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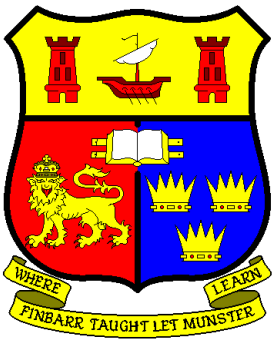

FUNDAMENTAL STUDY ON PERSPECTIVES FOR POST-HARVEST BIOPROTECTION OF CEREAL CROPS

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Declaration

I hereby declare that this thesis is my own work and effort, and that it has not been submitted for another degree, neither at the National University Ireland, Cork nor elsewhere. Where other sources of information have been used, they have been acknowledged.

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Signature

Abbrevations

(N)EW	(neutralised) electrolysed water
(U)SS	(ultra) superheated steam
3-ADON	3-Acetyldeoxynivalenol
3-HPA	3-hydroxypropion aldehyde
ACC	active chlorine concentration
AF	aflatoxin
AMP	antimicrobial peptide
BAC	benzalkonium chloride
CAPP	cold atmospheric pressure plasma
CD	circular dichroism
cfs	cell-free supernatant
cfu	colony forming unit
DAD	dioden array detector
DAS	Desoxyscirpenol
DIW	deionized water
DNA	deoxyribonucleic acid
DON	deoxynivalenol
DON3G	Deoxynivalenol-3-glycoside
DTAC	dodecyltrimethylammonium chloride
e-beam	electron-beam
EFSA	European Food Safety Agency
FA	fatty acid
FB_1	fusarenon B_1
FHB	*Fusarium* head blight
FUS-X	Fusarenone-X
GI	gluten index
HHP	high hydrostatic pressure
IC_{50}	half-inhibitory concentration
LAB	lactic acid bacteria
LDMR	low dose microwave radiation
MAP	modified atmosphere packaging
MIC	minimal inhibitory concentration
MRS	de Man, Rogosa, Sharp
MS	mass spectrometry
NIV	nivalenol

OD	optical density
OTA	ochratoxin A
OTB	ochratoxin B
PBS	phosphate buffered saline
Pcfs	PLA enriched cell-free supernatant
PDA	potatoe dextrose agar
Phe	phenylalanine
PLA	3-phenyllactic acid
PMRS	MRS + 1.5% Phe
PPA	phenylpyruvic acid
QAC	quaternary ammonium compound
RH	relative humidity
RP(U)HPLC	reverse phase (ultra) high performance liquid chromatography
RVA	rapid visco analyser
s/n	signal-to-noise-ratio
SDS	Sodiumdodecylsulfate
SFM	synthetic fungal medium
TPA	texture profile analysis
US	ultrasound
USDA	United States Data Agency
UV	ultra violet light
UV/VIS	ultraviolet/visible
ZEA	zearalenone

Abstract

As a result of the rapidly growing human population, reducing post-harvest crop losses of cereals due to microbial pests has major importance. Therefore, recent advances and emerging technologies for chemical, physical and microbial grain decontamination were reviewed as part of this thesis. It became evident that no single treatment can sufficiently decontaminate cereals without quality deterioration. This thesis investigates the spread of a fungal contamination during storage if the crop is treated inappropriately. During 6 weeks of storage, the ergosterol content (measure of fungal bio-mass) increased from <0.75 ppm to 44 ppm. Substantial levels of mycotoxins, such as deoxynivalenol and zearalenone were found in concentrations up to 953 ppb and 78 ppb, respectively. Also the grain quality was reduced due to the degradation of carbohydrates and proteins by fungal hydrolytic enzymes. Consequently, the gluten quantity and network strength was substantially lowered, inducing dough and bread quality deterioration. This was particularly expressed by the increased dough stickiness, bake loss and open crumb structure. The optimisation of the antifungal performance of *Lactobacillus reuteri* R29 due to increased accumulation of phenyllactic acid (PLA) or reuterin was investigated also. While both compounds efficiently inhibited spore germination of *Fusarium culmorum* (10^6 spores/mL) *in vitro*, only PLA could successfully extend the microbial shelf-life of bread (4 days, 100%). Furthermore, emerging microbial, physical and chemical methods of decontamination, were investigated during cereal storage. While microwave (2 min, 800 W), microwave+NaOCl (5%), sorbate and propionate (both 5%, 10 min), high pressure (10 min, 300 MPa, 30°C) treatment and vacuum packing fully inhibited fungal growth and mycotoxin accumulation, ultrasound, quaternary ammonium compounds, cold plasma and LAB were not successful. However, the dry microwave treatment induced protein denaturation, resulting in poor quality of the resulting dough and breads. Finally, plant derived antimicrobial peptides were studied at the example of the cowpea-thionin II. After extraction and partial purification, the peptide was identified by N-terminal sequencing. The antifungal activity against 3 major food spoilage fungi (10^4 spores/mL) was shown. The extract was found to be resistant against heat treatment but lost its antifungal activity in presence of cations. Finally, it was successfully used to protect stored wheat grains from fungal spoilage. Overall, this thesis provides a valuable contribution to the knowledge regarding fungal spoilage and the possibilities and actual shortcomings of diverse emerging approaches and technologies for food preservation.

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Chapter 1: Introduction

1.1 Introduction

As a result of the rapidly growing human population but limited agricultural space available, providing the globally required nutrition is a continuously increasing challenge. From 2005 – 2050 an increased global crop demand of 100% - 110% was predicted by Tilman et al. (2011). Furthermore, an increased food consumption per-capita was estimated also (Tilman et al., 2011). Besides, recent studies have predicted that current approaches to increase the yield of agricultural crops, such as cereals, will not be sufficient to satisfy the nutritional demands of future generations (Ray et al., 2013). Hence, the reduction of food losses and waste is mandatory to meet these future demands

Among the most frequently grown and consumed foods are cereals and cereal products. Since centuries, cereals play an essential role in human nutrition and hence cover a surface of about 700 million ha worldwide with over 2000 million tonnes of grains produced. Global trade is boosted to a record, where wheat is one of the most important cereals with an estimated global production over 140 million tons with approximately 51 million hectares of harvested area (USDA, 2017), with as much as 25% of the production worldwide being processed into bread (McGee, 2004). As a result, the reduction of crop losses, using efficient and publically accepted methods, would be a substantial contribution to feeding the global population.

The main reason for crop losses of cereals is microbial spoilage caused by a wide variety of organisms, including bacteria, yeasts and filamentous fungi. Apart from in-field spoilage, post-harvest crop losses, mainly due to filamentous fungi, cause enormous economic losses. Approximately 15% of the annual cereal harvest is lost to microbial pests (Freita-Silva, de Oliveira and Freire Júnior, 2014), resulting in an economic damage exceeding $300 million in the USA alone (Pitt and Hocking, 2012). However, due to the ubiquitous presence of microorganisms and fungal spores in the atmosphere it is impossible to avoid contamination completely. Instead, crops have to be treated appropriately after harvest to prevent microbial spoilage during storage and subsequent downstream processing. Otherwise spoilage fungi can develop in ecological niches and contaminate the entire crop (Champeil, Dore and Fourbet, 2004). As a result, grain quality deterioration and potential consumer health hazard due to the mycotoxins produced occurs (Bottalico and Perrone, 2002).

Mycotoxins are secondary metabolites of the fungal metabolism with severe acute and chronic toxicity towards humans and animals (Magan and Aldred, 2007). In addition, toxins produced by *Fusarium* spp., the most commonly found fungus in cereals and cereal products, are very heat resistant and water soluble (Lancova et al., 2008; Wolf-Hall, 2007). This explains how the toxins can survive food processing steps and accumulate in the final product. Fusarium toxins commonly found in cereal products include zearalenone (ZEA) and deoxynivalenol (DON) among others. In breakfast cereals, 8.7% and 56% of the samples tested were found to contain ZEA and DON, respectively (Marin et al., 2013). Furthermore, 7.5% and 56% of bread samples analysed were found to contain substantial amounts of ZEA and DON, respectively (Marin et al., 2013). The same study concluded that daily exposure in countries such as Spain, France or Germany can exceed the tolerable daily intake of these mycotoxins (Marin et al., 2013). In particular for children, this imposes a long term health risk. In addition, research has demonstrated that mycotoxin production is accelerated due to environmental stress, such as sub-lethal doses of preservatives and incomplete decontamination (Kabak, Dobson and Var, 2006). As a consequence, inefficient measures of post-harvest decontamination can promote fungal development and consumer health hazard due to mycotoxin production.

However, fungal spoilage can affect cereals in numerous other ways as well. Several constituents of the kernel have essential influence on the grain quality and technological performance during downstream processing. Apart from the production of fungal bio-mass and mycotoxins, the release of hydrolytic enzymes inducing the degradation of wheat proteins and carbohydrates has to be considered (Tucker and Talbot, 2001). These enzymes allow the fungus to penetrate deeper into the grain and release essential nutrients. The resulting degradation of storage proteins, the gluten proteins in particular, has a major impact on the grain properties. Therefore, grain viability and quality are reduced and the technological performance during downstream processing is compromised. The most common industrial use of wheat grains is for the production of bakery products, primarily bread. Therefore, the gluten proteins of the endosperm are of major importance to produce a high quality dough and final bread. It was previously shown by Nightingale et al. (1999) how proteases can compromise the quality of wheat dough and bread due to the degradation and weakening of the gluten network. Finally, starch and other carbohydrates which have to be considered for the baking performance are likely to be compromised by the fungal spoilage also. To-date only

little research has been conducted to understand the kinetics and mechanics of post-harvest fungal spoilage and the consequences low levels of microbial contamination can cause during downstream processing. Nonetheless, it is evident that the prevention of post-harvest spoilage is of major importance.

However, despite the clear need for efficient microbial decontamination, the consumer acceptance for conventional food preservatives is on a record low (Figiel and Kufel, 2016). Instead, more natural, “clean-label” products, meeting the highest quality and safety standards are requested. As a result, the food industries are in severe need to find and develop new approaches and methods to fulfil these demands. One such approach is the application of lactic acid bacteria (LAB) as bio-preservatives, which was extensively studied during the previous decades (Dalie, Deschamps and Richard-Forget, 2010). Alongside with the antifungal activity, LABs were also shown to have positive effect on the technological and organoleptic properties (Leroy and De Vuyst, 2004). Due to the complex nutritional requirements, LAB are found only in nutrient rich substrates, but antifungal activity in cereal substrates is reported (Axel et al., 2016). The antifungal activity of LAB is based on complex synergistic mechanisms which are not completely understood yet. However, carboxylic and phenolic acids are recognised as key antimicrobial compounds among others, including reuterin, fatty acids and proteinaceous compounds (Ryan, Dal Bello and Arendt, 2008). In order to encourage the industrial use of LABs as preservatives, the specific modification of the growth medium to enhance the antifungal activity is discussed. First studies to enhance the production and accumulation of 3-phenyllactic acid (PLA), a strong antifungal compound, were published by Rodriguez et al. (2012) and Valerio et al. (2016). The strain *Lactobacillus reuteri* R29 was previously demonstrated to have high antifungal activity *in vitro* and *in situ* (Axel et al., 2016; Oliveira et al., 2015). However, to-date no investigations regarding the optimisation of PLA accumulation of the strain have been conducted. In addition, certain strains of *Lactobacillus reuteri* express antimicrobial activity based on the production of 3-hydroxypropionaldehyde, also known as reuterin. Hence, the investigation of PLA and reuterin accumulation, as well as the application in a food system, can contribute valuable knowledge regarding the potential of *Lactobacillus reuteri* R29 and LAB in general for industrial application as bio-preservative.

Besides LAB, several other novel technologies are emerging and show great potential as preservatives with high consumer acceptance. These approaches also include chemical methods, such as cold plasma (Mir, Shah and Mir, 2016) and

microwaves (Aron Maftei et al., 2014), and physical decontamination, including high hydrostatic pressure (HHP) (Heinz and Buckow, 2009) and ultrasound (Bilek and Turantas, 2013). However, currently it is not clear how these new approaches compare to the already established ones and how much further optimisation is required. This is particularly important, as mycotoxin production is known to be promoted due to exposure to sub-lethal decontamination (Kabak, Dobson and Var, 2006). Hence, every application has to ensure that spoilage organisms are fully inhibited, to prevent a potential consumer health hazard. In addition to the prevention of microbial development and production of mycotoxins, also the treatments impact on grain quality parameter has to be investigated also. Heat treatments in particular are known to cause denaturation of proteins and inactivation of enzymes. As a result, grain quality deterioration can occur as a side effect to the microbial decontamination.

Furthermore, plant derived antimicrobial peptides (AMPs) are discussed as very promising natural alternatives for conventional preservatives. Located in the seeds, these peptides are part of the defense mechanism of plants against environmental spoilage organisms. Therefore, these peptides are highly specific and effective against certain pathogens (Garcia-Olmedo et al., 1998). Commonly, plant AMPs are 5 – 8 kDa peptides, comprising of 45 to 54 amino acid residues and have an isoelectric point around 9 (Thery and Arendt 2018). The global fold of plant AMPs consists of one α-helix and one β-sheet, which are stabilised by disufide bonds (Lay, Brugliera and Anderson, 2003). The mode of action of plant defensins is manifold and not fully understood yet. It often involves binding of the peptide to the microbial cell membrane, followed by internalisation of the peptide or it remains on the surface to induce inhibiting mechanisms, such as modification of ion fluxes or the activation of enzyme pathways. One such peptide, previously reported as natural antibacterial agent, is the cowpea-thionin II (Cp-thionin II) (Franco et al., 2006). However, the antifungal activity against common food spoilage fungi would be of further interest. Furthermore, no studies investigating the antifungal activity in a food matrix and consumer health safety are available. A synthetic linear analogue of the peptide was recently characterised by Thery and Arendt (2018) and investigated for potential food application, showing promising results. With similar results for the natural peptide Cp-thionin II it would present a highly promising natural alternative to conventional preservatives.

The main objective of this thesis was firstly to gain further knowledge regarding the mechanisms and kinetics behind fungal infections and the resulting impact on the

gain quality and technological performance. Furthermore, emerging approaches for microbial decontamination were studied to determine their potential as food bio-preservatives and provide helpful knowledge towards industrial application.

Therefore, firstly the current state of knowledge regarding new emerging methods of physical (**chapter 2**), chemical and microbial (**chapter 3**) decontamination was reviewed and evaluated. Subsequently, the consequences of failed decontamination and mechanisms of fungal infection were investigated. The impact of the fungal infection on grain quality (**chapter 4**) and technological performance (**chapter 5**) were characterised also. Subsequently, the antifungal performance of *Lactobacillus reuteri* R29 was studied and optimised for the possibility of industrial use as natural post-harvest grain protective (**chapter 6**). The optimised *L. reuteri* R29, as well as various newly emerging chemical and physical approaches, including non-thermal plasma, electrolysed water, ultrasound and high hydrostatic pressure, were investigated for their suitability to protect cereal crops during storage from fungal spoilage and their impact on the grains technological performance (**chapter 7**). Lastly, an antimicrobial peptide was extracted from organic cowpea seeds, purified, characterised and studied for its antifungal activity *in vitro* and on cereal grains (**chapter 8**).

