worl, 5: 661-666 (1990).
- 25. Sobrova P, Adam V, Vasatkova A, Beklova M, Zeman L, and Kizek R, Deoxynivalenol and its toxicity. *Interdiscip Toxicol*,**3**: 94-99 (2010).
- Wagacha JM1, Muthomi JW.Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies. *Int J Food Microbiol*, **124**: 1-12(2008).

- 27. Zhu Y, Hassan YI, Watts C, and Zhou T, Innovative technologies for the mitigation of mycotoxins in animal feed and ingredients-A review of recent patents. *Anim Feed Sci Technol*, 216: 19-29 (2016).
- 28. Zucca P, Cocco G, Sollai F, and Sanjust E, Fungal laccases as tools for biodegradation of industrial dyes. *Biocatalysis*, **1**: 82-108 (2015).
## Chapter 4

# Reduction of Mycotoxins by Heat and UVC Irradations

#### **REDUCTION OF MYCOTOXINS BY HEAT AND UVC IRRADATIONS**

#### 4.1 INTRODUCTION

It is widely documented that although many mycotoxins are thermally stable compounds, some food processing, such as applying temperatures above 100°C, can reduce their amount (Raiola & Ritieni, 2014; Vidal, Sanchis, Ramos & Marín; Santini, Raiola, Meca & Ritieni, 2015; Raiola et al., 2015; Shanakhat et al., 2018). Among the known thermal processing techniques, variations on fluidized bed drying are extensively applied for particulate drying at industrial scales in both traditional and innovative processed products, since it is a cost-effective technique providing a high thermal efficiency (Jangam, 2011). Dry processing is reported in wheat flour to modify the functional properties of some constituents, affecting technological properties. For instance, dry heating of wheat flour at 120°C for 30 min increased the volume of Kasutera cake (Nakamura, Koshikawa & Seguchi, 2008). The use of heat treated flour increased the viscosity of cake batters compared with batter made with untreated flour, and the gel network in emulsions obtained from heat treated flours is reported to be stronger than that prepared from untreated wheat flour (Meza et al., 2011). Wheat flour treated in a fluidized bed drier in order to generate products with longer shelf life, finer texture, moist crumb and sweeter taste, showed a decrease of gluten extensibility with positive effects on baking quality as the gluten appeared to retain its moisture, whereas base flour gluten retained its extensibility and cohesion (Neill, Al Muhtaseb & Magee 2012).

In the literature, the application of ultraviolet (UV) irradiation has also been reported to be an efficient strategy for the destruction of mycotoxins in different agricultural products such as pistachio, groundnut and almond (Shanakhat et al., 2018; Jubeen, Bhatti, Khan, Zahoor-Ul-Hassan & Shahid, 2012; García-Cela, Marin, Sanchis, Crespo-Sempere & Ramos, 2015).Furthermore, due to its low cost and simplicity of application, UVC irradiation is already

used in the food industry for disinfection of the air, control of contamination on the surface of packaging (Begum, Hocking & Miskelly, 2009) and the destruction of mould spores in baked products (Magan & Aldred, 2006).

This part of the thesis project aims to explore the impact of heating and UVC irradiation on semolina and dough properties, after confirming the effectiveness of these methods for reducing mycotoxin contamination. In particular, colorimetric analysis, water absorption parameters and gluten yield were analyzed. Furthermore, differential scanning calorimetry (DSC) was adopted to evaluate the effect of treatments on starch gelatinization, while a Brabender Farinograph was used to test the behaviors of obtained doughs. Finally, microstructure of doughs was observed by scanning electron microscopy (SEM) to evaluate potential changes in the structure of starch and proteins after the physical treatments.

This is the first study that investigates technological changes and structural features in a cereal derivative after the application of physical methods for mycotoxin reduction.

#### 4.2 MATERIALS AND METHODS

#### 4.2.1 Materials

Durum wheat semolina was purchased at a supermarket in Naples (Italy); different lots were mixed together and placed into closed plastic containers during storage. Semolina particle size dimensions ranged between 100 and 300 µm.

#### 4.2.2 Thermal and UVC Treatments

The first physical treatment applied for mycotoxin detoxification was the heating of 300 g of semolina at high temperatures (100, 150 or 200°C) for different durations (15, 30 or 60 min) using a fluidized bed dryer (Sherwood Scientific Model MK11, UK). The temperature was adjusted controlling the front of the dryer and the air flow speed was set at level 4. Temperature and air velocity were verified before each experimental test by a digital thermometer (range -50 -  $300^{\circ}C \pm 1^{\circ}C$ ) and a hot wire anemometer (range 0.4-30 m/s ±3%) respectively.

The second treatment consisted of UVC irradiation (254 nm), applied for 15, 30, 60 or 120 min. A germicidal UV Lamp, with 30 W (G30T8) power providing UVC radiation at 254 nm, was obtained from Sankyo Denki, Japan. Semolina (100 g) was spread in two different trays (50x30 cm) to form a layer not more than 1 cm thick that was exposed to UV radiations at a distance of 15 cm. For each treatment, moisture content analyses were carried out according to ISO Standard No. 712.

#### 4.2.3 Chemical and Reagents

All the standards and chemical reagents were purchased from Sigma Aldrich (Milan, Italy); HPLC grade organic solvents were from Merck (Darmstadt, Germany). The individual stock solutions of mycotoxins were prepared by diluting 1 mg of each mycotoxin in 1 mL of acetonitrile. Individual stock standards were diluted to obtain working solutions at different concentration levels and stored at -20°C until the analysis.

#### 4.2.4 Mycotoxin Extraction and LC/MS Analysis

QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction was based on that reported by Tolosa, Font, Mañes&Ferrer (2017). A C17:0 standard was added to the samples prior to extraction, to perform reliable quantification of analytes with correction of errors due to the presence of other chemicals that co-extracted from the matrix. Samples from each treatment were extracted in triplicate.

Separation was carried out using an HPLC system (Agilent 1200 Infinity) coupled to a mass spectrometer (Agilent G6420 Triple Quadrupole) equipped with an Agilent API-Electrospray ionization source. Agilent MassHunter Workstation B.07.00 software was used for data acquisition and analysis. The LC/MS conditions were as reported by Sobolev et al. (2018). With this method the following mycotoxins were analysed simultaneously: trichothecenes (DON; 3-acetyl-deoxynivalenol (3-ADON), nivalenol (NIV); T-2 toxin; and HT-2 toxin), aflatoxins (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>), fumonisins (FB<sub>1</sub>, FB<sub>2</sub>), OTA, and enniatin B (ENB). Quantification was performed with authentic standards where the transition of each compound was monitored on calibration curves with at least 5 points. The ratios of the MRM transition were used to draw the calibration curves.

The lower limit of detection (LOD) and lower limit of quantification (LOQ) were respectively assessed as the 3 and 10 folds the ratio between the standard error of the intercept and the slope of the calibration curve. The results were expressed as mean value ( $\mu$ g mycotoxin/kg semolina) ± standard deviation.

#### 4.2.5 Technological Properties of Semolina

#### 4.2.5.1 Color

The reflectance colorimeter was the Chroma Meter CR-200 (Minolta) equipped with a pulsed xenon arc lamp. Absolute measurements in L\*, a\*, b\* (CIE 1986) coordinates in the Munsell color system were taken using D65 lightning. Samples to be analyzed were placed into a granular material attachment. Results were the average of three determinations. The colorimeter calibration parameters L\*, a\*, and b\* were L=97.07, 0.27 and 1.83, respectively.

#### 4.2.5.2 Total Phenols Determination and Antioxidant Power

For those physical treatments that successfully reduced mycotoxin content, total phenols amount was estimated and potential oxidative effects were examined by ABTS [2,2 azinobis (3-ethylbenzothiazoline-6 sulfonic acid)] radical scavenging test and FRAP (Ferric Reducing Ability of Plasma) test.

Extraction from samples and ABTS analysis were carried out as reported by Pellegrini et al (2006), while FRAP test and total phenols determination were performed as Benzie & Strain (1996) and Singleton et al (1965) respectively.

#### 4.2.5.3 Hydration Indices

Water absorption index (WAI), water solubility index (WSI) and swelling power (SP) of control and treated semolina samples were determined following the method of Anderson, Conway, Pfiefer & Griffin (1969).

According this procedure, WAI, was determined as:

 $WAI = \frac{weight \ of \ sediment}{weight \ of \ sample}$ 

#### Chapter 4

While, WSI was calculated as:

$$WSI = \frac{weight \ of \ soluble \ solids}{weight \ of \ sample}$$

and SP was calculated as:

$$SP = \frac{weight of sediment}{weight of sample \times \frac{(1-WSI)}{100}}$$

#### 4.2.5.4 Water Sorption Isotherm

The sorption characteristics of semolina have been determined by a gravimetric method using a Surface Measurement SystemsR automated Dynamic Vapour Sorption (DVS1000) which is a controlled atmosphere microbalance. The mass variation durum wheat semolina was measured as a function of time over a range of values of relative humidity by mixing dry and saturated vapour gas flows. The mass variation of the sample and the time are recorded.

Water adsorption isotherms were determined at the constant temperature of 30°C at a nitrogen flow of 375 standard cm3. Samples (8.32-9.75 mg) were loaded and pre-equilibrated at 0% equilibrium relative humidity of the air by a continuous flow of dry air. Samples were then equilibrated at successive levels of relative humidity (from 10% to 95% in 11 steps). For each equilibrium relative humidity level, the equilibrium conditions were defined when the change in sample mass as a function of time was lower than 0.002% min-1 (Hebrard et al, 2003).

#### 4.2.6 Gluten Parameters

The gluten extraction was carried out according to the AACC method38-12.02 (2005). Briefly, doughs were prepared by using a Farinograph-AT Brabender (Duisburg, Germany). Obtained doughs were held in water for 40 min and successively washed under stream of running water until starch was washedout and the water was clear. The obtained mass was wet gluten. The wet gluten yield (WGY) was calculated as (Kaushik, Kumar, Sihag & Ray, 2015):

$$WGY = \frac{weight of wet gluten}{weight of semolina} \times 100$$

The dry gluten yield (DGY) was determined by drying the wet gluten into freeze dryer (ALPHA 1-2 LD plus-Fisher Bioblock Scientific) as (Kaushik, Kumar, Sihag& Ray, 2015):

$$DGY = \frac{weight of dry gluten}{weight of semolina} \times 100$$

The moisture content of wet gluten was determined by drying the wet gluten into an oven at 105°C until the constant weight was achieved (Kaushik, Kumar, Sihag& Ray, 2015).

The water absorption capacity (WAC) of dried gluten was determined according to the method described by AACC (2005). Gluten sample (500 mg) was immersed in water (10 mL), mixed, kept for 60 min in continuous shacking and centrifuged at  $2000 \times g$  for 30 min. The supernatant was discarded and sediment was weighed. WAC was determined as (Kaushik, Kumar, Sihag& Ray, 2015):

$$WAC = \frac{weight \ of \ sediment}{weight \ of \ sample} \times 100$$

#### 4.2.7 Farinograph Analysis

Farinograph curves of both control and treated semolina, were acquired using a farinograph fitted with 50 g mixing bowl, according to the AACCmethod 54-21.02 (2000) methods. Water absorption (WA), dough development time (DDT), dough stability (DS), degree of softening (DOS) and elasticity (E) were determined from farinograms and the results were expressed as the average value of three replicates for each sample (Singh, Singh & MacRitchie, 2011). At the end of each test, the moisture content of the obtained dough was determined according to ISO Standard No. 712.

#### 4.2.8 Differential Scanning Calorimetry Analysis

Thermal transitions of starch and/or proteins of each dough sample, both at the development time and after 30 min of mixing, were studied using DSC TA Instrument Q 200 (New Castle, DE). Dough samples (10 mg) were equilibrated at 30°C for 5 min, and then heated at 5°C/min up to 150°C. All the samples were placed into a steel hermetic DSC pan that was covered with a lid, crimped together with the pan. Four replicates were scanned for each condition and temperature of onset (To), temperature of peak (Tp) and the enthalpy ( $\Box$ H), associated to each phase transition were determined.

#### 4.2.9 Microstructure

Scanning electron microscopy (SEM) was carried out for doughs obtained at the development time and after 30 minutes of mixing in the farinograph. Lyophilized samples were dissected and mounted on specimen stubs and Au-coated by DC sputtering (AGAR B7340) in order to make the specimen conductive. The coated specimens were then observed using a LEO EVO 40

scanning electron microscope (Zeiss, Germany), with a 20kV acceleration voltage. Representative micrographs from all the samples were selected.

#### 4.2.10 Statistical Analyses

All the parameters evaluated in three trials and three analytical replicates were expressed as mean value  $\pm$  standard deviation. Differences among control and treated samples were determined by Anova (Duncan's test) at a significance level of 0.05, using SPSS (Statistical Package for Social Sciences) Package 6, version 15.0 (SSPS Inc., Chicago, IL, USA).

#### 4.3 **RESULTS AND DISCUSSION**

#### 4.3.1 Degradation of Myctoxins

Several mycotoxins were detected in the semolina used for this study (Table 1). In particular, the most abundant mycotoxin was represented by enniatin B (ENB) ( $1002.91\pm23.53 \mu g/kg$ ) followed by NIV ( $763.96\pm49.05\mu g/kg$ ) and DON ( $121.55\pm10.00\mu g/kg$ ). Mean levels of T-2 and HT-2 were  $4.20\pm0.80$  and  $19.10\pm1.50 \mu g/kg$  respectively, while AFB<sub>1</sub>, AFG<sub>1</sub> and OTA mean values ranged between  $1.20\pm0.30$  and  $2.18\pm0.92 \mu g/kg$ . Furthermore, mean level of contamination for FB<sub>1</sub> and FB<sub>2</sub> were  $3.45\pm1.21$  and  $2.60\pm1.03 \mu g/kg$  respectively (Table 1).

μg/kg
121.55±10.00
n.d.
763.96±49.05
4.20±0.80
19.10±1.50
2.18±0.92
n.d.
1.20±0.30
n.d.
1.95±0.26
3.45±1.21
2.60±1.03
1002.90±23.53

Table 1: Level of detected mycotoxins in control semolina.

Mean  $(n=3) \pm$  standard deviation; n.d.= not detectable.

After heat treatments under different temperature and duration, we found that among the ten mycotoxins detected in control semolina, about half declined in concentration after heating (Fig. 1) while for the rest of the molecules (NIV, T2, HT-2, AFB<sub>1</sub>, AFG<sub>1</sub>, and FB<sub>2</sub>) no significant reduction of contamination after treatments was observed (data not shown). In particular, heating at 200°C for 15 min was the most efficient treatment for ENB, DON, OTA, and FB<sub>1</sub>, with mean reductions equal to  $70.82\pm1.18$ ,  $68.07\pm6.79$ ,  $56.52\pm17.02$ , and  $59.09\pm0.75\%$  respectively.

For ENB (Fig. 1-a), a significant reduction ranging between 28.36±2.42% and 31.73±13.45% was found after treatment at 100°C for 15 and 30 min respectively, and no further reduction was observed after 60 min. With heat treatment at 150°C, a mean degradation of ENB from 60.63±3.53% to 65.73±5.48% at 15 and 60 min respectively was obtained. Therefore, for this mycotoxin, the effect of degradation seemed to be related mainly to the temperature instead of processing time. Previously, Serrano, Meca, Font & Ferrer (2013) investigated the stability of enniatins by simulating the processing of pasta cooking and found a high percentages of thermal degradation (from 82 to 100%). The reduction of ENB, which was present at high levels in the analyzed semolina, is of particular importance since there are still no legal limits for this mycotoxin, though its toxic effects are widely documented in the literature (Meca, Font & Ruiz, 2011). Indeed, several research groups have been working for several years to reduce ENB levels (Serrano, Meca, Font, Ferrer, 2013; Manzini, Rodriguez-Estrada, Meca&Mañes, 2013; Tolosa, Font, Mañes&Ferrer, 2013).

No significant contamination reductions were observed for DON after treatment at 100°C or 150°C (Fig. 1-b). This accords with previous studies that have found DON to be stable at 120°C, moderately stable at 180°C and degraded at 210°C (Milani & Maleki, 2014).

Treatment at 100°C for 15 or 30 min did not show any significant effect on OTA degradation, while a slight reduction was found when the heating was prolonged until 60 min (20.46±1.51%). At 150°C, a further detoxification was observed, until OTA content was reduced by 61.18±5.04% after 60 min of heating, which was not significantly different from the reduction reached after 30 min (Fig. 1-c). In a previous work, a 23.5% loss in OTA was found at 136°C, while at 196°C the average loss was 31%, and degradation increased up to 40% by extending the time dough was retained in the extruder (Scudamore, Banks & Guy, 2004).

A significant reduction until the mean value of  $69.97\pm18.51\%$ , was observed for FB<sub>1</sub> after treatment at 100°C for 30 and 60 min. Some degradation was observed at 150°C for 15 min (22.80±6.96%) (Fig. 1-d), and a further reduction was observed when the treatment was prolonged to 30 min ( $60.21\pm11.33\%$ ). Therefore, for FB<sub>1</sub>, the time of processing appears to be as important as the temperature of treatment, but after 30 min of treatment no further reduction was observed. Also in this case, our data confirm results reported in literature: extrusion cooking was reported to be able to reduce FB<sub>1</sub> up to 66.6% at 160°C in the presence of glucose (Milani & Maleki, 2014).



Fig. 1: Impact of thermal treatment on ENB (a), DON (b), OTA (c) and FB1 (d) levels (µg/kg).

The application of UVC irradiation for 15 min was sufficient to completely degrade AFB<sub>1</sub>, OTA and FB<sub>2</sub> while for NIV, reductions of 8.87%, 11.91%, 17.36% and 24.57% were observed with treatment durations of 15, 30, 60 and 120 min, respectively (Table 2). Our results confirm the report of Jubeen, Bhatti, Khan, Zahoor-Ul-Hassan & Shahid (2012), who investigated the effect of UV irradiation on aflatoxins in ground nuts and found a complete AFG<sub>1</sub> degradation in some samples and an AFB<sub>1</sub> reduction up to 96.5%. For the other detected mycotoxins, no significant changes were observed after the UVC treatment (data not shown).

	AFB <sub>1</sub>	ОТА	FB <sub>2</sub>	NIV
Time (min)			μg/kg	
15	n.d	n.d	n.d	696.19±22.43 <sup>b</sup>
30	n.d	n.d	n.d	672.94±35.47 <sup>b</sup>
"60	n.d	n.d	n.d	631.27±15.11 <sup>ab</sup>
120	n.d	n.d	n.d	576.22±8.11 <sup>a</sup>

 Table 2: Effect of UVC treatment on mycotoxins level.

Mean (n=3)  $\pm$  standard deviation; n.d. = not detectable. Different letters in the same column indicate significant differences (p<0.05).

Chapter 4

It is worth noting that, as for ENB, current European legislation does not place limits on NIV contamination in food stuffs, despite the reporting of toxic effects (Minervini et al, 2004), and that the issue of NIV contamination has been understood for some time (Bretz, Knecht, Göckler&Humpf, 2005). Moreover, the combined effect of mycotoxins may result in a greater toxicity than that found for individual compounds (Tammer, Lehmann, Nieber&Altenburger 2007). This issue is particularly relevant in Italy, given the high daily consumption of products based on semolina such as pasta and bread. Therefore, the degradation of multiple mycotoxins in a mixture could be interesting for the reduction of food safety risk.

However, for the evaluation of the effectiveness of physical treatments in detoxification of mycotoxins, the degradation products should be investigated. Given that this aspect is outside the aim of this work, a preliminary identification of DON degradation products revealed the presence of the norDON A, norDON B, norDON C, norDON D, norDON F and 9-hydroxymethyl DON lactone, in agreement with Bretz, Beyer, Cramer, Knecht & Humpf (2006). It is known from literature that the products derived from physical treatments of mycotoxins allowed an effective detoxification, such as in the case of fumonisins (Hahn et al., 2015), OTA (Bittner, Cramer, Harrer & Humpf, 2015) and AFB<sub>1</sub> (Wang et al, 2016).

#### 4.3.2 Technological Properties of Semolina

#### 4.3.2.1 Color

The color plays an important role in the definition of semolina and pasta quality. In food industries, the most common color measurement techniques are based on the color-space system  $L^*$ ,  $a^*$ ,  $b^*$  as defined by the Commission Internationale de l'Eclairage (CIE).

Table 3 shows the colorimetric indices (L\*, a\*, b\*) determined in control and treated semolina samples. L\* values ranged from 82.76±0.20, for semolina treated at 200°C for 15 min, to 90.20  $\pm 0.05$  in the sample treated at 100°C for 15 min. In general, after the thermal treatments, no burned particles are present in semolina with the exception of treatment at 200°C for 15 min. The lower L\* value, which quantifies the lightness of the product, is the result of the browning due to the intense thermal treatment, as confirmed by the higher value of red index (a\*) that is strictly linked to the Maillard reaction (Cavazza et al., 2013; Pasqualone et al., 2014). Therefore, despite this treatment being highly efficient for the reduction of some mycotoxins, it is not suitable at an industrial level. Semolina yellowness is determined by the b\* value, and is closely related to the carotenoid pigments. A lower b\* value, equal to 19.51±0.37, was observed in semolina treated at 150°C for 60 min. Both L\* and b\* values are similar with those reported by previous studies (Cavazza et al., 2013), while, in our study a\* values are significantly lower; this dissimilarity could be attributable to the different varieties of semolina used for these investigations.

Treatment	Time (min)	$\mathbf{L}^{*}$	a <sup>*</sup>	b <sup>*</sup>
Control		88.89±1.17 <sup>bc</sup>	-4.45±0.01 <sup>c</sup>	24.68±0.78 <sup>ef</sup>
	15	90.20±0.05 <sup>d</sup>	-3.89±0.04 <sup>d</sup>	23.33±0.41 <sup>de</sup>
100°C	30	$89.25 \pm 0.22^{d}$	$-4.34 \pm 0.05^{\circ}$	26.32±0.34 <sup>g</sup>
	60	88.61±0.66 <sup>cd</sup>	-4.06±0.13 <sup>d</sup>	22.44±0.63 <sup>cd</sup>
	15	90.09±0.05 <sup>d</sup>	-3.15±0.03 <sup>f</sup>	21.41±0.16 <sup>b</sup>
150°C	30	$88.82{\pm}0.46^{cd}$	-3.60±0.27 <sup>e</sup>	21.56±0.41 <sup>bc</sup>
	60	89.48±0.09 <sup>d</sup>	-2.62±0.22 <sup>g</sup>	19.51±0.37 <sup>a</sup>
200°C	15	82.76±0.20 <sup>a</sup>	$0.19{\pm}0.08^{h}$	$24.80{\pm}0.42^{\rm f}$
	15	88.26±0.26 <sup>cd</sup>	-5.14±0.13 <sup>b</sup>	28.40±0.53 <sup>h</sup>
	30	$86.41 \pm 0.48^{b}$	-5.61±0.19 <sup>a</sup>	$28.92{\pm}0.40^{h}$
UVC	60	$85.82{\pm}0.54^{b}$	-5.60±0.04 <sup>a</sup>	$27.95 \pm 0.90^{h}$
	120	$89.29 \pm 0.27^{d}$	$-3.79 \pm 0.06^{d}$	$23.22 \pm 0.18^{f}$

**Table 3**: Colorimeter results of control and treated semolina.

Mean (n=3)  $\pm$  standard deviation; Different letters in the same column indicate significant differences (p<0.05).

Chapter 4

#### **4.3.2.2** Total Phenols Determination and Antioxidant Power

For those physical treatments that successfully reduced mycotoxin content, potential oxidative effects were examined, by analyses of total phenols and antioxidant activity using FRAP (Ferric Reducing Ability of Plasma) and ABTS (2,2 azinobis (3-ethylbenzothiazoline-6 sulfonic acid)). Phenol content did not show a significant change after all the studied treatments. Nevertheless, the FRAP test showed an increase of antioxidant capacity from 13% to 32% in UVC treated samples, which could be attributed to the breaking of chemical bonds, and the consequent release of antioxidant molecules from matrix after the UVC treatment; on the other hand, a FRAP reduction by 37.19% after the 150°C/30 min treatment was also observed. A similar trend was shown from ABTS results (Table 4).