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Chapter 2: Recent advances in Physical Post-Harvest Treatments for Shelf-Life Extension of Cereal Crops

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2.1 Abstract

As a consequence of the rapidly growing global population and limited agricultural area, sufficient supply of cereals for food and animal feed becomes increasingly challenging. Consequently, it is essential to reduce pre- and post-harvest crop losses. Extensive research, featuring several physical treatments, has been conducted to improve cereal post-harvest preservation, leading to increased food safety and sustainability. Various pests can lead to post-harvest losses and grain quality deterioration. Microbial spoilage due to filamentous fungi and bacteria is one of the main reasons for post-harvest crop losses and mycotoxins can induce additional consumer health hazards. In particular, physical treatments have gained popularity, making chemical additives unnecessary. Therefore, this review focuses on recent advances in physical treatments with potential application for microbial post-harvest decontamination of cereals. The treatments discussed in this article are evaluated for their ability to inhibit spoilage microorganisms and degrade mycotoxins without compromising the grain quality. All treatments evaluated in this review have the potential to inhibit grain spoilage microorganisms. However, each method has some draw-backs, making industrial application difficult. Even under optimised processing conditions, it is not likely that cereals can be decontaminated of all naturally occurring spoilage organisms with one single treatment. Therefore, future research should aim for the development of a combination of several treatments that harness their synergistic properties without leading to grain quality deterioration. For the degradation of mycotoxins, the same conclusion can be drawn. In addition, future research has to investigate the fate of degraded toxins, to assess the toxicity of their respective degradation products.

2.2 Introduction

In times of a rapidly growing worldwide population, sufficient nutritional supply to humanity becomes increasingly challenging. Due to their long tradition as staple of the human diet and livestock feed globally, agricultural crops such as cereals will have a key role in satisfying this increasing nutritional need. However, the agricultural area on the world is limited and thus it is difficult to expand cereal production. Considering that approximately 15% of all cereals worldwide are lost due to microbial pests (Freita-Silva, de Oliveira and Freire Júnior, 2014), the most sensible approach to encounter this issue is to increase food safety and sustainability to reduce economic losses. Microbial spoilage, pre- and post-harvest, counts as one of the main factors for crop losses all over the world. Various strategies to prevent microbial contamination in the field have been investigated and reviewed by Oerke (2006). However, even the best management practices cannot completely eliminate the risk of contamination. Due to the permanent and ubiquitous presence of microorganisms and fungal spores in the environment, cereals always carry a certain microbial load when harvested. Also climatic conditions that are not under human control, such as temperature and humidity, may be crucial for contamination with moulds (Siciliano et al., 2016). Therefore, appropriate post-harvest crop treatment, before and during storage, is equally as important as pre-harvest strategies in preventing microbial spoilage. Thus this review is focused on research regarding post-harvest treatments exclusively.

Depending on the climatic conditions during growth, grains carry a microbial load with a high diversity of potential spoilage organisms when harvested. In addition, post-harvest contamination during transport is possible. This microbial load consists of bacteria, yeasts and filamentous fungi, belonging to many different genera. The activity of these micro-organisms during storage, and therefore the shelf life of the crop is dependent on a range of different factors, as illustrated in Figure 1. Amongst the most influential parameters are the moisture content and water availability during storage. In consequence, grains are usually stored at low moisture contents of 12 – 13% and a water activity of <0.70 (Magan et al., 2003). However, cereals are usually traded as wet weight and thus inefficient drying systems can lead to microbial spoilage during storage. Furthermore, even if dried properly, some xerophilic species of *Aspergillus* can still develop during storage, resulting in quality deterioration and mycotoxin accumulation (Oliveira, Zannini and Arendt, 2014).

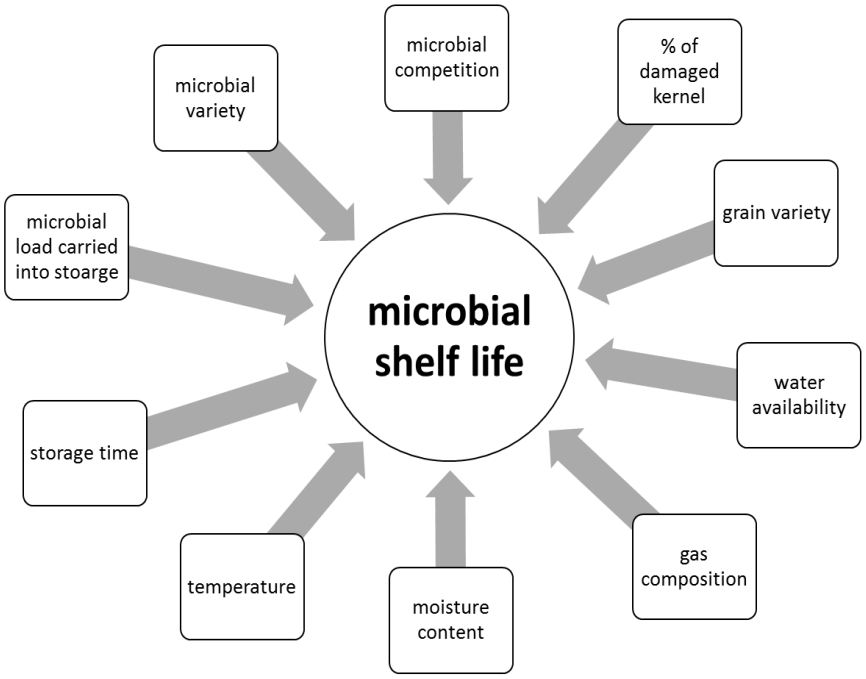


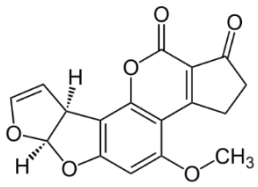
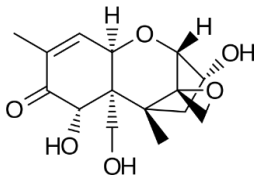
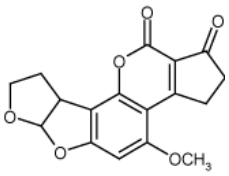
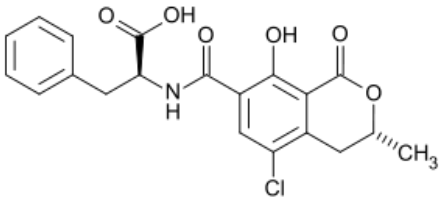
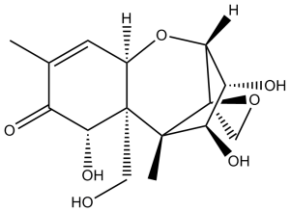
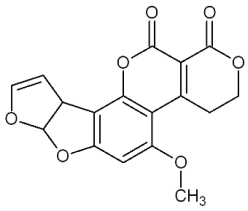
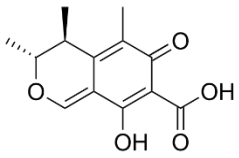
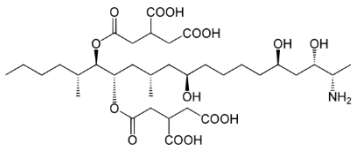
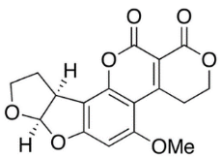
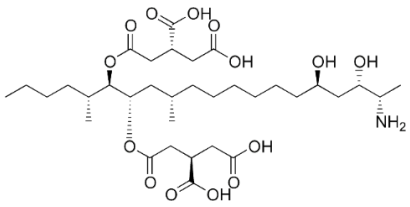
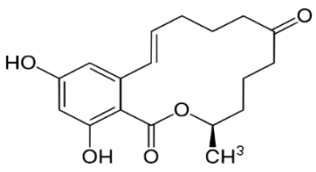
Figure 1: Biotic and abiotic factors influencing the microbial shelf-life of cereal grains during storage.

In addition, conventional storage systems, such as silos, are often cost intensive and inflexible regarding the volume. In these rigid systems, due to inappropriate size for the amount of grains stored, the environmental conditions in the headspace cannot be controlled. Thus it is likely that suitable conditions arise which promote microbial growth and production of toxic metabolites (Magan and Aldred, 2007). As shown in a recent study conducted by Schmidt et al. (2016), if the conditions during storage are suitable, a minimal fungal field contamination can rapidly evolve into a serious consumer health hazard.

The biggest microbial threat during storage is displayed by filamentous fungi, mostly belonging to the genera *Aspergillus* and *Penicillium*. This is mainly due to their relative tolerance to low water activities and the production of mycotoxins, which are secondary fungal metabolites with toxic effects on humans and animals. In addition, these fungi induce a loss of grain nutritional value, produce off-odours and result in reduced baking and milling quality (Magan et al., 2003). Although *Fusarium* spp. are typical field pathogens and unlikely to develop during storage, previously produced and accumulated mycotoxins remain a serious issue during storage and processing of cereals (Audenaert et al., 2012). Hence, in addition to the living organisms, a broad variety of mycotoxins have to be countered during post-harvest treatments, as the prevention of their production is not always possible. Table 1 shows the most

commonly reported mycotoxins of cereal crops and the producing fungal genera. It has been reported that approximately 25% of the global cereal crops, translating to over 500 million tons per annum, are contaminated with mycotoxins and thus present a potential consumer health hazard (Cheli et al., 2013). In contrast to fungal mycelia, mycotoxin contaminated grains often do not vary visually from clean ones and are therefore difficult to identify and eliminate.

Table 1: Chemical structures of mycotoxins commonly found on cereal grains sorted by the producing fungal species.

***Aspergillus* spp.**	***Aspergillus* spp./ *Penicillium* spp.**	***Fusarium* spp.**
		
Aflatoxin B_1		Deoxynivalenol
		
Aflatoxin B_2	Ochratoxin A	Nivalenol
		
Aflatoxin G_1	Citrinin	Fumonisin B_1
		
Aflatoxin G_2		Fumonisin B_2
		
		Zearalenone

Previous studies have established that mycotoxins are primarily located in the husk layers of grains. A study conducted by Vidal et al. (2013) showed that the bran of wheat obtained from a Spanish market contained substantial amounts of various mycotoxins, mainly deoxynivalenol (DON) and zearalenone (ZEA). The authors concluded that the production of whole grain products from these wheat samples would constitute a substantial consumer health hazard. However, since wheat bran is rich in dietary fibre, there is a high interest in exploiting its application in food and feed products due to its potential health promoting properties. In addition to these *Fusarium* toxins, different toxins produced by storage fungi, namely aflatoxins (AFs), ochratoxin A (OTA) and citrinin are commonly found as contaminants in cereals (Table 1). Finally, bacterial spoilage organisms must also be considered to ensure sufficient grain shelf life. Although most bacteria are unlikely to grow under conditions commonly applied to grain storage, however, their presence can result in significant quality deterioration during subsequent processing or in the final product (Magan and Aldred, 2006).

In recent years, the consumer desire for more natural, less processed foods with less or no chemical additives, has increased enormously, however, the requirement for the maintenance of the highest safety standards remains. This increases the emphasis on physical and microbiological treatments to control post-harvest microbial spoilage in cereals (Balasubramaniam, Martínez-Monteagudo and Gupta, 2015). In addition, the application of physical and microbiological decontamination methods, incorporated into the necessary food processing procedures, yet also enabling a “clean label”, have gained increasing interest from both industry and researchers. Various approaches for bio-preservation, in particular using antifungal lactic acid bacteria (LAB) have been investigated and reviewed elsewhere (Crowley, Mahony and Van Sinderen, 2013; Oliveira, Zannini and Arendt, 2014; Pawlowska et al., 2012). The focus of this article is instead on the recent advances in novel physical decontamination methods.

Physical grain treatments, including dry and wet heat, ionizing and non-ionizing irradiation, high hydrostatic pressure and modified atmosphere packaging are critically reviewed, regarding their suitability to eliminate viable forms and spores of both food spoilage bacteria and fungi commonly found on cereals. Furthermore, the potential of the treatments to remove previously produced mycotoxins, and the impact on the grain quality, viability and technological performance are evaluated

based on the existing literature. This allows the identification of future research needs as well as possibilities for industrial application of the treatments discussed. It should further be mentioned that classical physical treatments such as milling, sorting and hulling are not covered in this review, as these methods are well established and industrially applied. Therefore, they are not largely the subject of recent research.

2.3 Modified Atmosphere Packaging (MAP)

Modified atmospheres have been investigated for the storage of cereals intended for food and feed. While fungi responsible for grain deterioration during storage are considered to be obligate aerobes, many are actually microaerophilic. Hence, they can develop in niches where other species cannot grow and therefore dominate in grain ecosystems. In many cases the oxygen level must be <0.14% and the carbon dioxide level >50% to achieve significant growth inhibition (Magan and Lacey, 1984; Taniwaki et al., 2001). Certain species, such as *P. roqueforti* and some *Aspergillus* spp., are able to grow and infect grains even at >80% carbon dioxide, if at least 4% oxygen are present. In addition, post-harvest systems to prevent grain deterioration also entails the use of (oxygen-free) nitrogen (Gupta, Sinha and Atwal, 2014). It must be noted however, that results obtained in different sample systems are very difficult to compare, as the tolerance to low oxygen and high carbon dioxide levels is highly dependent on the sample matrix and water availability. Low water availability is thereby reported to increase the sensitivity of microorganisms to the modified atmosphere. While MAP is used to control microbial spoilage and insect pests in moist grains during storage, different threats require different treatment conditions.

Exposure of various toxigenic fungi to ozone gas (60 µmol/mol) for up to 120 min resulted in significantly reduced growth and spore germination for *Fusarium graminearum*, *F. verticillioides*, *Penicillium citrinum*, *Aspergillus parasiticus*, and *A. flavus*. However, the efficiency of the treatment was strongly dependent on the specific strain. *F. graminearum* was found to be particularly sensitive, as its growth was totally inhibited after exposure for 40 min, whereas *F. verticillioides* growth after 120 min of exposure was only slightly reduced (Savi and Scussel, 2014).

However, very little research on modified atmosphere packaging has been performed in recent years. This can be attributed to various reasons. Firstly, storage conditions under high carbon dioxide and low oxygen levels are difficult to apply for a conventional silo storage system. Also environmental conditions, such as temperature, moisture content and water availability determine the gas composition required to achieve sufficient microbial inhibition. Possibly the biggest draw-back of this technology is the fact that the microorganisms are not killed and therefore can induce product spoilage during subsequent processing. In addition, the removal of in-field produced mycotoxins, or the inactivation of microbial enzymes is not possible and microbial inhibition is substantially dependent on the fungal or bacterial strain. Also, as cereals after harvest carry a wide range of microorganisms it

appears very difficult predict the success on a specific crop. As a consequence, recent research to prevent post-harvest microbial spoilage has shifted towards novel and more flexible methods for application.

2.4 Thermal Treatments

Table 2: The effects of different heat (wet and dry) treatments on microbial load and mycotoxin content.

Target organism / Toxin	Treatment	Sample matrix	Technological impact	Reference
Natural microbial load	Dry air 9d/100°C	Various cereals	No impact	Lan (2006); Lehtinen et al. (2003)
Natural microbial load	Steam 60min/82°C	corn	No impact	Rose et al. (2012)
Natural microbial load	Steam 210-250°C /15sec	Wheat, barley, rye	Not investigated	Bari et al. (2015)
F. graminearum	dry air 15d/60°C; 5d/70°C; 2d/80°C	Wheat	No impact	Gilbert et al. (2005)
F. graminearum	Dry air 5d/90°C	Wheat	Reduced seed viability	Gilbert et al. (2005)
F. graminearum	Dry air 21d/60°C; 9d/70°C; 5d/80°C	Barley	Reduced viability for 9d/70°C; 5d80°C	Gilbert et al. (2005)
Aspergillus spp, *Penicillium* spp, *Fusarium* spp, *E. coli*, *L. monocytogenes*, *Salmonella* spp	Steam 170 – 200°C / <60sec	Various cereals	No impact	Ban and Kang (2016); Chang et al. (2015); Hu et al. (2016)
Geobacillus stearothermophilus spores	Steam 20min/160°C	Dried spore pellet – sand mixture	Not investigated	Cenkowski et al. (2007)
DON (50%reduction)	Steam 6min/185°C	wheat	Not investigated	Cenkowski et al. (2007)

Thermal treatments, including pasteurisation and sterilisation techniques, are the most regularly used treatment for the inactivation of microorganisms and enzymes in the food industry (O'Donnell et al., 2010). For post-harvest application of heat

treatments various approaches, such as hot water dips, hot dry air or superheated steam, using different time-temperature combinations, are possible (Klein and Lurie, 1991). Table 2 summarises heat treatments reported to have been successful for microbial inactivation or toxin degradation and the side-effects on the sample.