Samples	Phenols	FRAP	ABTS	
	(mg/100g)	(µmol TE/100g)	(µmol TE/100g)	
Control	10.64±0.66 <sup>a</sup>	$86.08 {\pm} 8.18^{ m b}$	$75.66 \pm 2.02^{b}$	
UV 15 min.	$11.52 \pm 0.24^{b}$	$100.45{\pm}16.97^{bcd}$	89.59±14.39 <sup>c</sup>	
UV 30 min.	$10.42 \pm 0.30^{a}$	$97.47 \pm 8.47^{bcd}$	$95.66 \pm 6.04^{\circ}$	
UV 60 min.	11.99±0.61 <sup>b</sup>	$107.77 \pm 19.32^{cd}$	86.35±7.98 <sup>c</sup>	
UV 120 min.	$10.66 \pm 0.66^{a}$	$113.39{\pm}11.74^{d}$	86.89±4.76 <sup>c</sup>	
150°C/60 min.	$10.72 \pm 0.70^{a}$	$54.06 \pm 6.05^{a}$	$64.45 \pm 7.52^{a}$	

#### **Table 4:** Antioxidant activity of semolina

Mean (n=3)  $\pm$  standard deviation; Different letters in the same column indicate significant differences (p<0.05).

On the basis of degradation data of mycotoxins and colorimetric results, samples of semolina treated at 150°C for 30 min and by UVC for 120 min were selected for the subsequent analyses.

#### 4.3.2.3 Hydration and Gluten Indices

Both control and UVC treated semolina showed mean moisture content equal to  $11.0\pm0.11\%$ , while thermally treated sample at 150°C for 30 min was totally dried.

Table 5 shows the hydration and gluten indices of semolina before and after UV and heat treatments. WAI in control semolina was equal to 2.17±0.08, which was comparable to the value in UVC treated semolina, while a significant increase of 18.89% was observed in the thermally treated sample. Since WAI indicates the amount of water absorbed by starch, the observed increase in WAI in the thermally treated sample could indicate that the starch absorbed more water as a consequence of damage to the structure of starch granules during the high temperature treatment. The increase of SP (18.72%) after the treatment, from the initial value of 232.76±8.90, also supports this hypothesis. In fact, SP reflects the uptake of water in starch granules by amylopectin, which is considered to contribute to water absorption and swelling (Blazek & Copeland, 2008). WSI is often adopted as an indicator of degradation of molecular components and it is a measurement of the amount of soluble components released from the starch (Anderson, Conway, Pfiefer & Griffin, 1969). Contrary to previous evaluated parameters, WSI value was not significantly different in treated samples from the initial value of 0.068±0.001 found in the control.

Moisture content of gluten was not significantly changed by UVC and temperature treatments compared to the control (66.18%±0.49), while WAC of gluten was slightly increased in UVC

129

treated (319.56±6.23%) compared to the control (302.97%±0.21), and no significant changes were observed in thermally treated sample. The increase of WAC in UVC treated gluten sample could be explained by hypothesizing an increased reactivity of the protein fraction, absorbing a higher amount of water in the network. Moreover, values obtained for WAC in gluten samples confirm that the increase of WAI and SP in thermally treated semolina was attributable to the higher retention of water by presumably damaged starch, since we have not observed an increase of water absorption in the gluten fraction, but rather a slight, non-significant reduction.

WGY was comparable in control and thermally treated samples  $(38.61\pm1.67\%)$  and 40.78%, respectively) with a slight decrease in UVC treated samples  $(34.36\pm0.20\%)$ , while DGY was slightly increased in thermally treated samples  $(14.66\pm0.30\%)$  compared to the control  $(13.04\pm0.38\%)$  and UVC treated samples  $(12.09\pm0.89\%)$ . This result can be explained by assuming that, after the treatment, gluten became easier to wash and retained less starch, according with evidences reported by Neill, Al-Muhtaseb & Magee (2012) on heat treated base flour processed by fluidized bed drier.

	Control	150°C/30 min	UVC/120 min
WAI	2.17±0.08 <sup>a</sup>	2.58±0.13 <sup>b</sup>	2.06±0.03 <sup>a</sup>
WSI	$0.068 {\pm} 0.001^{a}$	$0.066{\pm}0.003^{a}$	$0.069 \pm 0.006^{a}$
SP	232.76±8.90 <sup>a</sup>	276.34±15.19 <sup>b</sup>	221.88±1.87 <sup>a</sup>
Moisture of gluten (%)	66.18±0.49 <sup>a</sup>	$64.25 \pm 0.36^{a}$	$66.24{\pm}2.8^{a}$
WAC (%)	302.97±0.21 <sup>a</sup>	295.88±2.66 <sup>a</sup>	319.56±6.23 <sup>b</sup>
WGY (%)	$38.61 \pm 1.67^{b}$	$40.78{\pm}0.95^{b}$	34.36±0.20 <sup>a</sup>
DGY (%)	13.04±0.38 <sup>ab</sup>	14.66±0.30 <sup>b</sup>	12.09±0.89 <sup>a</sup>

**Table 5:** Hydration and gluten indices for control and treated semolina

Mean (n=3) $\pm$ standard deviation. Different letters in the same row indicate significant differences (p<0.05).

WAI: Water Absorption Index; WSI: Water Solubility Index; SP: Swelling Power; WAC: Water Absorption Capacity; WGY: Wet Gluten Yield (WGY); DGY: Dry Gluten Yield;

#### 4.3.2.4 Water Sorption Isotherm

The water soption isotherm can give informations about the nature, the structure of the matrix, and the mechanisms of physiosorption, since sorption properties can be influenced by biochemical factors and/or physicochemical properties of particles that could affect the aptitude of semolina to be extruded. It is reported that the hydration of semolina particles is mainly a physical phenomenon and, at the first step, water cannot penetrate into particles through pores, while after adsorption of water on the surface of the semolina particles, water molecules penetrate inside the particle by diffusion (Hèbrard et al (2003).

Fig 2 shows the sorption isotherms of control (untreated) and thermally treated (150°C for 1h) semolina. Our profiles are very similar to those reported by previous study on durum wheat, although only a few publications studied sorption isotherms of semolina (Hèbrard et al (2003), Murrieta-Pazos et al, 2014).

Analyzed samples present type II sorption isotherm curves with the shape of a "S", according to the IUPAC classification which is typical of finely divided non-porous solids or macro-porous materials (Zeng et al, 2011; Murrieta-Pazos et al, 2014). As reported by Murrieta-Pazos et al, 2014, the curves can be divided in three different parts: from 0 to 20% RH the monolayer formation is represented, from 20 to 80% there is a linear portion, that represents the formation of binds among water and semolina components, and from 80 to 95% water presents weak binding. The curves of treated samples are almost perfectly coicident with the control, so the analyzed physical treatments seem to have no effect on the water content of semolina for each equilibrium relative humidity of the air, since they did not affect the ability of semolina components to establish interactions with water.

132

**Fig. 2**: Water sorption isotherms of semolina: a) control (untreated), and b) thermally treated (150°C for 1h)



b) Thermally treated (150°C 1h)

#### 4.3.3 Farinograph Analysis

Fig. 3 and Table 6 show data obtained from Brabender farinograph. Water absorption (WA) is a common farinograph parameter that allows one to quantify the exact content of water necessary to obtain a specific value of dough consistency (by convention, corresponding to 500 Brabender Units (BU)). In accordance with reports by other authors (AbuHammad, Elias, Manthey, Alamri & Mergoum, 2012), in control semolina this value was 55.5%, while for semolina treated at 150°C for 30 min, and at UVC for 120 min we found that the water amount was equal to 87.2% and 58.0% respectively (Table 6).

Mean moisture content of dough obtained from control semolina was equal to  $43.65\pm0.21\%$ , which was comparable to the value found in UVC treated samples ( $44.00\pm0.12\%$ ), while in samples treated at 150°C for 30 min, the mean value was significantly different ( $45.33\pm0.21\%$ ).

These data strongly indicate a high impact of thermal treatment on the structure and properties of semolina dough.

Dough development time (DDT) increased significantly in thermal treated semolina ( $8.98\pm0.66$  min) compared to the control ( $3.93\pm1.07$  min), while in UVC treated semolina this DDT was equal to  $2.45\pm0.11$  min. DDT values observed for control semolina are similar to those reported by a previous characterization study of sixteen durum wheat cultivars carried out by AbuHammad, Elias, Manthey, Alamri & Mergoum (2012) that found values ranging between 2.9 and 5.7 min. The increase of DDT in heat treated semolina might be explained considering the lack of gluten network formation. Other authors also found an extension of DDT in dough from flour heated at 100°C for 5 h, which did not develop within a mixing time of 8 min (Van Steertegem, Pareyt, Brijs & Delcour, 2013). In addition, the cross-linked protein network

surrounding the starch granules can reduce their water uptake with significant consequences on the total structure.

Dough stability was significantly reduced after both heating  $(11.32\pm1.07 \text{ min})$  and UVC treatment  $(7.35\pm0.21 \text{ min})$  compared to the control  $(18.19\pm2.53 \text{ min})$ . The dough softening was not significantly different between control  $(9.66\pm1.52 \text{ BU})$  and thermally treated semolina  $(9.00\pm2.82 \text{ BU})$  while it was higher in UVC treated semolina  $(43.50\pm2.12 \text{ BU})$ .

Treatment	WA (%)	DDT (min)	DS (min)	DOS (BU)	E (BU)
Control	$55.5\% {\pm} 0.05^{a}$	$3.93{\pm}1.07^{b}$	$18.19 \pm 2.53^{\circ}$	$9.66 \pm 1.52^{a}$	$113.00 \pm 4.00^{\circ}$
150°C/30 min	$87.2\% \pm 0.07^{c}$	$8.98 \pm 0.66^{\circ}$	$11.32{\pm}1.07^{b}$	$9.00{\pm}2.82^{a}$	$82.00 \pm 4.36^{a}$
UVC/120 min	$58.0\% {\pm} 0.03^{b}$	2.45±0.11 <sup>a</sup>	7.35±0.21 <sup>a</sup>	$43.5 \pm 2.12^{b}$	$101.33{\pm}6.65^{b}$

 Table 6: Farinograph parameters of control and treated semolina

Mean (n=3)±standard deviation; Different letters in the same column indicate significant differences (p<0.05).

WA: Water Absorption; DDT: Dough Development Time; DS: Dough Stability; DOS: Degree Of Softening; E: Elasticity The elasticity values showed significant differences among three analyzed doughs, with the highest level in the control ( $113\pm4.00$  BU), followed by UVC treated samples ( $101.33\pm6.65$  BU) and thermally treated samples ( $82.00\pm4.36$  BU).

Taken together, these data demonstrate that thermally treated semolina required more time to develop into dough, but this dough was more stable compared to that obtained from UVC treated semolina. In addition, the observed increase of the WA value is related to the higher water uptake of starch granules, as a consequence of the cross-linked protein network loss surrounding them. Protein aggregation and starch surface modifications after heat treatment lead to changes in starch-protein and starch-starch interactions, also affecting the rheological properties of doughs (Mann, Schiedt, Baumann, Conde-Petit & Vilgis, 2014).

Moreover, we observed the lowest DDT, also compared to the control, in the UVC exposed sample, which may be attributable to increased reactivity of protein components after the treatment; on the other hand, the protein network that formed during the mixing was less stable, as indicated from the lower stability time and the high degree of softening.

The protein content and composition of durum wheat have a major impact on dough rheological properties and pasta cooking quality. In particular a highly significant inverse relationship between semolina protein content and DDT has been demonstrated (Aalami, Rao & Leelavathi, 2007). The properties of dough are widely related to the quantity and quality of gluten including glutenin, responsible for the elastic properties, and gliadin, which contributes to the extensibility: higher glutenin content is related to greater gluten strength. In fact, when semolina is mixed with water, the glutenin and gliadin proteins start modifying their shape and trigger several complex phenomena: water molecules become linked to the protein molecules, breaking the pre-existing

137

bonds between them and forming new bonds. Consequently, the protein molecules start stretching out in a disorderly manner, to form filaments.

The significant differences of farinograph parameters after the thermal treatment could reflect the chemical changes as a consequence of heating, since most proteins denature at a temperature range of 50 to 80°C (Mann, Schiedt, Baumann, Conde-Petit & Vilgis, 2014), reducing their solubility in water. In addition, heating leads to disulfide bond linked aggregates and conformational changes affecting gliadins and low molecular weight albumins and globulins (Guerrieri, Alberti, Lavelli & Cerletti, 1996), as evidenced by the loss of elasticity (equal to about 27.43%) and by the reduced dough stability in thermal treated samples compared to the control.

Furthermore, mixograph studies confirmed that the heat treatments significantly affected gluten hydration and likewise, dough development, indicating that flour particles hydrated more slowly, probably as a consequence of a more rigid structure as formed by the cross-linked gluten protein for the decreased level of free SH groups after the treatment (Van Steertegem, Pareyt, Brijs & Delcour, 2013).



Fig. 3: Farinograms of doughs obtained from control (a), thermally treated at 150°C/30 min (b) and UVC treated/120 min (c) semolina.

#### 4.3.4 Differential Scanning Calorimetry Analysis

DSC is a thermal analysis that measures the thermodynamic transitions of a material during heat treatment. This technique was applied in order to explain the changes associated to the major dough components that can affect the macroscopic properties as observed from farinograms after exposure to UVC and temperature treatments.

Table 7 reports the temperatures and enthalpy values observed during DSC analyses of dough prepared with control and treated semolina. All the samples showed the typical endothermal transition due to the gelatinization of amilopectin that appears at temperatures between 61.18 and 63.41°C for thermally treated and control samples respectively, while the enthalpy ranged between 0.84 and 1.11 J/g dried semolina and was not significantly different among the three analyzed samples. Gelatinization is partly shifted to higher temperature due to the melting of the remaining amylopectin crystallites (Moreira, Chenlo & Arufe, 2015), giving as result a second peak at a temperature between 85.53 and 88.73°C for thermally treated samples and the control, respectively. Enthalpy values ranged from 1.27J/g dried semolina in thermally treated samples to 1.60 J/g dried semolina in the control, with statistically significant difference. Another transition at higher temperature was observed in all three dough samples, at 114.12 and 116.03°C for thermally treated samples and the control respectively; this corresponds to the melting of amylose-lipid complexes (Moreira, Chenlo & Arufe, 2015). Also for this transition we observed a significant reduction of enthalpy in thermally treated samples; in fact, the energy required for the melting of amylose-lipid complexes depends on microstructural properties of both polymers. The temperatures and enthalpies we measured were comparable to those reported by Agyare, Xiong, Addo & Akoh (2006), who also observed three transitions. No additional information were obtained from semolina samples and no distinct DSC peaks were observed for dried and wet

gluten at 43% moisture when heated from 30 to 150°C (data not shown), which is consistent with results previously reported by other authors (Agyare, Xiong, Addo & Akoh, 2006).

### **Table 7**: Temperatures ( $T_o$ and $T_p$ ) and enthalpies ( $\Delta H$ ) from DSC analyses of dough at DDT obtained from control and treated semolina.

	StarchGelatinization					Malding of lividg with stough			
Treatmont		Step I			Step II		wiening	staren	
meatineit	To	T <sub>p</sub>	ΔH	To	T <sub>p</sub>	ΔH	To	T <sub>p</sub>	ΔH
	(°C)		(J/g <sub>dried</sub> semolina)	Iried (°C) (		(J/g <sub>dried</sub> semolina )	(°C)		(J/g <sub>dried</sub> semolina)
Control	55.87±1.06 <sup>ab</sup>	$63.41 \pm 0.72^{b}$	1.11±0.36 <sup>a</sup>	$75.54{\pm}0.87^{b}$	$88.73 \pm 1.09^{b}$	1.60±0.27 <sup>a</sup>	105.63±1.0 <sup>a</sup>	116.03±0.38 <sup>a</sup>	1.96±0.25 <sup>b</sup>
150°C/30 min	54.76±0.16 <sup>a</sup>	61.18±0.13 <sup>a</sup>	1.08±0.18 <sup>a</sup>	73.27±0.48 <sup>a</sup>	85.53±0.58 <sup>a</sup>	1.27±0.14 <sup>a</sup>	103.12±0.97 <sup>a</sup>	114.12±0.99 <sup>a</sup>	1.43±0.15 <sup>a</sup>
UVC/120 min	56.55±0.15 <sup>b</sup>	62.68±0.36 <sup>b</sup>	$0.84{\pm}0.20^{a}$	75.26±1.02 <sup>b</sup>	87.60±1.13 <sup>b</sup>	1.26±0.10 <sup>a</sup>	104.58±1.72 <sup>a</sup>	115.85±1.4 <sup>a</sup>	2.19±0.08 <sup>b</sup>

Mean (n=3)±standard deviation; Different letters in the same column indicate significant differences (p<0.05).

Chapter 4

#### 2.3.5 Microstructure

To evaluate the impact of both physical treatments and mixing on the microstructure, SEM analyses were conducted on doughs obtained from control, thermally treated and UVC treated semolina, at both the time of development and with overmixing. Fig. 4 shows the micrographs taken at 2000× magnifications. Dough from control semolina displayed an evident network attributable to the protein fraction connecting starch granules of heterogeneous dimensions, that are similar to those reported for wheat dough in literature (Fig 4-a; Ding & Yang, 2013). Similar microstructure was also shown in dough obtained from UVC treated semolina (Fig 4-b), although the gluten network appeared less continuous compared to the control. A further reduction of gluten continuity was revealed in dough obtained from thermally treated semolina (Fig.4-c), confirming the strong impact of heat application on the protein, such as was revealed by the change of dough mixing properties.

We also observed an effect of dough mixing time on the microstructure. The impact of overmixing was observed for all three treatments that were analyzed, since a reduction of interconnection among starch granules was evident as a consequence of the loss of gluten network continuity. In particular, this effect was more marked after overmixing of dough obtained from UVC treated semolina, confirming the mixing results, since this sample revealed the major DOS as previously described. In fact, the microstructure of this sample (Fig.4-d) shows an evident loss of protein network among starch granules as a consequence of the overmixing. In addition, damage to starch granules was more evident in thermally treated samples, confirming the previous considerations.

143



**Fig. 4**: Micrographs at 2000× of doughs from control (a-b), thermally treated at 150°C/30 min (c-d) and UVC treated/120 min (e-f) semolina, Obtained at DDT (left) and after 30 min of

mixing (right).
Chapter 4

#### 4.4 CONCLUSION

Analysed semolina contained 10 different mycotoxins, among which the most abundant were the unregulated ones, ENB and NIV, whose toxic effects are documented. For these two mycotoxins we obtained a significant reduction after thermal treatment (ENB) and UV irradiation (NIV). Other considered mycotoxins (OTA, AFB<sub>1</sub>, FB<sub>1</sub>, FB<sub>2</sub>) were also reduced as a consequence of physical treatments, confirming prior reports.

Treatment of semolina with heat and with UVC exerted a significant impact on dough properties, as revealed from some farinograph parameters, showing significant changes in DDT, WA, time of stability, degree of softening and elasticity. These data correlated well with some measures of semolina performance as well as WAI, SP, WAC of gluten, some parameters from thermograms and modifications in microstructure observed by SEM. As future perspective, it could be interesting to apply heating and UVC irradiation together and to study if changes observed in doughs significantly impact on industrial processing such as pasta making or baking, affecting the nutritional and technological properties of the final products. Since analyses of thermally treated semolina suggest a high degree of damage to the gluten network, remediating mycotoxin contamination via thermal treatment may be more suitable for derivative foodstuffs that do not need a strong gluten network, such as some types of biscuits characterized by a high crumbliness.

Chapter 4

#### 4.5 REFERENCES

- Aalami M, Rao UJSP and Leelavathi K, Physicochemical and biochemical characteristics of Indian durum wheat varieties: Relationship to semolina milling and spaghetti making quality. *Food Chem*, **102**: 993-1005 (2007).
- AbuHammad WA, Elias EM, Manthey FA, Alamri MS, and Mergoum M, A comparison of methods for assessing dough and gluten strength of durum wheat and their relationship to pasta cooking quality. *Int JFood Sci Technol*, 47: 2561-2573 (2012).
- Agyare KK, Xiong YL, Addo K, and Akoh CC, Dynamic rheological and thermal properties of soft wheat flour dough containing structured lipid. *J Food Sci*, 69: 297-302 (2006).
- Anderson RA, Conway HF, Pfiefer VF and Griffin EL, Roll and extrusion cooking of grains sorghum grits. *Cereal Sci Today*, 14: 372-376 (1969).
- Approved methods of the AACC, 11th ed. St Paul, MN: American Association of Cereal Chemists (2005).
- Begum M, Hocking AD, and Miskelly D, Inactivation of food spoilage fungi by ultra violet (UVC) irradiation. *Int J FoodMicrobiol*, **31**: 74-77 (2009).
- Bittner A, Cramer B, Harrer H, Humpf H-U, Structure elucidation and in vitro cytotoxicity of ochratoxin α amide, a new degradation product of ochratoxin A. *Mycotoxin Research*, **31**: 83-90 (2015).
- 8. Blazek J, and Copeland L, Pasting and swelling properties of wheat flour and starch in relation to amylose content. *Carbohyd Polym*, **71**: 380-387 (2008).
- 9. Bretz M, Beyer M, Cramer B, Knecht A, and Humpf H-U, Thermal Degradation of the Fusarium Mycotoxin Deoxynivalenol. *J AgrFood Chem*, **54**: 6445-6451 (2006).

- Bretz M, Knecht A, Göckler S, and Humpf H-U, Structural elucidation and analysis of thermal degradation products of the Fusarium mycotoxin nivalenol. *Mol Nutr Food Res*, **49**: 309-316 (2005).
- 11. Cavazza A, Corradini C, Rinaldi M, Salvadeo P, Borromei C, and Massini R, Evaluation of Pasta Thermal Treatment By Determination of Carbohydrates, Furosine, and Color Indices. *Food Bioprocess Tech*, 6: 2721-2731 (2013).
- 12. CIE Publication 15.2, Colorimetry, 2nd ed.; CIE Central Bureau Kegelgasse: Wien, Austria; 27-A-1030 (1986).
- Ding S and Yang J, The influence of emulsifiers on the rheological properties of wheat flour dough and quality of fried instant noodles. *LWT-FoodSci Technol*, **53**: 61-69 (2013).
- 14. European Commission (EC), Regulation no. 1881/2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of EuropeanUnion*, **L364**: 5 (2006).
- 15. García-Cela E, Marin S, Sanchis V, Crespo-Sempere A and Ramos AJ, Effect of ultraviolet radiation A and B on growth and mycotoxin production by *Aspergillus carbonarius* and *Aspergillus parasiticus* in grape and pistachio media. *Fungal Biol*, **119**: 67-78 (2015).
- Guerrieri N, Alberti E, Lavelli V and Cerletti P, Use of spectroscopic and fluorescence techniques to access heat induced molecular modifications of gluten. *Cereal Chem*, 73: 368-374 (1996).
- 17. Hahn I, Nagl V, Schwartz-Zimmermann HE, Varga E, Schwartz C, Slavik V, Reisinger N, Malachová A, Cirlini M, Generotti S, Dall'Asta C, Krska R, Moll W-D, Berthiller F, Effects of orally administered fumonisin B1 (FB1), partially hydrolysed FB1, hydrolysed

FB1 and *N*-(1-deoxy-D-fructos-1-yl) FB1 on the sphingolipid metabolism in rats. *Food Chem Toxicol*, **76**: 11-18 (2015).