2.4.1 Dry Heat Treatments

Being described as an alternative to chemical grain disinfection, several applications of dry heat in the form of hot air treatments were studied for the ability to control fungal spoilage without compromising the kernels viability (Gilbert et al., 2005). Differing results have been reported for the effects of dry heat treatments on the cereals sensory quality and nutritional value. Several authors reported no impact on technological performance and enzymatic activities after 9 days at up to 100°C (Lan, 2006; Lehtinen et al., 2003). In contrast, Gilbert et al. (2005) found a significant loss in seed viability after treatment for 5 days at 90°C.

For inhibition of various fungal strains on wheat grains, time-temperature combinations of 15 d/60°C, 5 d/70°C and 2 d/80°C, respectively, were found to inhibit fungal growth and spore germination completely without compromising the grains viability (Gilbert et al., 2005; Clear et al., 2002). When treating barley at the same temperatures, the fungi were completely inhibited after 21 d, 9 d and 5 d, respectively. But in contrast to wheat, after 9 d at 70°C, the germination capacity of barley was substantially reduced.

Although bacterial spoilage organisms, such as *Bacillus* spp. are unlikely to cause grain quality deterioration during storage, they have to be inactivated prior to processing of the grains to avoid subsequent product spoilage (Magan and Aldred, 2006). However, no studies investigating the heat inactivation of grain spoilage bacteria post-harvest in particular are available to date. In general, bacteria are reported to be less heat stable than fungal mycelia and it can therefore be assumed that they are also inactivated with the above mentioned antifungal treatments (Bond et al., 1970).

Thus dry heat treatment prior to grain storage shows potential for prevention of post-harvest microbial spoilage, based on the heat inactivation of the microbes, and the reduction of the grains moisture content and water availability which further supports microbial inhibition (Nielsen et al., 2004).

It has been reported that, depending on the processing conditions and sample matrix (i.e. presence or absence of yeast), a substantial reduction of mycotoxins occurs during heating. However, nowadays cereal flours are not always heat treated during processing and this approach is very inconsistent. Therefore, an efficient pre-storage treatment to degrade mycotoxins is essential (Miller and Trenholm, 1997). In general, conventional heat treatment is not suitable for detoxifying crops contaminated with mycotoxins. Commonly found mycotoxins, such as aflatoxins, trichothecenes, OTA and others reportedly possess great heat stability (>300°C) and thus cannot be degraded by dry heat without seriously damaging the treated cereal. To date, no studies reporting the total removal of mycotoxins by dry heat treatments *in vitro* or *in situ* are available. In addition, the reported partial degradation of different toxins by dry heat was always found to compromise the grain quality and viability. This indicates that dry heat can, only in combination with other treatments, serve as a sufficient tool for the degradation of mycotoxins (Bretz et al., 2005). In addition, the thermal degradation of mycotoxins into intermediate breakdown products of unknown identity and toxicity must also be considered (Vidal et al., 2015; Boudra, Lebars and Lebars, 1995).

All in all, from the literature available it becomes evident that depending on the target microorganism and cereal substrate, dry heat treatments have the potential to decrease the microbial load without compromising the grain quality. However, it is highly energy and time consuming, due to the long treatment times of up to several days. Furthermore, the conditions have to be adjusted carefully to achieve satisfying results for both microbial decontamination and grain quality. In addition, the sole use of dry heat is unsuitable for killing spores of heat resistant bacteria, as the temperatures applicable without killing the grains are too low. Hence, bacterial spores enter a dormant state and can germinate once the conditions become more suitable again, in particular during processing of the cereals (Chang et al., 2015). In terms of mycotoxin degradation, dry heat cannot serve as sole treatment to remove in-field produced mycotoxins efficiently. The temperatures required are not feasible to maintain the grains technological performance, decomposition is always incomplete and the resulting degradation products could display a health risk of yet unknown potential. Finally, it is noteworthy that the efficiency of hot air treatments is further compromised by the particle size of the treated sample. Thus, it is less efficient for the treatment of whole grains than for treating the milled product.

2.4.2 Wet Heat Treatments

Compared to conventional hot air treatments, the use of hot steam appears to be a more time and energy efficient approach for microbial decontamination and mycotoxin degradation (Table 2). In addition, food spoilage bacteria and fungi were found to be less heat resistant when in conditions with high water availability (Syamaladevi et al., 2016). Apart from the classic saturated water steam (up to 100°C), superheated steam (SS; up to 250°C) has recently gained much interest as a rapid, non-destructive and safe decontamination method (Ban, Yoon and Kang, 2014). Hence, recent advances and novel applications of SS will be the main focus of this section. SS, having higher enthalpy than saturated steam, can quickly transfer heat to the material being processed, resulting in rapid temperature increase in the sample. In addition, SS is reported to contribute to better product quality. The major advantages of using SS for food processing include better product quality (colour, shrinkage, and rehydration characteristics), reduced oxidation losses, and higher energy efficiency (Alfy et al., 2016).

Microbial decontamination from vegetative forms of food spoilage fungi (*Aspergillus* spp, *Penicillium spp*, *Fusarium* spp) and bacteria, such as *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Salmonella enteritidis* phage type 30 and *Listeria monocytogenes* on different cereals was achieved by several researchers using SS. Treatment times of less than 60 seconds with water steam temperatures of 170°C - 200°C were reported to be sufficient to reduce the microbial load below the respective limit of detection (Hu et al., 2016; Chang et al., 2015; Ban and Kang, 2016). At the same time none of those treatments resulted in noteworthy reduced sensory or nutritional quality. In contrast, corn treated with conventional steam at 82°C had to be exposed for 60 min in order to achieve a significant reduction of the microbial load by 4-log units (Rose et al., 2012). No results regarding the impact of the treatment on the grain quality were reported. In consequence, despite the ability of both approaches to decontaminate cereals, the use of superheated steam is a much more time efficient solution.

Compared to their vegetative forms, fungal and bacterial spore inhibition presents a much bigger challenge. As mentioned above, bacterial endospores, in particular, can lay dormant and subsequently germinate when conditions are favourable during downstream processing. Nonetheless, SS was found to be efficient at permanently killing spores of *Geobacillus stearothermophilus*. However, for spore killing exposure times of up to 20 min are required. This is significantly longer, compared

to the inactivation of vegetative microbes. Thus, during the first 5 min of treatment the biggest reduction in spore viability was detected, independent from the processing temperature (Cenkowski et al., 2007).

In addition, SS was also investigated for the removal of mycotoxins from different cereal matrices. Partial degradation of DON upon SS treatment was reported by Cenkowski et al. (2007). The authors found that increases in steam temperature and exposure time correlated with higher degradation rates for DON. Thus, the highest treatment temperature (185°C) combined with the longest treatment time (6 min) resulted in the highest reduction of DON, by 50%, which was found to be independent of the steam velocity. Furthermore, the reduction was found to be exclusively due to thermal degradation, rather than solubilisation and water extraction with the steam. However, no investigation of the impact of the treatment on the grains technological performance were undertaken in the study of Cenkowski et al. (2007). Likewise, the dry decomposition temperature of aflatoxins (approximately 270°C) is known to be significantly reduced under moist conditions (Jalili, 2015). Consequently, SS treatments present a much more efficient and promising approach for AF degradation than conventional dry heating.

In conclusion, the application of wet heat and superheated steam in particular found to be very effective in decontamination of cereals without compromising the grains technological quality and performance. However, despite extensive research undertaken future work is still required to optimise the processing parameters depending on the matrix present. The technological impact of treatments long enough to kill spores and degrade mycotoxins requires further investigations. Also the fate of mycotoxins thermally degraded by the SS still remains unclear. Thus, the degree of actual detoxification is still in question. Also the possibility of degrading other toxins, such as patulin or bacterial toxins requires closer investigation.

Finally, it is noteworthy that ultra-superheated steam (USS) has recently gathered some research interest. This technique employs temperatures of 400 – 500°C. Exposure of different cereals, namely wheat, barley and rye for 15 sec to USS (actual contact temperature 210 – 250°C) resulted in total inhibition of spoilage fungi and grain shelf-life extension without notable quality deterioration (Bari et al., 2015). However, to-date there are very few studies available which have investigated the application of USS for the microbial decontamination of food commodities. Thus, the optimal conditions of use and full potential of this method remain unclear. Further

research is needed to clarify the suitability of USS treatments to decontaminate and potentially detoxify cereal grains post-harvest.

2.5 Ionizing Irradiation

In general, ionizing irradiation approaches are based on short waves of electromagnetic energy that travel at the speed of light. All treatments discussed in this section share the basic properties of electromagnetic radiation. Ionizing radiation treatment, using either gamma rays or electron beam (e-beam) is well established as a rapid, efficient, safe and environmentally friendly technique for the reduction of food-borne diseases by destroying pathogenic and toxigenic microorganisms (Nemţanu et al., 2014). Their biggest advantage is the great penetrating power, which is inversely related to the frequency (Dev et al., 2012), the high efficiency against various food spoilage organisms and the absence of a temperature raise in the treated sample (Freita-Silva, de Oliveira and Freire Júnior, 2014). In addition, irradiation treatments for food processing purposes are unconditionally regarded as safe for dosages of up to 10 kGy (1 Gy = 1 Joule of irradiation energy/kg sample matter, with 1 $Joule$ = 1 $\frac{\mathrm{kg} \cdot \mathrm{m}^2}{\mathrm{sec}^2}$) (FAO/IAEA, 1982). Due to the unit of irradiation dosage containing the ratio of energy per time and sample matter treatments are commonly compared by means of dose and form of application only. Successful treatments with ionizing irradiation against various food spoilage organisms and toxins are summarised in Table 3.

Table 3: The effects of different ionizing irradiation treatments (gamma- and e-beam irradiation) on microbial load and mycotoxin content in various sample matrices.

Target organism / Toxin	treatment	Sample matrix	Technological impact	Reference
Natural fungal population	0.75 kGy gamma	millet	none	Mahmoud et al. (2016)
Natural microbial load	6 kGy e-beam	chestnuts	No effect on nutritional value	Carocho et al. (2014)
L. monocytogenes	3.3 kGy e-beam (soft electrons)	Alfalfa sprouts	No quality deterioration	Schoeller et al. (2002)
Aspergillus spp., *Alternaria* spp., *Fusarium* spp., *Curvularia* spp. *Helminthosporium* spp.	1.5–3.5 kGy gamma	wheat	Reduced quality for doses >2.5 kGy	Salem et al. (2016)

Fusarium spp	4 kGy gamma	Barley	Reduced quality	Aziz et al. (2007)
Fusarium spp	6 kGy gamma	Wheat, maize	Reduced quality	Aziz et al. (2007)
Aspergillus spp, *Penicillium* spp	10 – 15 kGy e-beam	Dry split beans	No quality deterioration (10 kGy)	Supriya et al. (2014)
Penicillium spp, *Fusarium* spp, *Aspergillus* spp	1.7 – 4.8 kGy e-beam	corn	Not investigated	Nemţanu et al. (2014)
Fusarium spp and DON	6 – 10 kGy e-beam	Barley, malt	Not investigated	Kottapalli et al. (2006)
OTA and aflatoxins	15 kGy gamma	Wheat and sesame	Not investigated	Akueche et al. (2012); Herallah et al. (2008); Jalili et al. (2012)
OTA	2 kGy gamma	Aqueous solution	-	Mehrez et al. (2016)
DON, ZEN, T-2, FB1	10 kGy gamma	Soy beans, corn, wheat	Not investigated	Hooshamad & Klopfenstein (1995)
FB_1	7 kGy gamma	Barley, wheat, maize	Not investigated	Aziz et al. (2007)
aflatoxins	1.5 kGy e-beam	Ground almond flour	Not investigated	Lanza et al. (2013)

DON: Deoxynivalenol; OTA: Ochratoxin A; ZEN: Zearalenone; T-2: Fusariotoxin T 2; FB_1: Fumonisin B_1

2.5.1 Gamma Irradiation

This section discusses the application of irradiation to decontaminate cereals, using gamma rays. These electromagnetic waves are usually produced by radioactive cobalt isotopes (^{60}Co). The use of gamma rays for irradiation treatments is characterised by their high penetration energy and short treatment times.

For millet grains exposed to gamma radiation, no significant decrease in fungal incidence or spore germination was reported for radiation doses up to 0.5 kGy.

However, at doses of 0.75 kGy or higher, the rate of fungal incidences and spore germination sharply decreased by over 80% and 2-log units, respectively (Mahmoud et al., 2016). Salem et al. (2016) applied gamma irradiation, ranging from 0.5 – 3.5 kGy to wheat grains prior to storage. A dosage of 1.5 kGy was found to be sufficient for at least a 90% reduction of *Aspergillus* spp., *Alternaria* spp., *Fusarium* spp., *Curvularia* spp. and *Helminthosporium* spp., immediately after the treatment. However, after 6 months of subsequent storage, the degree of inhibition (compared to the untreated control) was significantly reduced for all species, apart from *Fusarium* spp. The authors further reported that higher irradiation doses resulted in higher inhibition rates. In particular, 3.5 kGy, resulting in total inhibition directly after the treatment, also showed total inhibition after 6 months against all fungi, apart from *Aspergillus* spp (96.4%). In contrast, Aziz et al. (2007) reported higher irradiation doses of 4 kGy for barley and 6 kGy for wheat and maize, necessary for total inhibition of *Fusarium* spp. Analysis of selected physical, chemical and rheological properties of these grains, prior to and after storage, showed that from the technological performance point of view, irradiation dosages higher than 1.5 – 2.5 kGy were not feasible. This also correlates with the findings reported by other researchers (El-Naggar and Mikhaiel, 2011; Melki and Marouani, 2010).

From the literature available it becomes evident that the radio sensitivity of different fungal species appears to differ significantly, depending on the reference consulted. These discrepancies are likely to result from countless influencing factors which require further research. These factors include the form of fungal contamination (mycelium or spores) and the moisture contents of spores or commodities. While moist conditions promote fungal growth and spore germination, dry spores are considered to be more irradiation resistant. Furthermore, the age of spores and the nature of the matrix irradiated can have a big influence on the radio sensitivity. In addition, the temperature before, during and after irradiation, and the fungal strain, influence the efficiency of the treatment for each sample.

The most recent research on the application of gamma irradiation on cereals has focused on the degradation of mycotoxins in food and feed commodities. In particular, the degradation of aflatoxins (AFs) and ochratoxin A (OTA) was investigated by several researches. Degradation of OTA in aqueous solution due to gamma irradiation with 2 – 5 kGy has previously been reported by Deberghes et al. (1995). However, *in situ* degradation of mycotoxins, such as OTA was found to be much more difficult. After gamma irradiation of wheat and sesame seeds using 15 kGy, degradation of OTA, AFB_1, AFB_2, AFG_1 and AFG_2 with reduction rates of

23.9%, 18.2%, 11.0%, 21.1% and 13.6%, respectively, were found (Di Stefano et al., 2014; Akueche et al., 2012). However, application of lower irradiation doses in both studies showed no substantial reduction of AFs and OTA. Similar results on various cereal matrices, intended for food or feed use were also reported by several other authors (Aquino et al., 2005; Fareg, El-Baroty and Abo-Hagger, 2016; Herzallah, Alshawabkeh and Al Fataftah, 2008; Jalili, Jinap and Noranizan, 2012; Prado et al., 2003). However, it must be noted that none of these studies took the moisture content of the seeds into consideration, which appears to have a critical impact on the radio stability of the toxins. Therefore, the meaningfulness of the results is limited. In another study, soybeans, corn and wheat, with respective moisture levels of 9, 13, 17%, showed no substantial reduction in AFs or OTA, after irradiation dosages of up to 20 kGy (Hooshmand and Klopfenstein, 1995). Therefore, it can be concluded that the success of AF degradation due to gamma rays is not just dependent on the dose; the moisture content of the sample has a major impact on AF degradation. It is proposed that this is primarily due to the radiolysis of water, producing highly active radicals which can degrade AFs to compounds with lower biological activity (Jalili, 2015; Wang et al., 2011). Similar results were reported for the degradation of OTA *in vitro* and *in situ*. OTA showed great radiostability as dry substance, as irradiation with 8 kGy resulted in no noteworthy reduction. In contrast, when in aqueous solution (50 ng/mL) significant reduction was achieved with just 2 kGy. Similarly, the degradation in moist wheat kernels (16% moisture) was substantially higher than in dry ones (11% moisture) when irradiated with 8 kGy (Mehrez et al., 2016).

From the literature consulted, typical *Fusarium* toxins, such as DON, ZEN, T-2 and FB_1 appear to be more radio sensitive than AFs and OTA. DON, ZEN and T-2 toxin in soybeans, corn and wheat were significantly reduced by irradiation with 10 kGy (Hooshmand and Klopfenstein, 1995). Irradiation of barley, wheat and maize naturally contaminated with FB_1 resulted in a significant reduction (>85%) and total destruction of the toxin after exposure to 5 kGy and 7 kGy, respectively (Aziz et al., 2007).

Nevertheless, it has to be considered that irradiation results in degradation-products of unknown identity and toxicity. Wang et al. (2011) detected 20 radiolytic products of AFB_1 after irradiation in a water/methanol mixture. To-date, just 7 of these products have been identified and 6 are considered less toxic than AFB_1, due to the missing double bound on the terminal furan ring (Table 1). However, these results imply that, with current knowledge, it remains unclear if degradation of mycotoxins

really detoxifies the sample or if the resulting products are equally as toxic as the original substance. Furthermore, as the irradiation efficiency is highly dependent on the water availability, the application in dry food matrices appears to be limited. Although AFs and OTA belong to the most commonly found toxins in cereal crops, the fate of other contaminants, such as DON, NIV and ZEA requires further research.

Therefore, it can be concluded that gamma irradiation can reduce, and potentially fully inhibit, fungal and bacterial spoilage of grains during storage, thus avoiding the production of mycotoxins. However, the irradiation dosages required for total inhibition of common storage fungi, such as *Aspergillus parasiticus*, are highly dependent on the sample matrix and fungal load. Necessary doses reported range between 5 and 10 kGy. Unfortunately, such high irradiation dosages cause severe damage to the treated grains and are therefore, from a technological point of view, not feasible. On the other hand, simply reducing the microbial load is not sufficient either, as even minimal fungal contamination can lead to growth during storage. This ultimately results in huge economic losses and a potential consumer health hazard, as demonstrated by the authors in a previous study (Schmidt et al., 2016). Therefore, gamma irradiation alone cannot serve as an efficient tool for cereal grain preservation during storage, but could potentially be applied in combination with other treatments. However, for treatment of cereals purposed for animal feed, higher irradiation dosages are allowed. Thus, gamma irradiation appears as a promising approach to decontaminate animal feed.

2.5.2 Electron Beam Irradiation

The use of e-beam irradiation has several advantages over the use of conventional gamma irradiation. Table 4 summarises the comparison between gamma and e-beam irradiation. The main advantages include faster operation, lower irradiation dosages and the use of electricity to generate the electron, rather than radioactive materials, making the technology more flexible and easier to use. On the down-side however, its penetration power is lower, leaving it more as a tool for surface disinfection (Freita-Silva, de Oliveira and Freire Júnior, 2014). The use of e-beam irradiation in the food industry in general, including the technological background and mode of action was recently reviewed by Freita-Silva, de Oliveira and Freire Júnior (2014). Thus, this part of the review will focus exclusively on recent advances

in microbial decontamination using e-beam irradiation. With the exception of Europe, e-beam irradiation has been accepted and is widely applied for the treatments of various food product worldwide, such as fruits and grains; approximately 81,593 t are treated annually, but just 11 t of these are from Europe (Kume et al., 2009).

Table 4: Comparison of gamma (^{60}Co) and e-beam irradiation, adopted from Freita-Silva, de Oliveira and Freire Júnior (2014).

Parameter	Gamma irradiation	e-beam irradiation
Irradiation Time	slow	Fast
Doses (kGy)	Higher doses	Lower doses
Source	Radioactive material	Electricity to generate electrons
Flexibility	Inflexible (cannot be turned off)	More flexible (can be turned off)
Penetration	Good penetration	Lower penetration power

Microbial decontamination and mycotoxin degradation *in vitro* with potential of application *in situ* has been previously reported (D'Ovidio et al., 2007), but the irradiation dose necessary for sufficient decontamination depends on the type and species of grains to be treated (Lung et al., 2015). When dry split beans were subjected to e-beam irradiation (0, 2.5, 5, 10, 15 kGy) to control storage moulds, irradiation resulted in a dose-dependent decrease in fungal contaminants. High irradiation doses (10 and 15 kGy) resulted in complete absence of fungi and undetectable levels of aflatoxins B_1 and B_2. In contrast, un-irradiated beans carried *Aspergillus niger* at the highest level (33–50%), followed by *A. flavus* (14–20%) and *Penicillium chrysogenum* (7–13%). For total inhibition of fungal incidence irradiation doses of 10 and 15 kGy, were found to be necessary. Irradiated split beans (10 kGy) also showed improved shelf-life up to six months without quality deterioration (Supriya, Sridhar and Ganesh, 2014). In contrast, on raw corn under comparable conditions, doses as low as 1.7, 2.5 and 4.8 kGy were found to be sufficient to fully inhibit the growth of *Penicillium* spp., *Fusarium* spp., and *Aspergillus* spp., respectively (Nemţanu et al., 2014). The researchers concluded that with sufficient optimisation of the processing parameters, e-beam irradiation can have great potential in microbial decontamination of cereals. For the reduction of natural

microbial load on chestnuts, 6 kGy were reported to be sufficient for decontamination, while nutritionally valuable constituents remained unaffected (Carocho et al., 2012; Carocho et al., 2013; Carocho et al., 2014). Interestingly, e-beam irradiation of *Fusarium* spp.-infected barley with 6–10 kGy showed no significant reduction in fungal incidence and DON contents in the fresh barley. However, the resulting barley malt was reported to have significantly reduced DON content and fungal occurrence (Kottapalli, Wolf-Hall and Schwarz, 2006). In another study conducted by Stepanik et al. (2007) wheat grains and dried distillers grain were irradiated with up to 55 kGy, which resulted in a maximum DON reduction of 17%.

To date, no studies investigating the fate of mycotoxins exposed to e-beam irradiation are available. This is likely due to the lower energy value of this irradiation, compared to gamma irradiation (Table 4). Thus, e-beam irradiation is unlikely to create enough energy for mycotoxin degradation. This applies, in particular, if the toxins are not exclusively located on the grain surface, but also in the inner layers. Therefore, the application on ground almond flour, with an dose as low as 1.5 kGy was found to be sufficient for total degradation of aflatoxins (Lanza et al., 2013).

In conclusion, e-beam treatment shows potential to reduce the microbial load and content of in-field produced mycotoxins. However, depending on the sample matrix, microbial load and target organism, the irradiation dose necessary is likely to exceed that generally permitted i.e. 10 kGy (FAO/IAEA, 1982), leading to legislative difficulties. Furthermore, the impact of various e-beam treatments on the grains sensory and nutritional properties have to be investigated further. Finally, difficulties such as the even treatment of the sample surface and the low penetration energy of the e-beam have to be considered before any industrial application.

A variation of conventional e-beam irradiation is the use of so called “soft electrons”. The term “soft” refers to the low energy of the electrons fired at the sample. This results in less impact on the sample in terms of sensory and nutritional value. On the other hand, this approach can serve solely as a surface treatment, as the e-beam does not have sufficient energy to penetrate into deeper layers of the sample. The use of 3.3 kGy applied as soft electrons was reported to successfully eliminate *L. monocytogenes* from alfalfa sprouts (Schoeller, Ingham and Ingham, 2002). However, due to the low penetration power, a small particle size and an even sample surface are crucial to ensure uniform treatment. Consequently, the

irradiation treatment of soybeans was found to be more difficult, as 17 kGy was insufficient to eliminate the natural microbes present on the surface (Kikuchi et al., 2003). Thus, soft electrons show, due to the difficult applicability, very little potential for industrial post-harvest decontamination of cereal crops but could have potential for flour treatment after milling.

2.6 Non-Ionizing Irradiation

Table 5: The effects of different non-ionizing (light, microwave, ultrasound) treatments on microbial load and mycotoxin content in various sample matrices.

Target organism / Toxin	Treatment	Sample matrix	Technological impact	Reference
Different food spoilage bacteria and fungi	US >60W/cm^2	Aqueous solution	-	Chemat et al. (2011)
A. parasiticus	Microwave: 900W, 2.45GHz, 1 -5min	Aqueous solution	-	Fang et al. (2011)
Aspergillus spp and *Penicillium* spp	US: 6min, 60°C, 20 – 39W/cm^2	Culture medium	-	Herceg et al. (2015)
Aspergillus spp	51.2J/g pulsed white light	wheat	15% reduced seed viability	Aron Maftei et al. (2014)
Aspergillus spp	Microwave: 120sec, 2,450MHz, 1.25kW	Cereals and nuts	Not investigated	Basaran and Akhan (2010); Kabak et al. (2006)
Bacillus subtilis	1.0 J/cm^2 * 10 pulses light with 200 – 1100nm	spices	No quality deterioration	Nicorescu et al. (2013)
OTA, OTB, citrinin	Light: 455nm/470nm for 5d	Aqueous solution	-	Schmidt-Heydt et al. (2012)
Aflatoxins	UV-light: 265nm for 15 – 45min	nuts	Not investigated	Jubeen et al. (2012)
Trichothecenes	US >200W/cm^2	corn	No quality deterioration	Lindner and Hasenhuti (1996)

Recent advances in terms of non-ionizing irradiation treatments for microbial decontamination and detoxification with possible application for cereals will be reviewed in this section. Successfully applied treatments for the various target organisms or toxins are summarised in Table 5. Due to the non-ionizing character of the treatments discussed here, the impact on the grain quality is likely to be negligible. In addition, the consumer acceptance for ionizing irradiation is relatively low. Despite the primary reason being misinformation of the consumers (Farkas and Mohácsi-Farkas, 2011), use of non-ionizing irradiation should increase the consumer acceptance of the final products.

2.6.1 Ultra-Violet (UV) Light

The antimicrobial properties of UV-light are well investigated and, taking surface disinfection as an example, long established. However, the conventional application of UV-light in the form of continuous exposure has numerous disadvantages. As the sanitizing effect is primarily attributed to the high ionizing energy of vacuum-UV (wavelength <180 nm) the consumer acceptance is very low. The induced DNA damage, responsible for the microbial inhibition, also occurs in the sample which results in a substantial loss of quality. In addition, depending on the water content, substantial internal heating of the sample can occur. In order to avoid these unwanted side-effects, more recent research focused on the application of pulsed UV-light. Here, the treatment is carried out with numerous short flashes of light with a broad wavelength spectrum. Although the inhibitory effect is still attributed to the UV-spectrum of the light (wavelength 200 – 400 nm), microbial inhibition can be achieved with non-ionizing UV (>180 nm). At the same time the undesired side-effects are substantially reduced. Thus, pulsed UV-light presents a novel, non-thermal antimicrobial treatment with potential application for food preservation. Therefore, this section is focused exclusively on the application of non-ionizing UV-light. Microbial inactivation as a result of exposure to pulsed light, *in vitro* and *in situ*, has been reported by several researchers and comprehensively reviewed by Oms-Oliu, Martín-Belloso and Soliva-Fortuny (2010).

Successful inhibition of food spoilage fungi and bacteria with pulsed light applied to various food matrices, including milk, honey and fruits, was reported by Oms-Oliu, Martín-Belloso and Soliva-Fortuny (2010). However, to-date only one study investigating the application of pulsed light for pre-storage decontamination of

cereals is available. Aron Maftei et al. (2014) reported up to a 4-log unit reduction for naturally occurring *Aspergillus* spp. in wheat grains. Treatment was carried out with 40 flashes of broad spectrum white light (180 – 1100 nm) with an overall energy release of 51.2 J/g. The authors further reported that the same treatment with light of a narrower wavelength spectrum (305 – 1100 nm and 400 – 1100 nm, respectively) resulted in significantly less fungal inhibition.

Although pulsed UV-light causes less damage to the sample than continuous UV-irradiation, significantly reduced seed viability was reported nonetheless. Alongside the reduction of *Aspergillus* spp. from 10^5 to 10^1 cfu/g, the seed viability was significantly reduced by 15% (Aron Maftei et al., 2014). Consequently, despite showing the potential for microbial decontamination, further optimisation of the processing conditions is required to improve the efficiency and make the treatment potentially suitable for industrial application.

The inhibition of common food spoilage bacteria on the other hand was found to be more difficult, as in general, reductions of no more than 1-log unit could be achieved without substantial impact on the sample quality Oms-Oliu, Martín-Belloso and Soliva-Fortuny (2010). Furthermore, no studies investigating the inhibition of bacteria commonly found on cereals or in cereal matrices are available. However, Nicorescu et al. (2013) achieved up to a 1-log unit reduction of *Bacillus subtilis* in liquid medium and artificially contaminated spices. Despite being equal to a 90% reduction in the bacterial population, after the treatment approximately 10^4 cfu/g remained on the samples. Thus, the need for further research to improve the antibacterial properties of pulsed UV-light becomes evident.

The possibility of photodegradation of mycotoxins *in vitro* and *in situ,* using UV- and visible light, has also been investigated by several researchers. Treatment of different mycotoxins (OTA, OTB and citrinin) in aqueous solution with light of various wavelengths (455, 470, 530, 590, and 627 nm) for 5 days resulted in significant degradation of all three toxins after exposure to the 455 nm and the 470 nm light (Schmidt-Heydt et al., 2012). In particular, light of wavelength 455 nm was found to be very efficient in terms of mycotoxin degradation. Subsequently, 455 nm light was applied *in situ* on artificially fungal infected wheat kernels. After 5 days of exposure the OTA and OTB levels were reduced by >90% compared to the untreated control (Schmidt-Heydt et al., 2012).

In addition, the total aflatoxin content of various nuts could be substantially reduced by treatment with UV-light (265 nm) for 15 – 45 min (Jubeen et al., 2012). However,

numerous factors were found to have a significant impact on the level of aflatoxin reduction. The most influential factors included the moisture content of the nuts, the toxin targeted, the exposure time and the type of nut. The authors also reported higher resistance of AFB_1 and AFG_1 to the UV-light compared to AFG_2. For all toxins, increasing sensitivity towards the treatment could be attributed to increasing moisture levels and exposure time.