- 18. Hebrard A, Oulahna D, Galet L, Cuq B, and Abecassis J, Hydration properties of durum wheat semolina: influence of particle size and temperature.7th International Symposium on Agglomeration, *Powder Technology* (pp 211-218) Albi France: Elsevier Publishing (2003)
- 19. ISO 712:1985 Cereals and cereal products Determination of moisture content (Routine reference method)
- Jangam SV, An overview of recent developments and some R&D challenges related to drying of foods. *Dry Technol*, 29: 1343-1357 (2011).
- 21. Jubeen F, Bhatti IA, Khan M Z, Zahoor-Ul-Hassan MZK and Shahid M, Effect of UVC, Irradiation on Aflatoxins in Ground Nut (*Arachishypogea*) and Tree Nuts (*Juglansregia*, *Prunus dulcis* and *Pistacia vera*). J Chem Soc Pakistan, **34**: 1366-1374 (2012).
- 22. Kaushik R, Kumar N, Sihag MK and Ray A, Isolation, characterization of wheat gluten and its regeneration properties. *J FoodSci Technol*, **52**: 5930-5937 (2015).
- 23. Magan N, and Aldred D, Managing microbial spoilage in cereal and baking products. In: Blackburn, C de W (Ed), *Food Spoilage Microorganisms* Cambridge (pp 194-212), England: Woodhead Publishing (2006).
- 24. Mann J, Schiedt B, Baumann A, Conde-Petit B, and Vilgis TA, Effect of heat treatment on wheat dough rheology and wheat protein solubility. *Food Sci Technol Int*, **20**: 341-351 (2014).

- 25. Manzini M, Rodriguez-Estrada MT, Meca G, Mañes J, Reduction of beauvericin and enniatins bioaccessibility by prebiotic compounds, evaluated in static and dynamic simulated gastrointestinal digestion. *Food Control* **47**: 203-211 (2015).
- 26. Meza BA, Chesterton AKS, Verdini RA, Rubiolo AC, Sadd PA, Moggridge GD, and Wilson DI, Rheological characterisation of cake batters generated by planetary mixing: Comparison between untreated and heattreated wheat flours. *J Food Eng*, **104**: 592-602 (2011).
- Milani J, and Maleki G, Effects of processing on mycotoxin stability in cereals. J Sci Food Agric, 94: 2372-2375 (2014).
- 28. Minervini F, Fornelli F, Flynn KM, Toxicity and apoptosis induced by the mycotoxins nivalenol, deoxynivalenol and fumonisin B1 in a human erythroleukemia cell line. *Toxicol in Vitro*, 18: 21-28 (2004).
- 29. Moreira R, Chenlo F, and Arufe S, Starch transitions of different gluten free flour doughs determined by dynamic thermal mechanical analysis and differential scanning calorimetry. *Carbohyd Polym*, **127**: 160-167 (2015).
- 30. Murrieta-Pazos I, Galet L, Gaiani C, and Scher J, Evolution of particle structure during water sorption observed on different size fractions of durum wheat semolina. *Powder Technology*, 255: 66-73 (2014).
- 31. Nakamura C, Koshikawa Y, and Seguchi M, Increased volume of Kasutera Cake (Japanese Sponge Cake) by dry heating of wheat flour. *FoodSci Technol Res*, 14: 431-436 (2008).
- 32. Neill G, Al-Muhtaseb, AH, and Magee TRA, Optimisation of time/temperature treatment, for heat treated soft wheat flour. *J FoodEng*, **113**: 422-426 (2012).

- 33. Pasqualone A, Paradiso VM, Summo C, Caponio F, and Gomes T, Influence of drying conditions on volatile compounds of pasta. *Food BioprocessTech*, **7**: 719-731 (2014).
- 34. Raiola A, and Ritieni A, Good food and feed processing techniques. In Leslie JF and Logrieco AF.(Eds.), Mycotoxin reduction in grain chains: A practical guide (pp. 271– 279), New York: Wiley, Hoboken (2014).
- 35. Raiola A, Tenore GC, Manyes L, Meca G, and Ritieni A, Risk analysis of main mycotoxins occurring in food for children: An overview. *Food Chem Toxicol*, 84: 169-180 (2015).
- 36. Reverberi M, Ricelli A, Zjalic S, Fabbri AA, and Fanelli C, Natural functions of mycotoxins and control of their biosynthesis in fungi. *ApplMicrobiol Biotechnol*, 87: 899-911 (2010).
- 37. Santini A, Raiola A, Meca G, and Ritieni A, Aflatoxins, Ochratoxins, Trichothecenes,
  Patulin, Fumonisins and Beauvericin in finished products for human consumption. *J Clin Toxicol*, 5: 265 (2015).
- 38. Scudamore KA, Banks JN, and Guy RC, Fate of ochratoxin A in the processing of whole wheat grain during extrusion. *Food Addit &Contam*, **21**: 488-497 (2004).
- Serrano AB, Meca G, Font G, and Ferrer E, Degradation study of enniatins by liquid chromatography-triple quadrupole linear ion trap mass spectrometry. *Food Chem*, 141: 4215-4225 (2013).
- 40. Shanakhat H, Sorrentino A, Raiola A, Romano A, Masi P and Cavella S, Current methods for mycotoxins analysis and innovative strategies for their reduction in cereals: an overview. *J Sci Food Agric*, **98**: 4003-4013 (2018).

- 41. Singh S, Singh N, and MacRitchie F, Relationship of polymeric proteins with pasting, gel dynamic- and dough empirical-rheology in different Indian wheat varieties. *Food Hydrocolloids*, 25: 19-24 (2011).
- 42. Sobolev AP, Mannina L, Capitani D, Sanzò G, Ingallina C, Botta BS, Crestoni ME, Chiavarino B, Corradoni S, Locatelli M, Giusti AM, Simonetti G, Vinci G, Preti R, Toniolo C, Reverberi M, Scarpari M, and Di Sotto AA, Multi-methodological approach in the study of Italian PDO "Cornetto di Pontecorvo" red sweetpepper. *Food Chem*, 255:120-131 (2018).
- 43. Tammer B, Lehmann I, Nieber K, and Altenburger R, Combined effects of mycotoxin mixtures on human T cell function. *Toxicol Lett*, **25**: 124-133 (2007).
- 44. Tolosa J, Font G, Mañes J, Ferrer E, Mitigation of enniatins in edible fish tissues by thermal processes and identification of degradation products. *FoodChem Toxicol*, **101**: 67-74 (2017).
- 45. Van Steertegem B, Pareyt B, Brijs K, and Delcour JA, Impact of mixing time and sodium stearoyllactylate on gluten polymerization during baking of wheat flour dough. *Food Chem*, **141**: 4179-4185 (2013).
- 46. Vidal A, Sanchis V, Ramos, AJ, and Marín S, Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products. *Food Chem*, **178**: 276-286 (2015).
- 47. Wang B, Mahoney NE, Pan Z, Khir R, Wu B, Ma H, and Zhao L, Effectiveness of pulsed light treatment for degradation and detoxification of aflatoxin B1 and B2 in rough rice and rice bran. *Food Control*, **59**: 461-467 (2016).

48. Zeng M, Huang Y, Lu L, Fan L, Mangavel C and Lourdin D, Mechanical Properties of Thermo-moulded Biofilms in Relation to Proteins/Starch Interactions. *Food Biophys*,6: 49-57 (2011).

## Chapter 5

# Impact of Heat and UVC Treated Semolina on Pasta and Bread Making

### IMPACT OF HEAT AND UVC TREATED SEMOLINA ON PASTA AND BREAD MAKING

#### 5.1 INTRODUCTION

Traditionally, *Triticum turgidum, subsp. durum* is being used for semolina production which is the raw material for pasta making, since dough made from this type of wheat shows ideal structural properties for manufacturing parameters of the product. In southern Italy, durum wheat is used not only for pasta production, but also for bread making. In particular, in 2003, the bread of Altamura, thoroughly widespread, was recognized as a Protected Denomination of Origin (PDO) product, that is attributed to the foodstuffs originated and totally produced in a specific geographical area (Raffo et al, 2003). This product is obtained from re-milled semolina of single or combined Appulo, Arcangelo, Simeto and Duilio durum wheat cultivars from Altamura in Apulia, Southern Italy.

The semolina properties for pasta production are determined by the additive effects of protein content and gluten network in the semolina, therefore, durum wheat verities are in high demand because of its enhanced protein content and gluten quality (Mariani et al, 1995).

Re-milled semolina is characterized by smaller size of particles (70% below 180  $\mu$ m) and a higher hydration rate than semolina, while the main properties of Altamura bread are a long shelf life, linked to the higher water binding capacity of durum wheat flour, and a low loaf volume. In addition this product is appreciated by consumers for its typical sensory features and nutritional attributes due to the presence of higher protein content (Raffo et al, 2003) and carotenoid pigments with provitamin A activity (Pasqualone 2017).

Chapter 5

The sensory features include a thick brown crust with a typical toasted odor, coupled to a yellow and dense structure of crumb showing a high firmness and a coarse grain, accompanied by a marked sour taste and odor. The network structures and their behavior during the process depend on both proteins and state of flour starch and widely affect the physicochemical features of baking products. The texture of this type of products is associated to gas-retention ability during leavened and cooking, proportional to the number size and distribution of generated bubbles (Shibata et al, 2011). Therefore, changes consequent to pre-treatments under specific physicochemical conditions could produce technological variations interesting to investigate. About that, the most widely used procedure is the heat and UV treatment of flours at different time and temperatures.

These treatments can be applied to flours to reduce the moisture with a change of bacteriological properties, to prolong the shelf life or to decrease the risk of moulds development, to degrade some mycotoxins (Shanakhat et al 2019). In parallel, it is reported that these treatments on wheat flour could improve cake and bread quality, since they allow to obtain a slowed retrogradation of amylopectin, a finer texture, moist crumb and sweeter taste (Purhagen et al. 2011; Neill, Al-Muhtaseb, & Magee, 2012). Interestingly, denaturation of the proteins and enzymes in the treated flour increases batter expansion, preventing the collapse during baking and conferring higher volume and stability to the product (Sahin et al, 2008).

Dry heating of wheat flour at 120°C for 30 min increased batters viscosity, due to the formation of a stronger gel network (Meza et al., 2011) and the volume of Kasutera cake (Nakamura, Koshikawa & Seguchi, 2008). In addition, a positive effect on baking quality was found after treatment by fluid bed dryer, as a consequence of batter viscosity increase, linked to denatured gluten with reduced extensibility and a partial starch granule gelatinisation. (Neill, Al Muhtaseb

& Magee 2012). In a recent study, Shanakhat et al (2019) found a high impact of fluid bed drying treatment on semolina dough properties as revealed from some farinograph indices, well correlated with parameters obtained from calorimetric analysis and modifications in microstructure observed by SEM.

Although several studies were published about the effect of irradiations and heat treatments on flour, no information concerning the effect of these processing techniques on re-milled durum wheat and its technological properties during pasta and bread making are available.

This spart of the thesis project aims to investigate the technological performance of re-milled durum wheat from Altamura submitted to UVC irradiation and fluidized bed drying, applied at different time and temperature. In particular, the treated semolina has been used for pasta and bread processing. In the first case, the effects of the physical treatments were evaluated monitoring spaghetti quality parameters and microstructure. In the second case, the effects of the physical treatments of the semolina were analyzed both on the mixing and leavening phases, and on the characteristics of the bread.

#### 5.2 MATERIALS AND METHODS

#### 5.2.1 Materials

Durum wheat semolina purchased at a supermarket in Naples (Italy) was used for pasta making; re-milled durum wheat was purchased from a company in Altamura (Bari, Italy) for breadmaking. Both types of semolina were placed into plastic containers at room temperature until the analyses.

#### 5.2.2 Thermal and UVC Treatments

The thermal treatments were applied by heating 300 g of re-milled durum wheat at 90, 120, and 150°C for different time (5, 15, 30 min), using a fluidized bed dryer (Sherwood Scientific Model MK11, UK). The temperature was adjusted controlling the front of the dryer and the air flow speed was set at level 4. Temperature and air velocity were verified before each experimental test by a digital thermometer (range -50 -300°C±1°C) and a hot wire anemometer (range 0.4-30 m/s ±3%) respectively. For each treatment, moisture content analyses were performed according to ISO Standard No. 712.