To evaluate the potential use of photodegradation for the detoxification of cereals, the fate of the degraded mycotoxins has to be considered also. A pathway for the UV-light induced photodegradation of AFB_1 was proposed by Liu et al. (2010) after identification of the three main degradation products, using UHPLC-MS. Identification of the degradation products revealed that from the two most important parts of the molecule in terms of toxicity, namely the terminal furan ring and the lactone ring (Table 1), only the latter was effected by photodegradation. In addition, the authors reported first order reaction kinetics and found the process to be independent of the initial toxin concentration (within 0.2 – 5 ppm) but directly related to the irradiation intensity. Based on these results, a subsequent study investigated the cytotoxicity of UV-light degraded AFB_1 in aqueous solution (Liu et al., 2011). The authors assessed the toxicity of the three previously identified degradation products using the Ames-test. Interestingly, the cytotoxicity was found to be reduced by 40% compared to native AFB_1, but not fully eliminated. This leads to the question if photodegradation can serve as a suitable tool for mycotoxin detoxification. Considering that even after complete degradation of the toxin, 60% of the initial cytotoxicity remains, this suggests that alternative treatments are likely to be more suitable.

In conclusion, despite showing some potential for microbial decontamination and mycotoxin degradation, UV-light appears to be difficult to apply without effecting sample quality. Although the use of pulsed light, rather than continuous irradiation reduces the negative effects, to-date no complete inhibition without quality loss has been reported. This applies in particular for the decontamination of food spoilage bacteria. For application on cereal grains, the uneven sample surface, resulting in shadow spots on the grains makes a reliable application even more challenging. In terms of mycotoxin degradation, it was shown, that UV-light is capable of total degradation of toxins such as AFB_1, but the degradation products were found to remain cytotoxic. Therefore, it appears as unsuitable method for mycotoxin detoxification.

2.6.2 Microwave Treatments

Microwave technology is widely used in the food industry and offers several advantages, including safety, high efficiency, and environmental protection, but often affects food quality (Fang et al., 2011). Microwaves are defined as electromagnetic waves with frequencies ranging from 300 MHz to 300 GHz. The mechanism of microbial inhibition is primarily based on the internal heating of the sample due to the molecules movements in the pulsing electromagnetic field. This leads to denaturation of proteins, enzymes and nucleic acids. Thus, this implies the risk of losing enzymatic activities in the grain, activities which are essential for downstream processing steps (Fang et al., 2011). However, it is suggested that with optimised processing conditions, microwave treatment has the ability to fully inhibit microbial growth on cereal grains, without compromising the grains germination quality (Ursu, 2015). Therefore, the impact of various factors, such as moisture content, microbial load or sample matrix need to be investigated further to determine the optimised processing conditions for each cereal matrix.

Based on the literature available, microwave treatment for up to 10 min with an energy output of 1.45 kW with 2,450 MHz is reported to result in very minor reduction of total AFs including AFB_1 (Herzallah, Alshawabkeh and Al Fataftah, 2008). However, the possibility of inhibiting the growth and spore germination of *Aspergillus* spp. in cereals and nuts by 3-log units, without significant quality deterioration, was reported by different authors (Kabak et al., 2006; Basaran and Akhan, 2010). A treatment time of 120 sec with 2,450 MHz and 1.25 kW was found to be sufficient. Consequently, due to the fungal inhibition, the amount of mycotoxins produced was significantly lower also.

Furthermore, non-thermal inactivation of microorganisms due to repeated exposure to sub-lethal doses of high frequency microwaves has been reported (Fang et al., 2011). The lethal effect of low-dose microwave radiation (LDMR) on spoilage microorganisms was found to be due to disruption of the cell membrane and induced DNA damage, rather than protein denaturation. Thus the mechanism by which LDMR cause fungal death is evidently different from a conventional heating treatment (Fang et al., 2011). However, in a study conducted on *A. parasiticus* the severity of DNA injury was found to increase with rising temperature. Thus, the inactivation effect is still partially related to the processing temperature. However, to-date, there are very few studies available which have investigated this topic. Thus, this shows the need for future research in order to exploit this technology

further for establishing non-thermal microwave-based microbial hurdle processes that do not compromise the grain quality.

Overall, microwave treatment, based on internal heating, shows little potential for the microbial decontamination of cereals. This is primarily due to the heat induced damage of the sample. The same conclusion has to be drawn for the microwave induced degradation of mycotoxins, which possess high heat stability. However, the concept of non-thermal microbial inactivation appears much more promising. As research on this approach is in its infancy however, it is not yet possible to predict the full potential and applicability to industrial grain decontamination process until more extensive research has been conducted.

2.6.3 Ultrasonication

The term 'Ultrasound' (US) describes sonic waves with frequencies above the range audible for humans. In general, two categories are differentiated: high frequency ultrasound and power ultrasound. The former uses high frequencies of 2–20 MHz with low sound intensity (0.1–1 W/cm^2) and is predominantly used in food quality analysis and medical imaging. In contrast, power ultrasound, or high-intensity ultrasound, is characterised by low frequencies (20–100 kHz) but high sound intensity (10–1000 W/cm^2). Research on the inactivation of enzymes and microorganisms has predominantly focused on the application of high power ultrasound. It is considered as a promising, novel and non-thermal approach for microbial disinfection of various surfaces and food matrices (Feng, Yang and Hielscher, 2008). In contrast, high frequency US shows much less potential for microbial decontamination. Therefore, this review focuses on power Ultrasound exclusively.

Only a few studies have investigated the application of US alone for microbial decontamination and contrasting results are reported. Butz and Tauscher (2002) showed that US alone does not sufficiently inactivate food spoilage microorganisms, as the inactivating effects are not severe enough. In contrast, Chemat, Zill-E-Huma and Khan (2011) reported that, if the acoustic power applied is sufficiently high (>60 W/cm^2), even US alone can induce the rupture of cells and thus microbial inactivation. However, successful decontamination using US was achieved only under laboratory conditions and would be difficult to transfer to an industrial scale. In fact, most data available suggests that US alone is a very inefficient and energy

consuming treatment for microbial disinfection and therefore has to be used in combination with other treatments, such as sanitizing chemicals or heat (Bilek and Turantaş, 2013). Furthermore, the generation of such high-power US requires an immense energy input and is relatively inefficient compared to other techniques. This is further supported by O'Donnell et al. (2010), who described the challenges encountered by the industrial scale-up of US technology. In addition, the application of this technology to cereal grains could prove to be difficult, as the treatment has to be carried out in a liquid medium. Therefore, it would be crucial that the cavitation of the liquid around the grains is evenly distributed.

In general, the biggest potential of US is in combination with mild heat or sanitizing agents, which has been shown by several authors to have a synergistic effect (Scouten and Beuchat, 2002; Seymour et al., 2002). Herceg et al. (2015) achieved total inhibition of *Aspergillus* and *Penicillium* spp., after exposure to ultrasound for at least 6 minutes at 60°C when the applied power was ranging between 20–39 W/cm^2. However, these results were achieved in liquid sample matrices. Thereby, the US-waves can easily travel through the sample, resulting in an evenly distributed cavitation effect. But for possible application on cereal crops, it remains unclear if enough cavitation throughout the whole sample for a noteworthy disinfection effect can be generated. Thus, small sample size appears to be necessary to provide uniform cavitation. Furthermore, the grains would be required to be in a "washing solution", which produces a further challenge for the industrial use of this technology.

To-date, no studies investigating the application of US to decontaminate solid foods are available. Chemat, Zill-E-Huma and Khan (2011) recently reviewed the application of US in the food industry and its advances for decontamination of liquid food systems. However, chemical disinfection, supported by US is likely to result in synergistic effects. Thus, it appears to be a promising improvement over conventional chemical disinfection (reduction of treatment time and unpleasant side-effects) and, therefore, should be considered for further investigations. Ultrasound could be used to provide the energy necessary to form free radicals as reactive species and so support the disinfection efficiency of commonly used surface sanitizers. In particular, hydrogen peroxide or sodium hypochlorite based disinfection of food or food contact materials can be efficiently supported by US, creating more hydroxyl- and hypochlorite radicals, respectively. Furthermore, the use of US can substantially increase the speed and efficacy of conventional food preservation methods, such as sterilisation and pasteurisation. This would allow a

reduction in the processing time and temperature and therefore, reduce the undesirable side-effects, such as changes in taste, colour and nutritional value (Chemat, Zill-E-Huma and Khan, 2011).

Only very few studies investigating the impact of ultrasonication on mycotoxins have been conducted. Lindner and Hasenhuti (1996) reported the successful degradation of trichothecenes in contaminated corn, while the technological performance of the treated samples remained unchanged. On the other hand, as discussed earlier, for the combination of gamma irradiation with chemical sanitizers, the production of hydroxyl radicals can lead to chemical degradation of aflatoxins. In theory it should be possible to produce such radicals using US. However, to the best of the authors' knowledge, no studies investigating this have been conducted so far.

From the research published, it becomes evident that only a combination of US with heat or chemical sanitizers can sufficiently inactivate vegetative cells, spores and enzymes simultaneously. Only then, consistent and high product quality can be ensured, as the microbial enzymes can cause great damage to proteins and carbohydrates of the grains, even after killing of the vegetative cells (Schmidt et al., 2016).

2.7 High Hydrostatic Pressure (HHP)

Yet another emerging approach for the decontamination of food and feed products from spoilage microbes is the treatment with high hydrostatic pressure (HHP). Well established applications include the preservation of meat products, oysters, fruit juices, and many ready-to-eat foods. HHP is reported to have the potential to inactivate vegetative microorganisms and fungal spores at relatively low temperatures without compromising sensory and nutritional properties (Heinz and Buckow, 2009; Polydera, Stoforos and Taoukis, 2003). Thus it has the potential to be used in the expansion of the production of value added foods.

Microbial inactivation is reported for processing pressures ranging from 100 to 800 MPa and relatively short times (a few seconds to several minutes). Combined treatment with mild temperatures between 20 and 50°C to inactivate enzymes are reported also. The treatment conditions depend fundamentally on the food matrix and the microorganisms and enzymes targeted. However, it is noteworthy that this technology, applying the processing parameters commonly used in the food industry, cannot inactivate bacterial endospores (Ratphitagsanti, 2009). Pressures of at least 600 MPa with mild temperatures (60°C) are required for the killing of spores of most food spoilage bacteria. Certain strains of *Clostridium botulinum* and *Bacillus species* are reported to withstand hydrostatic pressures of up to 1000 MPa (Heinz and Buckow, 2009). Therefore, they present a much bigger challenge and cannot be inhibited by HHP alone, but which may be possible in combination with more severe heat treatment (Patterson, 2005).

For the inactivation of *Escherichia coli* K-12 and *Staphylococcus aureus* ATCC 6538 in cheese slurries, 20 min of 400 MPa at 30°C, and of 600 MPa at 20°C, respectively, were found to be sufficient (O'Reilly et al., 2000). Likewise, potential pathogenic food bacteria are inhibited due to HHP treatment. Studies on almonds, pressurised in water for 6 min at 414 MPa, followed by air drying at room temperature or 115°C for 25 min resulted in bacterial growth reduction of 4- and 6.7-log units, respectively (Willford, Mendonca and Goodridge, 2008). The authors concluded that HHP treatments show great potential for microbial inactivation if the sample is suspended in the pressurizing medium.

With regards to fungal inhibition by HHP, Bello et al. (2014) investigated the impact of HHP (300, 400 and 500 MPa, respectively) at 20°C for 10 min on fungal mycelia development, spore viability and enzymatic activity of *P. roqueforti*. Mycelia development was significantly reduced following all three treatments, but in direct

correlation with the pressure applied. Furthermore, the spore viability was notably reduced after exposure to 300 MPa and completely inhibited at higher pressures. Similarly, the total lipolytic activity of the samples decreased with increasing pressure. Similar results for the inhibition of different food spoilage fungi, such as *Penicillium* spp., *Fusarium* spp. and *Aspergillus* spp. in liquid medium and cheese were reported by other researchers (Black et al., 2007; O'Reilly et al., 2000). This suggests that HHP treatments are a promising option for the decontamination of cereal grains, in particular as the grains are known to withstand pressures of 400 MPa without sensory or grain quality deterioration. Thus, potential spoilage fungi and their hydrolytic enzymes could be completely inhibited, without the need for classic heat treatments or chemicals. However, asco-spores of heat-resistant moulds were reported to possess a high resistance to HHP also. Hence, pressure treatments routinely applied to foods do not result in sufficient inhibition. Interestingly, HHP appears to sensitize asco-spores to subsequent heat treatments. Thus, a combination of heat and pressure treatment appears very promising (Black et al., 2007).

Although the prevention of fungal growth is the best way to ensure mycotoxin-free crops, minor in-field contamination can result in mycotoxins present in otherwise good quality cereals. Thus, the *in vitro* and *in situ* degradation of mycotoxins is another relevant topic of research. To-date, very few studies have investigated the sensitivity of mycotoxins to HHP treatments. The degradation of patulin in fruit juices was reported after treatment with 600 MPa for 300 sec at 11°C (Hao et al., 2016). Unfortunately, there are no studies available regarding HHP treatment of cereals for microbial decontamination or degradation of mycotoxins. In addition, as observed previously by several researchers, the sensitivity of pathogenic and spoilage organisms and their metabolic products greatly depends on a number of factors, including the surrounding sample matrix, microbial strain, processing conditions and moisture levels (Black et al., 2007; Heinz and Buckow, 2009; Martínez-Rodríguez et al., 2014; O'Reilly et al., 2000; Patterson, 2005; Smith, Mendonca and Jung, 2009). Therefore, without sufficient studies performed on cereal grains, investigating typical cereal contaminants, it is impossible to predict the efficiency of HHP treatments for the microbial decontamination of cereal grains prior to storage.

In conclusion, the application of HHP appears to represent a very promising approach for ensuring microbial safety without the need for chemical preservatives. However, on the down-side, HHP treatments require the sample to be suspended in a liquid medium. Otherwise, the pressure applied cannot be distributed evenly,

leading to unsatisfactory results. This ultimately implies high moisture levels in the stored grains or would necessitate an additional drying step after the initial HHP treatment. HHP treatment has been shown to be effective in reducing the microbial load of foods for both pathogenic and spoilage microorganisms with minimal impact on the product quality. However, the fact that the treatment is unsuitable for in-line procedures and has to be performed in a batch process presents its biggest draw-back. Various parameters such as pressure, time, temperature, and pH have to be considered to optimise the process in terms of microbial inactivation but also considering final product quality (Torres et al., 2014). In particular, the combination of HHP with heating treatments requires further research to fully exploit its potential in the development of new products.

2.8 Conclusion and Future Trends

Scientific evidence for the potential of each treatment as a tool for microbial decontamination is available. In particular, novel technologies, not currently used in industry, were found to present several advantages over established ones. The use of superheated steam for instance combines a faster, more efficient microbial decontamination with less impact on the grain quality, compared to conventional saturated steam or hot air treatments. In addition, the use of e-beam irradiation, high hydrostatic pressure and microwaves, based on non-thermal inactivation mechanisms, presents several advantages over more established technologies, such as gamma rays or UV-light. However, due to their novelty, particularly in terms of microbial decontamination of cereals, the side-effects of these technologies have been sparsely investigated. Therefore, further research is required to obtain a deeper understanding on questions such as the impact on the treated grains and the effect on spoilage organisms after exposure to sub-lethal doses.

Another topic investigated in this review was the degradation of in-field produced and accumulated mycotoxins, to prevent a potential consumer health hazard. All treatments evaluated here showed some potential for reducing the mycotoxin content in cereals. However, due to their structural diversity (Table 1) and higher intrinsic resistance against many treatments, when compared to the living organisms, the reduction of the myxotoxigenic load was found to be a major challenge. In many cases, the treatment time and intensity must be much higher for mycotoxin degradation than for the inhibition of living organisms. Thus, successful degradation of these toxins without compromising the grains quality and seed viability is a major challenge. In addition, major concern related to the decay of mycotoxins are the degradation products released. The vast majority of research has examined the reduction in the level of the original parent mycotoxin, paying little attention to the degradation products or mechanism of degradation. Therefore, the identity of the degradation products and thus, their toxicity are unknown. As a consequence, the efficiency of mycotoxin degradation cannot be evaluated, as the released compounds may potentially be equal or even more toxic compared to the parent toxin. For future research it will be essential to understand the possible degradation mechanisms, in order to identify the resulting compounds. Only then it will be possible to assess their toxicity and evaluate the success and efficiency of the treatments discussed in this review. Furthermore, concerns regarding a possible

re-formation of the toxins during subsequent processing steps and the formation of masked mycotoxins due to the treatments merits greater research interest.