The second treatment consisted of UVC irradiation (254 nm), applied for 120 min. A germicidal UV Lamp, with 30 W (G30T8) power providing UVC radiation at 254 nm, was obtained from Sankyo Denki, Japan. 100 g of re-milled durum wheat semolina, spread in trays 50x30 cm, were exposed to radiations at a distance of 15 cm. For each treatment, moisture content analyses were carried out according to ISO Standard No. 712.

#### 5.2.3 Pasta Processing

For pasta processing, we selected two treatments (UVC for 120 min; heating at 150°C for 30 min) which have shown the reduction in mycotoxins (as reported in chapter 4). In order to obtain a semolina dough with 32% wet content, 1500 g of untreated and treated semolina (UVC for 120 min; heating at 150°C for 30 min) were put in a batch mixer (Kitchen Aid Inc.) for 15 min with warm water at the temperature of 45°C according to a protocol previously optimized in our laboratory. Semolina dough was processed into spaghetti using a laboratory single-screw extruder (Sercom-provided by Nestlè R&D Centre of Kemptthal) equipped with a screw of 350 mm length and 35 mm diameter. The mixture was placed into the feed section of the extrusion unit and feed into the extrusion barrel with regularity by a paddle mixer. The paddle speed during extrusion was set at 100 rpm and kept constant during process as such as dough composition and moisture level. Water hailing from an external thermostatic bath (Regoplas P140S, St. Gallen, CH) circulated in the external jacket of the extruder channel and kept barrel temperature constant (45°C). The bath was set to 54°C to have a die temperature of 45°C, since heat dispersion through the pipes system occurred. The selected die geometry was for the spaghetti production. Successively, spaghetti samples were collected and analyzed for quality performance.

#### 5.2.4 Pasta Quality

Quality performance evaluations were carried out on produced pasta (dried down at room temperature for 48 h). Effect of both thermal and UVC treatments were evaluated by the analysis of pasta quality parameters such as: increase of weight after immersion (g), increase of length (%), increase of diameter (%) moisture (%), increase of weight after cooking (%) starch loss (fresh) from cooked pasta and loss of soluble solids (g).

The water absorption was evaluated by immersing 100 g of pasta in 2 L of cold water. After 3 min of immersion, spaghetti were dried on paper and weighted: the amount of absorbed water was calculated as differences between initial and final weight. Similarly, the % increase in length and diameter of spaghetti was also measured as the difference of initial and final (after 3 min of immersion in cold water) length and diameter respectively. Starch analysis of pasta was evaluated by cooking test. Spaghetti (100 g) were cooked for 7 min (time necessary for the disappearance of the core), in 0.5 L of boiling water. The % increase in weight after cooking was calculated immediately as the difference between weight before and after cooking. For starch measurement, 100 mL of the water (in which pasta was cooked), taken and centrifuged at 10000 rotations for 35 min. The pellet obtained after centrifugation was evaluated as starch loss (fresh) from the pasta during cooking. The supernatant was separated, weighted and dried in an oven in vacuum at 50°C, until a constant weight was achieved to calculate the soluble solids. Three replicates for each measurement were carried out.

#### 5.2.5 Pasta Microstructure

Scanning electron microscopy (SEM) was carried to investigate the microstructure of spaghetti, prepared with both treated and untreated semolina. Lyophilized samples were mounted on specimen stubs and *Au*-coated by DC sputtering (AGAR B7340) in order to make the specimen conductive. Spaghetti were cut and oriented in both longitudinal and transverse directions before the *Au*-coating. The coated specimens were then observed by using a LEO EVO 40 scanning electron microscope (Zeiss, Germany), with a 20kV acceleration voltage. The representative micrographs from all the samples were selected.

#### 5.2.6 Bread Dough Mixing

Bread doughs, prepared with both heat treated and untreated semolina, were prepared by using a Farinograph-AT Brabender (Duisburg, Germany), fitted with 50 g mixing bowl. Re-milled wheat grain, 50 g; water, 36 g; NaCl, 0.65 g; yeast, 0.37 g were taken for dough preparation. Moisture content of doughs from treated semolina was adjusted at 47%, in comparison with control samples. For each blend, the farinographic development time was used as the mixing time. Doughs from treated and untreated semolina were prepared in triplicate and the consistency at the end of mixing, (the time of dough development+2 min)was measured. At the end of each test, the moisture content of the obtained dough was determined according to ISO Standard No. 712. Water activity of the dough samples was measured at 25 °C in an AquaLab (Decagon Devices, Inc, Pullman andEUA)

#### 5.2.7 Bread Dough Leavening

62 g of dough were taken just after mixing and placed on a flat surface where it could expand in every directions without constrains during a leavening stage. The dough was incubated at  $36 \pm 1^{\circ}$ C, 70 % U.R for 180 min. The following parameters were continuously and automatically recorded:

- internal humidity and temperature by means of data logger (Logger Escort mod. 10D8, Gamma Instrument s.r.l., Naples, Italy)
- Volume expansion by means of a camera Olympus<sup>®</sup> C-7070Wide ZOOM camera (Olympus, Milan, Italy) mounted on a photographic bench.

Dough evolution during leavening phase was studied by measuring the variation in time of the total volume of the sample by means of Image Analysis software (Image Pro Plus 6.1 for

Windows<sup>®</sup>, Media Cybernetics Inc.). The volume V of dough was calculated as reported by Romano et al. (2013). Each average value represents the mean of 3 - 7 independent measurements.

#### 5.2.8 Bread quality

Bread samples were prepared considering the treated semolina dough which had shown the best (120°C for 30 min) and the worst (150° for 30 min) behavior during leavening. A control bread sample was also prepared with untreated semolina. 2500 g of dough were prepared according to the recipe reported in the precedent paragraph. The dough was prepared in a mixer, using the farinographic development time as the mixing time. 800 g of dough prepared from each treatment was aliquoted in aluminum molds (25x15 cm) and the leavening was carried out according the conditions previously described for the measurements of volume dough expansion. The time of leavening for control and dough from thermally (120°C 30 min) treated semolina was the same (100 min). Whereas, time of leavening for dough obtained from semolina treated at 150°C 30 min was 70 min.

Baking was carried out in a conventional electric oven (Moretti Forni S.p.A., Pesaro, Italy) at 180°C for 110 min. Three lots were produced for each selected treatment and three bread loafs were obtained for each lot.

#### **5.2.8.1 Moisture Content**

The moisture content ofboth the crust and the crumb of bread samples, weredetermined in triplicate for each loaf by the AACC method (number 44-15.02, 1999). The results were calculated as percentage of water per sample weight (%).

#### 5.2.8.2 Water Activity

Water Activity of both the crust and the crumb the bread samples were measured at 25 °C in an AquaLab (Decagon Devices, Inc, Pullman andEUA), in triplicate for each bread loaf.

#### 5.2.8.3 Bubble Structure of the Loaf

Bread samples were cut into 20 mm thick slices using an electrical knife. Six samples were taken from the middle of the loaf. 2 D loaf slices images were analysed using an image analysis protocol as reported by Romano et al. (2013) with some modifications. Subsequent to cell detection feature extraction was performed for each sample analysed. Average gas bubble area fraction (AF) was calculated using the following equation:

$$AF(\%) = \frac{\sum_{i=1}^{n} A_{i}}{Ad}$$
(1)

Where n, was number of bubbles counted; Ad was area loaf section and Ai was bubble area.

Each result is the average of three different test bread production runs.

#### 5.2.8.4 Bread Hardness

Six slices 20 mm thick from each loaf were subjected to a compression test by means of an Instron Universal Testing Machine (Instron Ltd., mod. 4467, High Wycombe, GB), equipped with a 1 kN load cell. Cylindrical samples (diameter 16 mm, height 16 mm) were placed between parallel plates and compressed to a final deformation of 80%, at a crosshead speed of 60 mm/min. For each loaf, five measurements were performed. True stress–Hencky strain relationships were derived from load-displacement curves.

#### 5.2.9 Data Analysis

All the parameters were evaluated in three trials and three analytical replicates and expressed as mean value  $\pm$  standard deviation. Differences among control and treated samples were determined by using SPSS (Statistical Package for Social Sciences) Package 6, version 15.0 (SSPS Inc., Chicago, IL, USA). Significance was determined by Anova (Duncan's test) at a significance level of 0.05. (for me it is bettet to eliminate this space)

#### 5.3 **RESULTS AND DISCUSSION**

#### 5.3.1 Pasta Quality

Table 1 shows the data about pasta quality obtained from untreated and treated (UVC and thermal) semolina. Water absorption after immersion in cold water for 3 min determined an increase of weight equal to  $10.00\pm2.55\%$ ,  $12.22\pm0.68\%$  and  $20.41\pm0.02$  in spaghetti obtained from control and those obtained from UVC and heat treated semolina respectively. The increase in weight after immersion was not significantly different between the analysed samples of control and UVC treated, while for thermally treated semolina it was significantly higher than the control that could be attributed to damnaged structure of the starch after application of high temperature causing the increase of water absorption. We did not found a significant change in the % increase of length after the immersion in cold water for control ( $1.53\pm0.50$ ), UVC ( $1.15\pm0.83$ ), and thermally ( $0.87\pm0.12$ ) treated samples. Whereas, the % increase in diameter after immersion was significantly higher for the spaghettis obtained from the UVC ( $10.33\pm4.53\%$ ) and thermally ( $12.84\pm2.04$ ) treated semolina as compared to control ( $4.92\pm0.52$ ) samples.

The moisture content of pasta (after dried down at room temperature for 48 h) was not significantly different for UVC treated ( $16.17\pm0.06\%$ ) and control ( $17.68\pm0.09\%$ ), whereas for thermally treated the moisture content was higher to  $21.38\pm1.24\%$ . A decreasing trend was observed in % increase of weight after cooking,  $121.23\pm12.69\%$  and  $111.98\pm4.05$  for pasta from UVC and thermally treated semolina respectively compared to the control ( $128.30\pm3.26\%$ ), while the increase of the length was not different in all samples. The soluble solids in boiling water and fresh starch loss found in UVC treated sample was equal to  $0.61\pm0.21\%$  and  $7.33\pm0.51\%$  compared to the control ( $0.46\pm0.18\%$  and  $7.44\pm1.51\%$  respectively). While, in

thermally treated semolina the soluble solids  $(0.81\pm0.02\%)$  and strach loss from fresh pasta  $(9.02\pm0.76)$  was significantly higher than control.

Hydration of the pasta is verified as an effect of diffusion-controlled process, and the temperature-moisture conditions induce the gelatinization of starch, with an increase of starch solubilisation. Starch morphological changes range from a strong swelling and partial disintegration in the outer layer of the strand to a slight swelling in the centre (Sozer et al, 2010 in Kalnina 2015). From a macromolecular point of view, pasta can be assimilated to a large protein network with protein–protein crosslinks containing starch granules. Therefore, the differences of some quality values after immersion in water and cooking could be explained by a likely modification of protein-starch network after the UVC irradiation with a consequent release of starch from gluten network and the loss of moisture from thermally treated semolina.

The quality of the pasta obtained from the semolina treated at UVC was not significantly different from the control, as we have observed by the analysis of different quality parameters. Therefore, this treatment can be applied for the reduction of mycotoxins and enhancing the shelf life of semolina without having negative impact on the pasta processing and performance.

Table 1: Quality parameters of spaghetti prepared with untreated (Control), UVC (120 min) and

Parameters	Control	UVC 120 min	150°C 30 min
Weight increase after immersion (g)	10.00±2.55 <sup>a</sup>	12.22±0.68 <sup>a</sup>	20.41±1.52 <sup>b</sup>
Increase of length (%)	1.53±0.50 <sup>a</sup>	1.15±0.83 <sup>a</sup>	0.87±0.12 <sup>a</sup>
Increase of diameter (%)	4.92±0.52 <sup>a</sup>	10.33±4.53 <sup>ab</sup>	12.84±2.04 <sup>b</sup>
Moisture (%)	17.68±0.09 <sup>a</sup>	$16.17 \pm 0.06^{a}$	21.38±1.24 <sup>b</sup>
Increase of weight after cooking (%)	128.30±3.26 <sup>a</sup>	121.23±12.69 <sup>a</sup>	111.98±4.05 <sup>a</sup>
Starch loss (fresh) from cooked pasta	7.44±1.51 <sup>a</sup>	7.33±0.51 <sup>a</sup>	9.02±0.76 <sup>b</sup>
Loss of Soluble solids (g)	0.46±0.18 <sup>a</sup>	0.61±0.21 <sup>ab</sup>	0.81±0.02 <sup>b</sup>

heat (150°C 30 min) treated semolina.