For both purposes, microbial decontamination and detoxification, each treatment encompasses associated difficulties for the successful application to cereals. The even and homogeneous treatment of the whole sample appears to be the biggest challenge. In addition, the efficiency of a treatment depends on various influential factors. The sample matrix, target organism and in particular the moisture content and water availability were the most frequently observed influencing factors within the literature available. Thus, in order to make industrial application possible the treatment conditions and setting must be optimised for each crop. However, even under ideal process conditions it appears unlikely that one single physical treatment can result in sufficient microbial decontamination and detoxification without substantial grain quality deterioration. Consequently, the combination of several treatments appears to represent the most promising approach for optimal results. Future research should therefore focus on understanding and the optimisation of the synergies which are likely to be achievable through combinatory treatments. Then it will be possible to produce products that meet the highest standards in terms of food quality and safety.

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Chapter 3: Novel approaches for Chemical and Microbiological shelf life extension of cereal crops

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3.1 Abstract

Economic losses due to post-harvest fungal spoilage and mycotoxin contamination of cereal crops is a frequently encountered issue. Typically, chemical preservatives are used to reduce the initial microbial load and the environmental conditions during storage are controlled to prevent microbial growth. However, in recent years the consumers' desire for more naturally produced foods containing less chemical preservatives has grown increasingly stronger. This article reviews the latest advances in terms of novel approaches for chemical decontamination, namely application of cold atmospheric pressure plasma and electrolysed water, and their suitability for preservation of stored cereal crops. In addition, the alternative use of bio-preservatives, such as starter cultures or purified antimicrobial compounds, to prevent the growth of spoilage organisms or remove in-field accumulated mycotoxins is evaluated. All treatments assessed here show potential for inhibition of microbial spoilage. However, each method encounters draw-backs, making industrial application difficult. Even under optimised processing conditions, it is unlikely that one single treatment can reduce the natural microbial load sufficiently. It is evident that future research needs to examine the combined application of several treatments to exploit their synergistic properties. This would enable sufficient reduction in the microbial load and ensure microbiological safety of cereal crops during long-term storage.

3.2 Introduction

Cereal crops represent one of the most important food commodities worldwide. However, during growth and storage, cereals are exposed to countless biotic and abiotic stress factors. In particular, pathogenic and spoilage microorganisms, including bacteria, yeasts and filamentous fungi are major problems for the cereal industries. Approximately 15% of the annual global cereal production are lost due to these microbial pests (Freita-Silva, de Oliveira and Freire Júnior, 2014). In addition, if the contaminated crop is not disposed of, it can lead to acute and chronic food borne diseases and consumer health hazards. Inhibition of these spoilage organisms after harvest can prevent the crop from undergoing further spoilage and quality deterioration. But due to microbial metabolites that may accumulate, the potential consumer health hazard can remain. These metabolites, primarily mycotoxins, but also bacterial endotoxins, are not visible, unevenly distributed throughout the crop and not correlated with the level of microbial bio-mass found on the grains (Schmidt et al., 2016; Oliveira, Zannini and Arendt, 2014). Therefore, methods to prevent microbial spoilage and the production of toxins are essential to ensure high food safety and quality. In addition, the removal or degradation of accumulated toxins could further reduce economic losses and consumer health hazards.

At the time of harvest, grains carry a certain microbial load, consisting of various pathogenic and spoilage organisms. Numerous strategies are employed or still being researched into the reduction of such in-field contamination of cereal crops. This topic has been reviewed extensively by Oerke (2006). But due to the ubiquitous presence of bacterial and fungal spores in the environment, their presence on cereal crops cannot be avoided completely. In addition, post-harvest contamination, i.e. during transport or storage, has been reported (Magan and Aldred, 2007), demonstrating the importance of appropriate post-harvest crop treatments. Schmidt et al. (2016) recently demonstrated how minimal fungal in-field contamination can spread rapidly during storage if the conditions are suitable. The activity of contaminant microbes during storage and therefore the crop shelf-life is dependent on a range of different factors, including the water content and availability. To prevent microbial spoilage, cereals are commonly stored at low moisture content (12 – 13%) and water activity (<0.70) (Magan et al., 2003). However, some xenerophilic species of mycotoxigenic spoilage fungi, mainly *Aspergillus* spp., can still develop under these conditions (Oliveira, Zannini and

Arendt, 2014). In addition, other microbes have been found to develop on cereal crops stored in traditional silos or warehouses. This is primarily due to inappropriate vessel seize for the amount of stored grains, creating head spaces with more suitable conditions for spoilage microbes (Gregori et al., 2013).

The organisms most commonly responsible for cereal grain deterioration during long term storage are filamentous fungi. While *Fusarium* spp. are the predominantly occurring field contaminants, *Aspergillus* and *Penicillium* spp. are more likely to develop during storage (Audenaert et al., 2012). Secondary metabolites, i.e. mycotoxins, produced by these fungi (pre- or post-harvest) were found as contaminants in 25% of all cereals produced worldwide (Cheli et al., 2013). This is equal to over 500 million tons of contaminated cereals, being potentially a threat to health. The mycotoxins most frequently found to contaminate cereal crops and their producing fungal genera are shown in Figure 2. Despite being less common contaminants compared to filamentous fungi, spoilage bacteria and yeasts also contribute to the microbial load grains carry after harvest. Although these organisms are much less likely to develop during storage, they can survive in a dormant state and become problematic during subsequent processing. This results in poor product quality and potential consumer health hazards (Butscher et al., 2016). In consequence, good post-harvest management of cereal crops should reduce the natural microbial load substantially, but also degrade or remove harmful metabolites such as mycotoxins that may be present.

Apart from physical processing steps, such as de-hulling and drying, chemical disinfectants are most commonly applied to eliminate spoilage microbes. However, researchers have found numerous problems related to these treatments. As a consequence, the consumer desire for more natural, less processed foods, produced with less chemical preservatives, has grown increasingly stronger. In particular the use of bio-preservatives is highly emphasised and was therefore subject to extensive research in recent decades. This article will first evaluate the current situation regarding the use of conventional chemical disinfectants. Subsequently, alternative approaches for chemical decontamination of cereals, namely cold plasma and electrolysed water, will be evaluated based on recent research advances. In addition, research achievements in terms of bio-preservation will be reviewed and evaluated for their potential applicability to cereal crops. Finally, the possibility to remove previously produced and accumulated mycotoxins will be summarised. Thus, this article will evaluate novel approaches for microbial decontamination based on their suitability for cereal matrices.

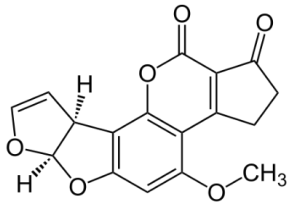
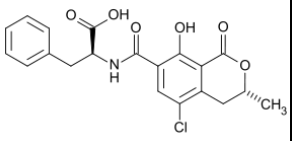
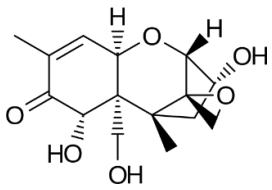
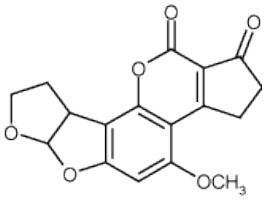
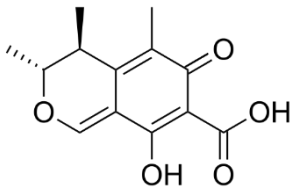
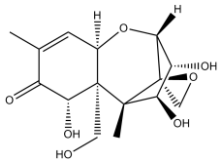
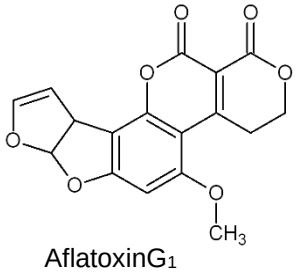
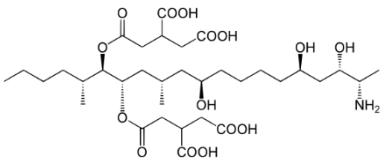
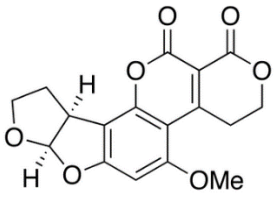
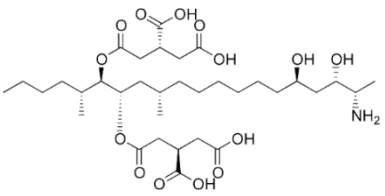
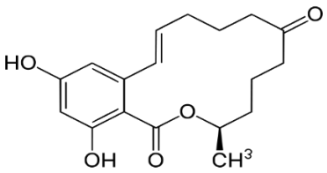
Aspergillus spp.	*Aspergillus* spp./ *Penicillium* spp.	*Fusarium* spp.
 Aflatoxin B_1	 Ochratoxin A	 Deoxynivalenol
 Aflatoxin B_2	 Citrinin	 Nivalenol
 AflatoxinG$_1$		 Fumonisin B_1
 Aflatoxin G_2		 Fumonisin B_2
		 Zearalenone

Figure 2: Chemical structures of mycotoxins commonly found on cereal grains sorted by producing fungus.

3.3 Conventional chemical decontamination

For long-term storage of cereal crops, physical post-harvest treatments, such as drying or de-hulling, and the adjustment of environmental conditions during storage are not sufficient to prevent microbial spoilage entirely (Magan et al., 2003). Thus, chemical disinfectants are employed routinely to reduce the microbial load on both the grains and all contact surfaces. Table 6 shows the minimal inhibitory concentrations (MICs) of commonly used preservatives and disinfectants against various spoilage organisms. Classic chemical treatments, including oxidising agents, antibiotics and organic acids (sorbic acid, propionic acid, benzoic acid and their salts) have a number of drawbacks. Negative effects of these synthetic disinfectants on the human health, in form of hypersensitivity, allergies and asthma, amongst others, have been reported and extensively reviewed by Anand and Sati (2013). Furthermore, several studies conducted in recent years provide evidence that pathogenic and spoilage microbes can develop resistance against commonly used chemical sanitizers. This applies in particular for quaternary ammonium compounds (QACs), but also the above mentioned organic acids and their respective salts (Schnürer and Magnusson, 2005; Voulgari et al., 2010).

Table 6: Minimal inhibitory concentration (MIC) values for selected commonly used chemical decontaminants and preservatives.

Quaternary ammonium compounds	Target organism	MIC-value	Reference
Benzalkonium chloride (BAC)	*Aspergillus* spp.	4 – 8 ppm	Sandle et al. (2014)
	Penicillium spp.	4 ppm	Sandle et al. (2014)
	Fusarium spp.	8 ppm	Sandle et al. (2014)
Didecyldimethymammonium chloride (DDAC)	*Escherichia. coli*	1.3 ppm	Yoshimatsu and Hiyama (2007)
	Enterococcus spp.	1 – 4 ppm	Schwaiger et al. (2014)
	Staphylococcus aureus	0.4 – 1.6 ppm	Ioannou et al. (2007)
Dequalinium (Bis-quaternary ammonium compound)	*Listeria monocytogenes*	8 ppm	Tischer et al. (2012)
	Candida albicans	5.5 µmol/L	Tischer et al. (2012)

Organic acid-based compounds	Target organism	MIC-value	Reference
Potassium sorbate	*Bacillus* spp., *Staphylococcus aureus*	1.0%	Stanojevic et al. (2009)
	Escherichia coli	0.5%	Stanojevic et al. (2009)
	Aspergillus flavus, Candida albicans	5.0%	Stanojevic et al. (2009)
	Fusarium oxysporum	0.725%	Stanojevic et al. (2009)
	Penicillium italicum	1.5%	Stanojevic et al. (2009)
Sodium benzoate	*Bacillus* spp., *Staphylococcus aureus*	1.0%	Stanojevic et al. (2009)
	Escherichia coli	0.5%	Stanojevic et al. (2009)
	Aspergillus flavus	5.0%	Stanojevic et al. (2009)
	Candida albicans	0.25%	Stanojevic et al. (2009)
	Fusarium oxysporum, Penicillium italicum	2.0%	Stanojevic et al. (2009)
Propionic acid	*Staphylococcus aureus, Escherichia coli*	0.25%	Haque et al. (2012)
	Aspergillus spp.	0.25%	Haque et al. (2012)
	Penicillium spp.	0.1 - 0.125%	Haque et al. (2012)
	Candida albicans	0.25%	Haque et al. (2012)

Quaternary ammonium compounds are commonly used disinfectants, in particular because of due to their ease of handling, short half-life time and high efficiency. However, the formation of resistance is a major issue in terms with these compounds (Buffet-Bataillon et al., 2012). Their antimicrobial spectrum of activity includes the prevention of growth of vegetative bacteria, yeasts, moulds, algae and viruses, as well as the inhibition of spore germination (Hegstad et al., 2010). The

main use of QACs is in the disinfection of food contact materials, but also for the sanitisation of silos and warehouses. Due to their universal use they are ubiquitous in the environment.

One of the most frequently used disinfectants is the QAC, benzalkonium chloride (BAC). It shows high efficiency against a broad variety of food spoilage bacteria and fungi. A study investigating the impact of various disinfectants *in vitro* against strains of *Aspergillus niger* and *Penicillium roqueforti* demonstrated the high potential of BAC (Korukluoglu, Sahan and Yigit, 2006). It was the most efficient agent compared to other QAC-based sanitizers, including sodium hypochlorite, formaldehyde, iodine, peracetic acid and isopropyl alcohol (Korukluoglu, Sahan and Yigit, 2006; Tortorano et al., 2005). When applied at a concentration of at least 1.0% (w/w), against 10^6 fungal spores, all spores were inhibited within 3 min of exposure. The potential of BAC is further highlighted by the low MIC values against common fungal contaminants *in vitro*. Values determined for *Aspergillus* and *Fusarium* spp. range between 4 and 8 ppm, and for *Penicillium* spp. between 2 and 4 ppm (Sandle et al., 2014). The spray treatment of shelled hazelnuts, artificially infected with *Aspergillus parasiticus*, using a BAC-solution (5% w/v in distilled H_2O) was found to efficiently inhibit fungal spore germination during 5 weeks of subsequent storage (Basaran, 2011). After exposure to the spray for 1 - 2 min, the effective BAC concentration on the samples was determined to be 0.05 - 0.1 mg/g hazelnut.

On the other hand, it is noteworthy that despite its presence in many sanitizers used for food contact materials, BAC is currently not permitted as a food additive (Kröckel, Jira and Wild, 2003). As a consequence, active concentrations in the food matrix have to remain at residual level, which is for many spoilage organisms equivalent to a sub-lethal dose. As pointed out by Buffet-Bataillon et al. (2012), repeated exposure of microbes with sub-lethal doses increases the risk of resistance formation. This phenomenon was investigated for *Pseudomonas* spp. against dodecyl-trimethyl-ammonium chloride (DTAC). Initially, DTAC was able to inhibit the bacterial growth efficiently. After repeated growth cycles under exposure to increasing levels of DTAC, the bacteria developed the ability to metabolise the disinfectant and use it as the sole source of carbon, nitrogen and energy for growth (Takenaka et al., 2007). This demonstrates how pathogenic and spoilage organisms cannot only develop resistance against disinfectants, but can even adapt to the point of using them as nutritional source. As a result the growth of such bacteria can pose a serious health hazard. Furthermore, QAC degradation results in concentration gradients in the environment, which are likely to further support the

development of bacterial resistance (Tezel and Pavlostathis, 2015). Similar mechanisms of resistance formation have also been reported for filamentous fungi against different antimicrobial agents, including food additives such as potassium sorbate (Sperber et al., 2009). The mechanistic background of resistance formation with a comprehensive evaluation of the topic was recently reviewed by Tezel and Pavlostathis (2015).

In conclusion, the literature available emphasises that routinely used microbial disinfectants cannot serve as long-term solutions for food preservation. Despite several indications for their efficiency in terms of inhibition of pathogenic and spoilage organisms, they also have several disadvantages. Many target organisms are able to develop resistance against these disinfectants. As a consequence, higher concentrations are required, which potentially results in consumer health hazards due to both spoilage organisms and preservatives alike. Furthermore, due to consumers increasing health awareness, the acceptance of chemical preservatives has decreased remarkably in recent years. Thus, food industries and researchers are required to develop novel methods of preservation to produce high quality, yet safe food. The following chapters of this article will evaluate novel approaches for food preservation with emphasis on the suitability for post-harvest preservation of cereal crops.

3.4 Novel approaches for chemical decontamination

In recent years extensive research has been conducted to develop novel approaches for the microbial decontamination of food commodities which do not carry the risk of microbial resistance formation. Two rapidly emerging technologies that would include several advantages for treated food and could easily be applied for microbial shelf life extension of cereals during storage are cold atmospheric pressure plasma (CAPP) and electrolysed water (EW) treatments. Both approaches can disinfect grains without the use of chemicals or leaving potentially harmful residues in the food, both of which would cause problems for the consumer. Thus, CAPP and EW are likely to receive higher consumer acceptance than conventional chemical treatments.

3.4.1 Cold atmospheric pressure Plasma

Due to recent advances in terms of microbial decontamination, cold atmospheric pressure plasma (CAPP) treatments find ever more applications in food industries. Plasma is referred to as the fourth state of matter. The overall system is a quasi-neutral gas but contains several active, unstable species. These include electrons, ions, radicals, electronically excited species and vacuum ultraviolet radiation (Mir, Shah and Mir, 2016). These active species can interact even with the surrounding air and thus, after the treatment, leave no residues on the samples. The antibacterial effects of plasma are already routinely used for disinfection in many areas, such as hospitals, food contact materials and dental medicine. Only in recent years have researchers started to focus on the direct decontamination of food commodities using CAPP. To generate the plasma, high voltage electricity is used to ionize different gases, such as nitrogen or synthetic air, imparting their reactive properties. Figure 3 shows a schematic of CAPP generation. As a dry, non-thermal process, CAPP has the potential to reduce the microbial load with minimal use of water and sensory impact on the treated foods (Niemira, 2012). Furthermore, the occurrence of resistance development upon exposure to sub-lethal doses has, in contrast to conventional chemical disinfectants, not been reported.

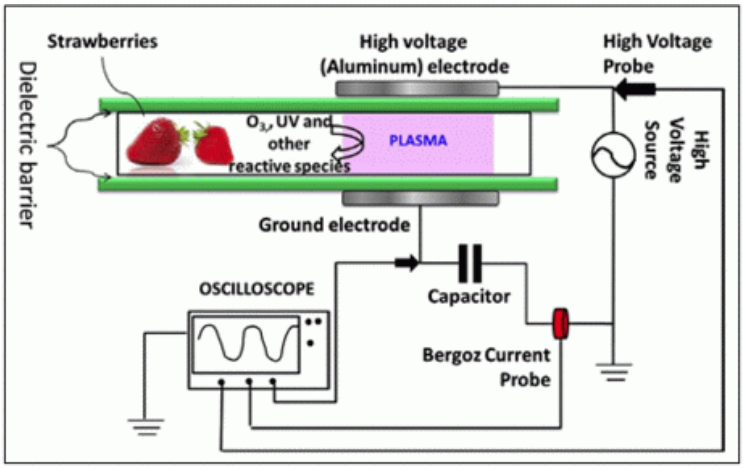


Figure 3: Schematic experimental set-up to generate cold atmospheric pressure plasma, using electric barrier discharge, as previously published by Misra et al. (2014).

In vitro treatment of different antifungal resistant *Candida* spp. spores in air and water with CAPP resulted in >90% spore inactivation after 10 min and 1 min of exposure, respectively (Daeschlein et al., 2011). The plasma was generated from a mixture of helium and oxygen (ratio: 98/2) and applied against 10^4 spores. Similar results were reported for the inactivation of cultures of *Candida albicans, Aspergillus* spp., *Penicillium* spp. and *Rhizopus* spp. on Sabouraud dextrose agar using an argon gas plasma jet (Sun et al., 2011; Matan et al., 2014). Sun et al. (2011) further reported that other fungal isolates of *Trichophyton interdigitale*, *Trichophyton rubrum* and *Microsporum canis* showed even higher sensitivity towards the plasma treatment. Electron microscopy of *Penicillium expansum* spores treated with CAPP *in vitro* revealed severe ruptures in the cell walls and hence cytoplasm leakage causing the cell death (Liang, Zheng and Ye, 2012). Moreover, the authors reported that fungal spore inhibition is directly proportional to the treatment time and thus follows first order kinetics.

To-date, most studies applying CAPP to cereal crops or flours have focused on the effects on grain quality and viability. For CAPP treated wheat grains, positive effects in terms of germination capacity, early plant growth (Filatova et al., 2013; Dobrin et al., 2015) and flour quality (Misra et al., 2015) were reported. The exact mechanisms behind these effects are not fully understood yet, but it is confirmed that plasma treatment induces seed surface modifications which enhance the water absorption and oxygen transmission (Filatova et al., 2013; Dobrin et al., 2015). However, other mechanisms, such as reaction with the ions, electrons and radicals present in the plasma or modifications due to the UV radiation emitted are

discussed also (Dobrin et al., 2015). One of the few studies investigating the potential of CAPP for microbial decontamination of cereals was conducted by Selcuk et al. (2008), using corona discharge plasma generated from air. After treatment for 20 min the spore viability of *Aspergillus parasiticus* and *Penicillium* spp. on various cereal substrates, namely wheat, barley, oat, rye and corn was decreased substantially from 10^7 to 10^4 cell-forming units (cfu)/g. The seed viability was found to remain unaffected by the treatment.

Similarly, the treatment of different nuts for 10 min using CAPP generated from air and sulphur hexafluoride resulted in a cfu-reduction of 2- and 5-log-units, respectively, for *A. parasiticus* (Basaran, Basaran-Akgul and Oksuz, 2008). Interestingly, plasma generated from sulphur hexafluoride showed higher efficiency in fungal growth inhibition, but less in terms of aflatoxin (AF) decomposition when compared to plasma generated from air. Hence, it is evident that different microbial challenges encountered after harvest require different treatment regimens to obtain the best results possible.

Research in recent years has primarily focused on the use of CAPP for degradation and detoxification of mycotoxins *in vitro* and *in situ*. Park et al. (2007) investigated the stability of aflatoxin B_1 (AFB_1), deoxynivalenol (DON) and nivalenol (NIV) (as dry substances) against microwave induced atmospheric pressure argon plasma. After treatment for 5 seconds, the amount of all three mycotoxins was below the limit of detection. Furthermore, cytotoxicity was tested using a mouse macrophage cell line. Cell viability remained at 100% upon exposure to the plasma treated toxins (5 sec), indicating the formation of non-cytotoxic degradation products. Similar results were reported for the degradation of AFs naturally produced by *Aspergillus* spp. *in vitro* and *in situ* (Siciliano et al., 2016).

Ouf et al. (2015) used cold double atmospheric pressure plasma to inhibit *Aspergillus niger* growth on date palm fruits and degrade mycotoxins commonly associated with this fungus. After 9 min of exposure to the plasma, fungal viability, as well as ochratoxin A (OTA) and fumonisin B_2 levels were substantially reduced. A subsequent study was conducted by the authors, using double atmospheric pressure cold plasma to degrade mycotoxins in washing water of mouldy cherries (Ouf, Mohamed and El-Sayed, 2016). Upon plasma treatment for 9 min, reduction rates of 88%, 66% and 72% were determined for aflatoxins, fumonisins and ochratoxins, respectively.

Antibacterial properties of cold plasma treatments in terms of food preservation have been investigated also. When almonds, artificially contaminated with *E. coli* O157:H7 and *Salmonella* spp., were treated for up to 20 seconds with CAPP generated from air, reductions of up to 1.34-log cfu/mL were achieved (Niemira, 2012). In addition, the author reported that plasma generated from nitrogen results in significantly lower antibacterial activity, compared to air as source gas. Treatment of a three strain cocktail of *Listeria monocytogenes* (*L. monocytogenes*) on sliced cheese and ham with CAPP (generated from helium gas) for up to 120 seconds resulted in bacterial reduction by 8- and 1.7-log, respectively (Song et al., 2009). These results highlight the impact of the food matrix being treated on the antibacterial efficiency of the plasma treatment. Furthermore, the efficiency of bacterial inactivation by CAPP treatments in relation to the initial bacterial load was investigated by Fernández et al. (2012). The authors reported a directly proportional relationship between the initial microbial load and the treatment time required for sufficient inactivation, demonstrating the first order inhibition kinetics.

Despite the literature highlighting the potential of CAPP for microbial decontamination and mycotoxin degradation, several influencing factors have to be considered before industrial application becomes feasible. These include exposure time, flow velocity, mechanism of plasma generation, source gas, sample matrix and target organism (Surowsky, Schlüter and Knorr, 2014). Lerouge et al. (2000) studied the influence of the source gas composition on the sterilisation efficacy against *Bacillus subtilis* spores *in vitro*. Of all compositions tested (O_2, O_2/Ar, O_2/H_2, CO_2 and O_2/CF_4) the combination of O_2/CF_4 was found to be the most efficient, while O_2 alone was least efficient in terms of spore inactivation. The literature available also makes it evident that *in vitro* experiments require much shorter treatment times compared to *in situ* trials. Hence, the treatment times necessary for cereals were found to be significantly longer than for water or air matrices (Surowsky, Schlüter and Knorr, 2014). In addition, the antagonistic properties of antioxidants have been reported, for example, in the case of the inactivation of *Aspergillus niger* spores in washing water of mouldy fruits (Ouf, Mohamed and El-Sayed, 2016). The authors concluded that the excited species in the plasma were consumed by reaction with the antioxidants, rather than with the fungal spores. On the other hand, synergistic effects of CAPP with antifungal agents, such as eugenol against *Aspergillus*, *Penicillium* and *Rhizophorus* spp. were reported by Matan et al. (2014).

In conclusion, the great potential of cold plasma treatments to decontaminate and detoxify food commodities such as cereal grains has been demonstrated in several studies without a negative impact on the sample quality. However, research has just started to explore all the factors that are essential for the success and efficiency of the treatment. Thus, this area requires further research in order to determine the optimal processing parameters, depending on sample composition and target organism or toxin. Also the fate of toxins degraded by plasma treatments is of interest, to ensure the treatment results in appropriate detoxification. In addition, it must be considered that CAPP is a treatment limited to the grain surface only. Thus, it is inefficient against field-borne pathogens which have already penetrated through the grain surface and their respective metabolites underneath the grain surface. Due to the uneven surface of cereals and the possibility of loose pieces of bran the treatments efficiency is compromised even further (Butscher et al., 2016). Finally, for the purpose of industrial application cost-effectiveness and adaptability have to be considered as well. Hence, it has to be considered as major drawback that the processing parameter need careful adjustment for each sample application. In order to become a true option for industrial decontamination more research from the engineering site is required to improve cost-effectiveness and adaptability of the system. Therefore, it appears unlikely that in the near future CAPP can serve as sole pre-storage treatment for cereals to extend the microbial shelf-life. Instead, industrial applications in combination with other treatments or post-processing treatment, i.e. for cereal flours, appear more likely.

3.4.2 Electrolysed water (EW)

Despite having a long tradition as a sanitizing agent in Japan, in Europe electrolysed water (EW) is an emerging new approach for microbial decontamination. Thus far, it is primarily used to sanitize food contact surfaces. It represents a cost effective, safe and easy to apply disinfection method for silos and warehouses, to increase the microbial safety of cereal grains during storage (Rahman, Khan and Oh, 2016). Figure 4 shows the schematic production of EW in an electrolysis chamber. It is generated by electrolysis of a diluted sodium chloride solution with anode and cathode separated by a non-selective membrane. This process results in alkaline EW (also known as electrolysed reduced water) in the anode chamber and acidic EW (also known as electrolysed oxidising water) in the cathode chamber. The acidic EW is reported to have great antimicrobial efficiency

when applied *in vitro* (Rahman, Khan and Oh, 2016). However, due to the low pH-value (<3.0) and corrosive nature, industrial applications are very limited. Therefore, neutralised EW (NEW, also known as low concentration or slightly acidic EW) presents an easier to handle but equally efficient solution (Rahman et al., 2012) and is therefore the main focus of this section. It is produced in a similar way to acidic EW but the cathode and anode chambers are not separated by a membrane, resulting in pH-values of 5 – 6.5. Thus, most of the chlorine is present in the form of hypochloric acid (HOCl) which has even higher antimicrobial activity, compared to the hypochlorite-anion (ClO^-) present at lower pH (Audenaert et al., 2012). In addition, form the three parameters defining the mode of action of EW (pH, oxido-reduction potential and available chlorine concentration) the available chlorine concentration (ACC) was found to have the biggest influence on the antimicrobial properties (Mokudai et al., 2012).

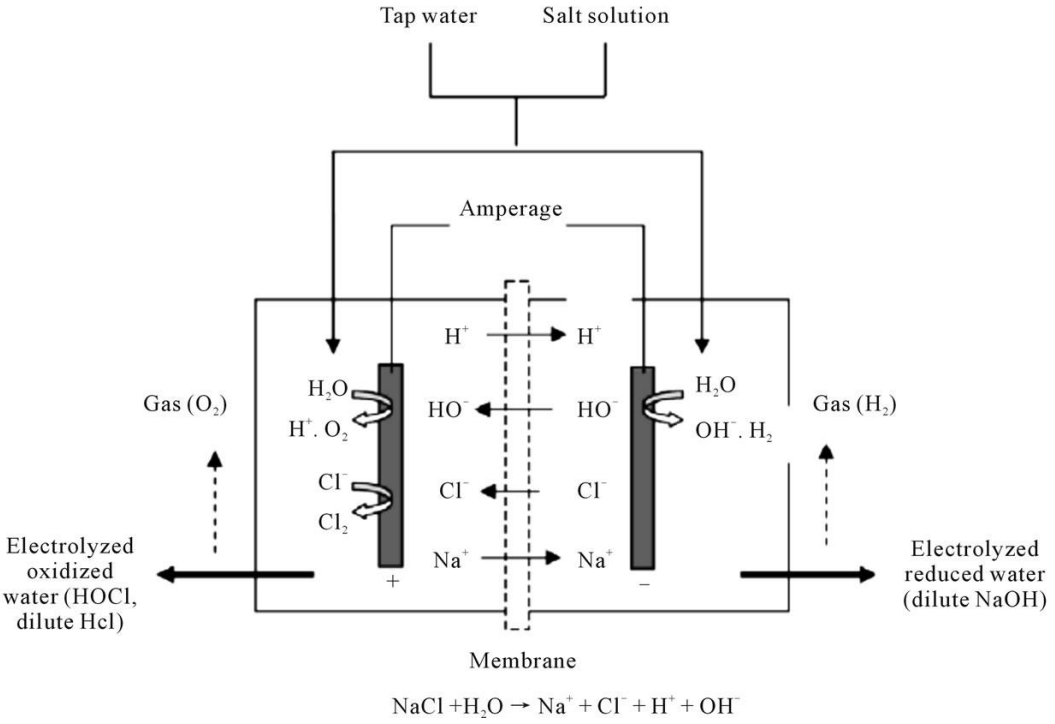


Figure 4: Schematic diagram of electrolyzed water generation system, as published previously by Hati et al. (2012).