Mean (n=3)  $\pm$  standard deviation; Different letters in the same column indicate significant differences (p<0.05).

#### 5.3.1.2 Pasta Microstructure

In Fig 1 the micrographs of uncooked spaghetti were shown in both longitudinal (fig.1 a-c) and transverse (fig.1 e-f) sections, obtained from control, UVC treated and thermally treated (150°C for 30 min) semolina, taken at 1900X magnifications. Pasta microstructure was the result of all the changes occurred during the making process, mainly affected by the protein and starch fraction. During extrusion, glutenins and gliadins molecules reorganize by stretching and straightening, because of the mechanical energy provided, forming a gluten network that trapped the starch granules. In fig 1 starch granules are well recognized from proteins, since all samples are characterized by starch granules embedded in a dense protein network.

In Fig 2 the micrographs of spaghetti cooked for 7 min (optimal time) were shown in both longitudinal (fig.2 a-c) and transverse (fig.2 e-f) sections, obtained from control, UVC treated and thermally treated (150°C for 30 min) semolina, taken at 1900X magnifications. The main phenomena involved during cooking are starch gelatinization and protein coagulation. The modifications after the cooking are expressed as the deformation of granules and the resulting discontinuities in the gluten net. This type of structure was observed both in the surface and in transverse section, as observed by Stuknyte et al, 2013. Same of the samples (Fig.2 a,b,c,d) shown a pronounced morphological modification of starch granules, that presented a shape of a flatted disk, also observed by Cocci et al. (2008), probably due to a greater degree of starch granules and protein network, the structure is compact enough. In both uncooked and cooked spaghetti, apparent differences of the sample structures are not evidenced between pasta obtained from semolina control and UVC treated semolina.

Fig 1: Microstructure of uncooked spaghetti in longitudinal section (on the left) and transverse section (on the right) obtained from control (a and d); UVC treated (b and e) and thermally

treated (c and f) semolina, taken at 1900X magnifications



(f) Heat treatedSpaghetti (transverse)1900 X

**Fig 2**: Microstructure of cooked spaghetti in longitudinal section (on the left a-c) and transverse section (on the right d-f) obtained from control; UVC treated and thermally treated (150°C for 30





(c) Heat treatedSpaghetti (longitudinal)1900 X

(f) Heat treatedSpaghetti (transverse)1900 X

#### 5.3.2 Bread Dough Mixing

Table 2 represents the moisture content (%), water activity (Aw) and consistency of the bread dough at the end of mixing obtained from the control and thermally treated semolina.. The moisture content of the dough obtained from control semolina was equal to 47.56±0.11%, which was comparable to the values of all other treated doughs. The amount of water used for the preparation of dough from the thermally treated semolina was adjusted to reach the same moisture content in doughs;therefore, when the treatments were more sever (120°C and 150°C), a higher amount of water in dough preparation was necessary to reach the same moisture content.

Another trend was observed for the water activity (Aw) of dough, from the level of  $0.908\pm0.016$  in control, increased, after heating treatment, at 90°C, to the values of 0.926, 0.929, 0.945 after 5, 15, 30 min; at 120°C to the values of 0.929, 0.951 and 0.930 after 5, 15, 30 min; and at 150°C to the values of 0.918, 0.984 and 0.936. The consistency at the end of mixing of dough obtained from Brabender farinograph, indicating a high impact of thermal treatment on the structure and properties of durum wheat doughs. The increase of dough consistency at the end of mixing of was directly proportional to the intensity of thermal treatment; mean value was equal to 244.66±2.51 BU in control, and increased from  $307.00\pm18.33$  to  $328.40\pm63.35$  BU in doughs obtained from samples treated at 90°C from 5 to 30 min respectively, the mean value incrreased260.15±25.31 to 429.5±26.16 BU in doughs obtained from re-milled durum wheat treated at 120°C from 5 to 30 min respectively, and from  $351.66\pm19.62$  to  $465.66\pm26.40$  BU in doughs obtained from samples treated at 150°C from 5 to 30 min respectively.

Mixing behaviour of dough reveals that flour particles hydrated more slowly as a consequence of a more rigid structure formed by the cross-linked gluten protein for the decreased amount of free SH groups after the treatment. The significant differences of dough consistency after the thermal treatments are due, in particular, to the proteins denaturation starting at a temperature range of 50 to 80°C, with a consequent reduction of their solubility in water (Mann, Schiedt, Baumann, Conde-Petit & Vilgis, 2014).

**Table 2**: Moisture, water activity, consistency of bread doughs prepared with untreated and heat treated semolina

Sample	Moisture Content	Water activity	Consistency
	(%)		(UB)
Control	47.56±0.11 <sup>b</sup>	0.908±0.016 <sup>a</sup>	244.66±2.51 <sup>a</sup>
90°C 5 min	46.20±0.01 <sup>ab</sup>	$0.926 \pm 0.007^{abc}$	307.00±18.33 <sup>abc</sup>
90°C 15 min	47.51±0.38 <sup>b</sup>	0.929±0.006b <sup>cd</sup>	328.40.5±63.35 <sup>bc</sup>
90°C 30 min	47.41±0.49 <sup>b</sup>	0.945±0.029 <sup>cde</sup>	325.5±31.81 <sup>abc</sup>
120°C 5min	47.57±0.10 <sup>b</sup>	0.930±0.008b <sup>cde</sup>	264.00.33±60.50 <sup>ab</sup>
120°C 15 min	47.31±0.55 <sup>b</sup>	0.951±0.023 <sup>e</sup>	260.15±25.31 <sup>ab</sup>
120°C 30 min	46.66±0.29 <sup>ab</sup>	$0.929 \pm 0.05^{bcd}$	429.5±26.16 <sup>de</sup>
150°C 5 min	47.27±0.25 <sup>b</sup>	0.936±0.004b <sup>cde</sup>	351.66±19.62 <sup>bcd</sup>
150°C 15 min	46.45±0.42 <sup>ab</sup>	$0.984 \pm 0.004^{\text{f}}$	359.50±13.43 <sup>cd</sup>
150°C 30 min	47.69±0.04 <sup>b</sup>	0.918±0.002 <sup>ab</sup>	465.66±26.40 <sup>e</sup>

Mean (n=3)  $\pm$  standard deviation; Different letters in the same column indicate significant differences (p<0.05).

#### 5.3.3 Bread Dough Leavening

The most apparent physical change related to the development of leavening in the dough is the increase of its volume (Romano et al., 2013). The volume expansion ratio of the dough was investigated by means of Image Analysis protocol at the end of leavening times. Fig. 3 represents the volume expansion ratio of semolina dough obtained from control and thermally treated semolina at the end of leavening. Significant increase in volume of doughs was observed from semolina treated at 90°C (5 min) and 120°C (5, 15, 30 min) as compared to control. While, decrease in volume for the doughs produced from the semolina treated at 150°C (5, 15, 30 min).

Although it is assumed that mainly starch properties are affected during heat treatment (Keppler et al., 2018), it does affect gluten extensibility (Neill and Al-Muhtaseb, 2012). Van Steertegem et al. (2013) reported crosslinking of protein in flour particles as a result of flour heat treatment. Therefore the effect of different heat treatments evaluated on the bread doughs during the leavening could be explained by the formation of gluten aggregates in the flour treated at a very higher temperature at 150°C (5, 15, 30 min), resulting in decreased protein contents and lower network strength in dough as reported in a study of Mann et al (2014) that heat treatment can modify the interactions between gluten and starch network of dough.Gluten is a complex molecule consisted of glutenin (polymeric), which plays a role in dough's elasticity and strength (Khatkar, 2006) and gliadin (monomeric), which is responsible for dough extensibility and viscosity (Wieser et al, 2006). The volume expansion in dough depends on an appropriate balance of glutenina and gliadin (Khatkar et al, 1995). The insufficient gluten elasticity can result in decreased volume of dough, while increase in elastic gluten leads to higher volume (Hoseney, 1994). The effects of low volume expansion were more pronounced in the doughs obtained from the semolina treated at very high temperature (150°C), while for treatments at lower temperatures has shown an increase in volume expansion. These changings were marked by the modifications in gluten starch network due to heat treatments.

**Fig. 3**: Effects of heat treatments (90°C, 120°C, 150°C for 5, 15 and 30 min)onvolume expansion ratio of semolina dough at the end of leavening



#### 5.3.4 Bread quality

Table 3 shows the quality parameters of bread such as moisture content and water activity of crumb and crust of bread obtained from control durum wheat and from treated sample at 120°C and 150°C for 30 min. We selected these two treatments of longer time (30 min) because it is known from literature and our study (chapter 4) that longer time of treatment have better effect on mycotoxins degradation and meanwhile they have shown the same improvement of leavening like similar treatments of shorter time (fig. 3)

The mean moisture values in crumb are similar for all the samples (from 47.00% in bread from treated sample at 150°C for 30 min to 47.66% in control), as well as moisture in crust (from 22.82% in control to 23.77% in bread from treated sample at 120°C for 30 min). Also the activity water in both crumb and crust was not significantly different in bread from treated samples compared to the control, where the values were 0.957 and 0.881 respectively.

The moisture levels reported in baked wheat soft flour bread were 43.48% in crumb and 18.16% in crust (Gao et al, 2015). These differences are associated to the higher water binding capacity of durum wheat flour, known to be responsible of the prolonged shelf life of the product and a softer crumb. This feature, due to the presence of damaged starch after the re-milling and to the high amount of proteins, is associated to higher productive yield for bread production: it is reported that from a quintal of re-milling durum wheat about 130 kg of bread are obtained. This property can also be due to the high content of ferulic acids, known for its antioxidant, antimicrobial, anti-inflammatory, and anti-cancer activities, that is twice compared to that present in soft flour (Quaglia et al 2001).

174

**Table 3**: Moisture content and water activity of the bread (crumb and crust) from control(untreated) and thermally treated semolina at 120°C 30 min and 150°C 30 min

Sample	Moisture of crumb (%)	Moisture of crust (%)	Aw crumb	Aw crust
Control	47.66±0.82 <sup>b</sup>	22.82±2.16 <sup>a</sup>	0.957±0.006 <sup>a</sup>	0.881±0.187 <sup>a</sup>
120°C 30 min	47.63±0.40 <sup>b</sup>	23.77±2.67 <sup>a</sup>	0.957±0.004 <sup>a</sup>	0.864±0.483 <sup>a</sup>
150°C 30 min	47.00±0.32 <sup>a</sup>	23.02±2.12 <sup>a</sup>	$0.954 \pm 0.006^{a}$	$0.869 \pm 0.188^{a}$

Mean (n=3)  $\pm$  standard deviation; Different letters in the same column indicate significant differences (p<0.05).

Image analysis was carried out to examine the effect of heat treatment on gas bubble area fraction (AF) in bread crumb. In figure 4 characteristic images of central slices of breads made with: a) untreated semolina (control), b) treated semolina at 120 °C (B) for 30 min, c) treated semolina at 150 °C (C) for 30 min are reported.

**Fig. 4:** Characteristic images of central slices of breads made with: a) untreated semolina (control), b) treated semolina at 120 °C (B) for 30 min, c) treated semolina at 150 °C (C) for 30

min



Chapter 5

A statistically representative sample of bubbles can be identified from the images by using an image analysis procedure. This approach is simple, direct and often the easiest way to get reliable quantitative information based on digital images. Results of the object analysis of breads are illustrated in Table 4. The bread quality was evaluated by gas bubble area fraction (AF) and the crumb firmness measurements in the bread loaf.

Table 4 shows AF (gas bubble area fraction) and the hardness of bread from control (untreated) and thermally treated semolina at 120°C 30 min and 150°C 30 min. The gas bubble area fraction in control bread had a mean value of  $29.6\pm1.4$  %, showing a significant difference from the treated samples. A decrease to a value of  $26.7\pm0.3$  % and  $17.7\pm1.5$ % in gas bubble area was observed in bread slices obtained from semolina treated at 120°C 30 min and 150°C 30 min respectively. A significant decrease in the gas bubble area as compared to control can be explained as the negative effect of higher temperature ( $150^{\circ}$ C for 30 min), which has lower the ability of gluten network in thermally treated semolina, to retain the carbon dioxide produced during the process of fermentation (Barak et al, 2013). Baking properties are affected by the quantity of the gluten present in the flour (Gomez et al, 2011), which plays an important role in determining both, the crumb appearance and firmness of cereal-based products (Demirkesen et al, 2010).

Hardness was measured as the stress required to deform the bread of 30 %; hardness of the control bread had a mean value of  $0.004\pm0.00$  N/mm<sup>2</sup>, significantly increased to  $0.005\pm0.001$  and  $0.020\pm0.007$  N/mm<sup>2</sup> for bread produced from the semolina treated at  $120^{\circ}$ C 30 min and  $150^{\circ}$ C 30 min respectively.

These results were well correlated to the data obtained from image analysis, since the crumb hardness was also significantly increased in the bread obtained from the thermally treated semolina, thus, lowering the bread quality. The reasons of higher crumb firmness could be explained due to poor gluten quality and lower loaf volume. Previous studies have reported the negative impact of heat treatment on the gluten proteins (Barak et al, 2013), and that the bread quality can also be influenced by the rheological properties of the doughs (Gras et al, 2000) as well as other components present in the wheat flour (Dowell et al, 2008; Edward et al, 2007).

The quality of the bread obtained from the semolina treated at  $120^{\circ}$ C for 30 min was not significantly different (p< 0.005)from the control, as we have observed by the bubble area fraction and hardness. Therefore, this treatment can be applied for reduction of mycotoxins and the improvement in shelf life of semolina based products without having any negative impact on the technological properties.

**Table 4:** Gas bubble area fraction (AF%) and hardness of the bread from control (untreated) andthermally treated semolina at 120°C 30 min and 150°C 30 min.

Sample	AF (%)	Hardness
		$(N/mm^2)$
Control	29.6±1.4 <sup>c</sup>	$0.004 \pm 0.001^{a}$
120°C 30 min	26.7±0.3 <sup>b</sup>	0.005±0.001 <sup>a</sup>
150°C 30 min	17.7±1.5 <sup>a</sup>	0.020±0.007 <sup>b</sup>

Mean (n=3)  $\pm$  standard deviation; Different letters in the same column indicate significant differences (p<0.05).

#### 5.4 CONCLUSIONS

Technological performance of semolina submitted to heat and UVC irradiation at different time and temperature was analysed. In particular, the impact of these treatments was investigated during pasta and bread processing. For pasta production, we examined the impact of heat and UVC irradiations on its quality parameters and no significant change in pasta quality was observed for UVC treatment. We did not found any apparent differences of the sample structures of both uncooked and cooked spaghetti obtained from control; heat and UVC treated semolina, as evidenced by microstructures observed through SEM analysis.

The impact of heat treatment on the technological parameters of bread baking was studied, by investigating the dough consistency at the end of mixing, leavening behaviour of dough and the bread loaf characteristics. We found an improvement in the leavening phase of dough obtained from the thermally treated semolina at 120°C for 30 min. The treatments of higher temperature and longer time were investigated for bread baking characteristics due to their potential role in mycotoxins degradation, confirming our prior study. The quality of the bread obtained from the semolina treated at 150°C for 30 min was impaired by high temperature, as a consequence of damage of the starch and gluten network. We did not found any significant change in the quality of bread obtained from the semolina treated at 120°C for 30 min, as revealed by the moisture content, water activity of bread crumb, bubble area fraction and Instron analysis.

Therefore this treatment can be applied for reduction of mycotoxins and in the improvement of shelf life of semolina based products without having any negative impact on the technological properties.
## 5.5 **REFERENCES**

- Barak S, Mudgil D, and Khatkar BS, Relationship of gliadin and glutenin proteins with dough rheology, flour pasting and bread making performance of wheat varieties. *LWT* -*Food Sci Technol*, **51**: 211-217 (2013).
- 2. Coccia E, Sacchetti G, Vallicellia M, Angiolonia A, and Rosa MD, Spaghetti cooking by microwave oven: Cooking kinetics and product quality. *J Food Eng*,**85**: 537-546 (2008).
- Demirkesen I, Mert B, Sumnu G, and Sahin S, Rheological properties of gluten free bread formulations. *J Food Eng*, 96: 295-303 (2010).
- 4. Dowell FE, Maghirang EB, Pierce RO, Lookhart GL, Bean SR, and Xie F, Relationship of bread quality to kernel, flour and dough properties. *Cereal Chem*,**85**: 82-91(2008).
- Edwards NM, Preston KR, Paulley FG, Gianibelli MC, Mc-Caig TN, and Clarke JM, Hearth bread baking quality of durum wheat varying in protein composition and physical dough properties. *J Sci Food Agric*, 87: 2000-2011 (2007).
- Gao J, Wong JX, Chu-ShernLim J, Henry J, and Zhou W, Influence of bread structure on human oral processing. *J Food Eng*, 167: 147-155 (2015).
- Gomez A, Ferrero C, Calvelo A, Anon M C, and Puppo MC, Effect of mixing time on structural and rheological properties of wheat flour dough for bread making. *Int J Food Prop*, 14: 583-598 (2011).
- Gras PW, Carpenter HC, and Andersen RS, Modelling the developmental rheology of wheat-flour dough using extension tests. *J Cereal Sci*, **31**: 1-13 (2000).
- Hoseney RC, Principles of cereal science and technology (2nd ed.). St. Paul, Minnesota, USA: American Association of Cereal Chemists, Inc. (1994).

- 10. ISO 712:1985 Cereals and cereal products Determination of moisture content (Routine reference method).
- 11. Kalnina S, and Rakcejeva T, Investigation of physicochemical, sensory characteristics, cooking and texture properties of whole grain pasta. *Chemine Technol*, **1**: 66 (2015).
- Keppler S, Bakalis S, Leadley C, Sahi S, and Fryer P, Evaluation of dry heat treatment of soft wheat flour for the production of high ratio cakes. Food Res Int, 107: 360-370 (2018).
- Khatkar BS, Bell AE, and Schofield JD, The dynamic rheological properties of glutens and gluten subfractions from wheats of good and poor bread-making quality. *J Cereal Sci*,22: 29-44 (1995).
- 14. Khatkar BS, Effects of high Mr glutenin subunits on dynamic rheological properties and bread making qualities of wheat gluten. *J Food Sci Technol*,**43**: 382-387 (2006).
- Mann J, Schiedt B, Baumann A, Conde-Petit B and Vilgis TA, Effect of heat treatment on wheat dough rheology and wheat protein solubility. *Food Sci Technol Int*, **20**: 341-351 (2014).
- Mann J, Schiedt B, Baumann A, Conde-Petit B, and Vilgis TA, Effect of heat treatment on wheat dough rheology and wheat protein solubility. *Food Sci Technol Int*, **20**: 341-351 (2014).
- 17. Meza BA, Chesterton AKS, Verdini RA, Rubiolo AC, Sadd PA, Moggridge GD and Wilson DI, Rheological characterisation of cake batters generated by planetary mixing: Comparison between untreated and heat-treated wheat flours. *J Food Eng*, **104**: 592-602 (2011).

- Nakamura C, Koshikawa Y, and Seguchi M, Increased volume of Kasutera Cake (Japanese Sponge Cake) by dry heating of wheat flour. *Food Sci Technol Res*, 14: 431-436 (2008).
- 19. Neill G, Al-Muhtaseb AAH, and Magee TRA, optimization of time/temperature treatment, for heat treated soft wheat flour. *J Food Eng*, **113**: 422-426 (2012).
- 20. Neill G, Al-Muhtaseb AH and Magee TRA, Optimisation of time/temperature treatment, for heat treated soft wheat flour. *J Food Eng*,**113**: 422-426 (2012).
- 21. Pasqualone A, Laddomada B, Centomani I, Paradiso VM, Minervini D, Caponio F, and Summo S, Bread making aptitude of mixtures of re-milled semolina and selected durum wheat milling by-products. *LWT - Food Sci Technol*, **78**: 151-159 (2017).
- 22. Purhagen JK, Sjöö ME, and Eliasson A-N, The use of normal and heat-treated barley flour and waxy barley starch as anti-staling agents in laboratory and industrial baking processes. *J Food Eng*, **104**: 414-421 (2011).
- 23. Quaglia M, Chenon K, Hall AJ, De-Lorenzi E, and Sellergren B, Target Analogue Imprinted Polymers with Affinity for Folic Acid and Related Compounds. *J Am Chem Soc*, **123**: 2146-2154 (2001).
- 24. Raffo A, Pasqualone A, Sinesio F, Paoletti F, Quaglia G, and Simeone R, Influence of durum wheat cultivar on the sensory profile and staling rate of Altamura bread. *Eur Food Res Technol*, **218**: 49-55 (2003).
- 25. Romano A, Cavella S, Toraldo G, and Masi P, 2D Structural imaging study of bubble evolution during leavening. *Food Res Int*,**50**: 324-329 (2013).

- 26. Sahin S, Cake batter rheology. In: Sumnu, S.G., Sahin, S. (Eds.), Food engineering aspects of baking sweet goods (pp 99-117). Florida, USA: CSR Press, Boca Raton (2008).
- 27. Shanakhat H, Sorrentino A, RaiolaA, Reverberi M, Salustri M, Masi P, and Cavella S, Technological properties of durum wheat semolina treated by heating and UV irradiation for reduction of mycotoxin content. *J Food Process Eng*, 13006 (2019).
- 28. Shibata M, Sugiyama J, Tsai CL, Tsuta M, Fujita K, Kokawa M, and Araki T, Evaluation of viscoelastic properties and air-bubble structure of bread containing gelatinized rice. *Proced Food Sci*, 1: 563-567 (2011).
- 29. Sozer N, Rheological properties of rice pasta dough supplemented with proteins and gums. *Food Hydrocoll*, **23**: 849-855 (2009).
- 30. Steertegem BV, Pareyt B, Slade L, Levine H, Brijs K, and Delcour JA, Impact of heat treatment on wheat flour solvent retention capacity (SRC) profiles. *Cereal Chem*, 90: 608-610 (2013).
- 31. Stuknyte M, Cattaneo S, Pagani MA, Marti A, Micard V, Hogenboom J, and De-Noni I, Spaghetti from durum wheat: Effect of drying conditions on heat damage, ultrastructure and in vitro digestibility. *Food Chem*, **149**: 40-46 (2014).
- 32. Wieser H, Bushuk W, and MacRitchie F, The polymeric glutenins. In C. W. Wrigley, F. Bekes, and W. Bushuk (Eds.), Gliadin and glutenin. The unique balance of wheat quality (pp. 213-240). St. Paul, Minnesota, USA: American Association of Cereal Chemists, Inc. (2006).

## PUBLICATIONS

- Shanakhat H, Sorrentino A, Raiola A, Romano A, Masi P and Cavella S, Current methods for mycotoxins analysis and innovative strategies for their reduction in cereals: an overview. *J Sci Food Agric*, **98**: 4003-4013 (2018).
- Shanakhat H, Sorrentino A, Raiola A, Reverberi M, Salustri M, Masi P and Cavella S, Technological properties of durum wheat semolina treated by heating and UV irradiation for reduction of mycotoxin content. *J food process eng*, e13006 (2019).

https://doi.org/10.1111/jfpe.13006

## POSTER PRESENTATION

• Hina Shanakhat, Susan P. McCormick and Matthew G. Bakker, Enrichment Cultures

with the Ability to Biotransform Deoxynivalenol. National Fusarium Head Blight Forum.

December 2 - 4, 2018 St. Louis, MO USA.