The *in vitro* antimicrobial activity of NEW against various plant pathogens, including *Fusarium oxysporum*, *Botrytis cinerea*, *Alternaria* spp., *Colletotrichum* spp. and *Monilinia longicolla* has been reported previously (Huang et al., 2008). However, it was also shown that, due to the high reactivity and low specificity of HOCl, the

sanitizing effect of NEW can be compromised by the presence of organic matter. As low as 0.3% (w/v) of organic matter substantially reduced the antimicrobial activity of NEW (Zhang et al., 2016a). Thus, it remains unclear if the antifungal activity *in vitro* can be transferred into a food system, such as cereal grains.

Various successful applications of NEW in food industries, including fish, meat, fruit and vegetable processing are established already (Rahman, Khan and Oh, 2016). Few studies in relation to agricultural products, however, have been conducted so far. Nevertheless, the antimicrobial properties of NEW were reported for the decontamination of various food commodities, including brown rice (Liu et al., 2013), millet (Li et al., 2015), radish (Zhang et al., 2016a) and mug bean sprouts (Rui et al., 2011). Furthermore, when applied during sprouting, NEW showed positive effects on the sprouting capacity of radish and mug beans (Zhang et al., 2016; Rui et al., 2011). Unfortunately, no studies investigating the application of NEW to improve the seed viability in combination with microbial disinfection of cereal grains are yet available. If NEW could also promote the germination of cereal grains, for instance for malting purposes, it would have the potential to perform as “clean-label” disinfectant with technological benefits. Therefore, it should be of particular interest for the cereal industries to investigate the potential of NEW further.

To-date, most studies investigating the antimicrobial activity of EW focused on the bactericidal activity. The application of NEW on radish sprouts reduced the populations of several food spoilage bacteria by >90% after 3 min exposure, namely *E. coli*, *L. monocytogenes*, *Enterobacteriacea* and *Pseudomonas* spp. (Zhang et al., 2016b). NEW was found to be even more effective against various strains of *E. coli* and *Campylobacter jejuni* than conventional chemical disinfectants, such as sodium hypochlorite or chlorine water with the same ACC (Park, Hung and Brackett, 2002; Jadeja, Hung and Bosilevac, 2013). Likewise, 3 min *in vitro* treatment of *Bacillus subtilis* and *Bacillus cereus* endospores with NEW with an ACC of 80 ppm fully inhibited the spore germination in both liquid medium and solid carrier (Zhang et al., 2016b). The efficiency of NEW is reported to increase further when applied in combination with other naturally produced antimicrobial substances, such as calcium lactate (Rahman, Wang and Oh, 2013) or if used at slightly elevated temperatures (Mansur and Oh, 2015). Rising the processing temperature from 25 to 40°C significantly increased the sanitizing effect against various common food spoilage bacteria (Mansur and Oh, 2015). In addition, the possibility of synergistic effects between NEW and ultrasonication (US) or thermosonication (TS) is gathering more research interest. Forghani and Oh, (2013) found a doubled log-

reduction of different spoilage bacteria when the NEW treatment was followed by 3 min US treatment and subsequent washing in distilled water, compared to each of the treatments alone. Furthermore, the combination of TS with NEW in a similar manner resulted in substantially increased antibacterial performance compared to the individual treatments (Mansur et al., 2014; Mansur and Oh, 2015). However, researchers have just started to explore the possibilities of combining NEW with existing methods. Hence, a lack of *in situ* investigations is still evident and requires more research to exploit the full potential of NEW.

While the rapid degradability of EW has the advantage that no residues remain on the samples after treatment, it also represents a substantial drawback in terms of storage stability. Both NEW and acidic EW showed significantly reduced bactericidal activity against *E. coli* and *L. monocytogenes* isolates after just 4 days of storage, even under ideal conditions (Rahman et al., 2012). After 21 days, the bactericidal activity was lost completely. Hence, EW has to be used fresh, directly after generation, in order to achieve the best sanitizing results.

One of the very few reports regarding the antifungal performance of NEW was conducted by Zhang et al. (2016a). The authors reported >90% inhibition of environmental yeasts and moulds on radish sprouts after washing with NEW (ACC: 40 ppm) for 3 min. The use of NEW to control the fungal load on wheat grains showed that, depending on the fungal isolate, NEW has equal or even better efficiency at inhibiting *Fusarium* spp. compared to a common fungicide (prothioconazole + fluoxastrobin) (Audenaert et al., 2012). However, the same study also reported an increased accumulation of DON if the NEW treatment was at a sub-lethal level for the fungus. Total spore germination inhibition of *A. flavus* (approximately 10^7 spores/mL) *in vitro* was reported for NEW and acidic EW after 90 and 120 seconds of exposure, respectively (Xiong, 2012; Xiong et al., 2010). For both samples the ACC was adjusted to 30 ppm. Thus, NEW was found to demonstrate even higher antifungal activity than acidic EW. The authors concluded this is due to the higher concentration of hydroxyl radicals present in NEW. However, as described above, NEW also contains more HOCl than the more acidic EW which mainly contains the de-protonated anion.

While studies on microbial decontamination primarily feature the application of NEW, in terms of mycotoxin degradation acidic EW was found to deliver more promising results. It was reported to significantly reduce AFB_1 levels by 85% in artificially contaminated peanuts after 15 min soaking time (Zhang et al., 2012).

Thereby, the high concentration of available chlorine was identified as the predominant reason for the toxin decomposing effect. Furthermore, a soaking time of 15 min at a temperature between 25 and 45°C was found to increase the treatment efficiency substantially. In a similar study conducted by Xiong (2012) comparable results were found and in addition, the AFB_1 degradation product was identified as the non-mutagenic or cytotoxic, 8-chloro-9-hydroxy-AFB_1. Furthermore, it is noteworthy that essential nutrients in the treated peanuts were found to be unaffected by the EW treatments. Similarly, the use of alkaline EW resulted in almost complete removal of AFB_1 from different plant oils, after washing for 5 min at 20°C (Fan et al., 2013).

In conclusion, EW in general, and NEW in particular, have been shown by several researchers to exhibit very strong antimicrobial activity combined with little to no impact on the treated sample. It does not involve the use of hazardous chemicals, is produced in an environmentally friendly manner and is rapidly degradable, leaving no residues on the samples. Furthermore, it was reported to promote mug bean sprouting. To-date, it has not been investigated if this effect also occurs on cereal grains, but should be a consideration i.e. for brewing industries, as disinfectant with potential technological benefits during malting. However, very little research regarding the antifungal activity and the ability to remove mycotoxins has been conducted to-date and therefore requires further investigations. The risk of increased mycotoxin accumulation after treatment with sub-lethal doses and the fate of toxins other than AFB_1 requires more research attention, to ensure high product quality and safety. Also, the extremely low storage stability must be considered before possible application. Moreover, studies exploring the possibilities for application on cereal matrices and synergisms with other treatments, such as US, are required. Only then can a conclusion regarding the potential of EW to replace conventional chemical decontamination treatments be drawn.

3.5 Possibilities for post-harvest bio-protection

Another approach to satisfy this increasing consumer desire for less chemical preservatives while maintaining or increasing food quality and safety is the use of bio-preservatives. The highest potential for commercial use as bio-preservatives is attributed to lactic acid bacteria (LAB). This is, in particular, due to their century long tradition of use in the production of fermented foods and their high antimicrobial activity (Axel et al., 2016; Crowley et al., 2013; Dalié et al., 2010; Oliveira et al., 2014; Oliveira et al., 2015b; Pawlowska et al., 2012). Therefore, the following section will primarily focus on the potential application of selected LAB strains or their respective antimicrobial metabolites as bio-preservatives and their suitability for cereal crops.

Bio-preservatives can be applied using two different approaches. The food matrix can be inoculated with the bacterial culture, in order to suppress the growth and development of spoilage organisms. This is primarily based on the production of active compounds *in situ* and competition for nutrients and space. On the other hand, antimicrobial metabolites can be produced *in vitro* and subsequently applied to the sample, without the presence of the living cells. Table 7 summarises successful applications of bio-protective cultures and their respective metabolites in different food matrices. Both approaches would ideally result in extended shelf life and microbial safety with less impact on the sensory and nutritional properties than conventional chemical preservatives or physical processes (Gálvez et al., 2010; Schillinger, Geisen and Holzapfel, 1996). This section will evaluate recent advances for both approaches, with their perspectives and possible drawbacks for their future application as post-harvest cereal treatments. It also should be mentioned that plant based antifungal peptides lately received a lot of research interest as promising biocontrol agents. However, as the topic is extensively reviewed elsewhere (Goyal and Mattoo, 2016; Maróti, Downie and Kondorosi, 2015; Vriens, Cammue and Thevissen, 2014) the authors decided to exclude it from the present review.

Table 7: Selected genera of lactic acid bacteria cultures reported to inhibit microbial spoilage organisms.

Bio-preserving microorganism	Spoilage organism inhibited	Method of application	Sample matrix	Reference
Leuconostoc citreum spp.	*Cladosporium* sp., *Neurospora* sp., *Penicillium* sp.	Protective culture	Rice cake	Baek et al. (2012)
	Penicillium sp., Aspergillus sp.	Protective culture	Milk bread roll	Le Lay et al. (2016a)
Leuconostoc mesenterodies sp.	*Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*	LAB cell-free supernatant	Ornithine decarboxylase broth	Ozogul et al. (2015)
Weissella confusa spp.	*Cladosporium* sp., *Neurospora* sp., *Penicillium* sp.	Protective culture	Rice cake	Baek et al. (2012)
Lactobacillus plantarum spp.	*Penicillium solitum*, *Aspergillus versicolor*, *Cladosporium herbarum*	Protective culture	Cottage cheese	Cheong et al. (2014)
	Aspergillus fumigatus, *Penicillium* spp.	Protective culture	Gras silage	Broberg et al. (2007)
	Fusarium culmorum	Protective culture	Barley malt wort	Peyer et al. (2016)
	Aspergillus niger, *Penicillium* spp., *Fusarium culmorum*	Protective culture	Wheat sourdough	Ryan et al. (2008)
	Escherichia coli	Phenolic acids	MRS medium	Sánchez-Maldonado et al. (2011)
	Penicillium sp., *Aspergillus* sp.	3-phenyllactic acid	MRS medium	Cortés-Zavaleta et al. (2014)
	Aspergillus niger, *Endomyces fibuliger*, *Penicillium roqueforti*	3-phenyllactic acid, polyporic acid	MRS medium	Valerio et al. (2016)
	Aspergillus spp., *Penicillium* spp.	Various monohydroxy FAs	MRS medium	Sjögren et al. (2003)

Lactobacillus plantarum spp.	*Aspergillus* spp., *Penicillium* spp.	Various monohydroxy FAs, 5-oxododecanoic acid	Malt extract agar	Ryu et al. (2014)
	Fusarium sp.	Methyl-FAs	Stored maize	Deepthi et al. (2016)
	Penicillium roqueforti, *Aspergillus fumigatus*	Cyclo(L-Phe-L-Pro), cyclo(L-Phe-trans-4-OH-L-Pro)	MRS medium	Ström et al. (2002)
	Candida albicans	Cis-cyclo(L-Val-L-Pro), cis-cyclo(L-Phe-L-Pro)	Potato dextrose agar	Kwak et al. (2014)
	Aspergillus spp.	Antifungal peptide	Malt extract broth	Muhialdin et al. (2016)
	Aspergillus niger, Rhizopus stolonifer, *Mucor racemosus*, *Penicillium chrysogenum*	Antifungal peptide	Stored wheat grains	Gupta and Srivastava (2014)
	Fusarium oxysporum, *Aspergillus flavus*	Cell-free supernatant	vegetables	Sathe et al. (2007)
Lactobacillus reuteri spp.	*Clostridium* spp.	reuterin	milk	Ávila et al. (2014)
	Escherichia coli O157:H7, *Salmonella enterica*, *Yersinia enterocolitica*, *Aeromonas hydrophila, Campylobacter jejuni*	reuterin	milk	Arqués et al. (2011)
	Clostridium tyrobutyricum	reuterin	cheese	Gomez-Torres et al. (2014)
	Clostridium spp.	reuterin	milk	Ávila et al. (2014)

Lactobacillus reuteri spp.	*Escherichia coli* O157:H7, *Salmonella enterica*, *Yersinia enterocolitica*, *Aeromonas hydrophila, Campylobacter jejuni*	reuterin	milk	Arqués et al. (2011)
	Clostridium tyrobutyricum	reuterin	cheese	Gomez-Torres et al. (2014)
	Listeria monocytogenes	reuterin	salmon	Montiel et al. (2014)
	Cladosporium sp., *Wallemia* sp.	Protective culture	Pound cake	Le Lay et al. (2016a)
	Fusarium culmorum	Cell-free supernatant	Barley malt	Oliveira et al. (2015b)
	Environmental moulds	Protective culture	Wheat sourdough	Axel et al. (2016)
	Escherichia coli	Phenolic acids	MRS medium	Sánchez-Maldonado et al. (2011)
	Penicillium sp., *Aspergillus* sp.	3-phenyllactic acid	MRS medium	Cortés-Zavaleta et al. (2014)
	Zymoseptoria tritici	LAB cell-free supernatant	Wheat grains	Lynch et al. (2016)
Lactobacillus amylovorus DSM 19280	*Fusarium culmorum*	Cell-free supernatant	Barley malt	Oliveira et al. (2015b)
	Environmental moulds	Protective culture	Wheat sourdough	Axel et al. (2016); Belz et al. (2012)
	Aspergillus fumigatus, Fusarium culmorum	Protective culture	Wheat sourdough	Ryan et al. (2011)
Lactobacillus brevis spp.	Environmental moulds	Protective culture	Wheat sourdough	Axel et al. (2016)

Lactobacillus brevis spp.	*Aspergillus niger*, *Endomyces fibuliger*, *Penicillium roqueforti*	3-phenyllactic acid, polyporic acid	MRS medium	Valerio et al. (2016)
	Zymoseptoria tritici	LAB cell-free supernatant	Wheat grains	Lynch et al. (2016)
	Environmental moulds	C18:1 monohydroxy acids	Rolls, cake	Le Lay et al. (2016)
Lactobacillus spicheri sp.	*Cladosporium* sp., *Wallemia* sp.	Protective culture	Pound cake	Le Lay et al. (2016a)
Lactobacillus hammesii spp.	*Aspergillus niger*, *Penicillium roqueforti*	Hydroxy-fatty acids	Wheat sourdough	Black et al. (2013)
	Escherichia coli	Phenolic acids	MRS medium	Sánchez-Maldonado et al. (2011)
Lactobacillus fermentum spp.	*Listeria monocytogenes*, *Escherichia coli*, *Bacillus cereus*	Protective culture	Chicken meat	Maragkoudakis et al. (2009)
	Aspergillus oryzae, *Aspergillus niger*	LAB cell-free supernatant	bread	Muhialdin et al. (2011)
	Aspergillus sp., *Fusarium* sp., *Penicillium* sp.	Antifungal peptide	MRS medium	Gerez et al. (2013)
	Penicillium sp., *Aspergillus* sp.	3-phenyllactic acid	MRS medium	Cortés-Zavaleta et al. (2014)
	Aspergillus niger, *Endomyces fibuliger*, *Penicillium roqueforti*	3-phenyllactic acid, polyporic acid	MRS medium	Valerio et al. (2016)
	Escherichia coli	Phenolic acids	MRS medium	Sánchez-Maldonado et al. (2011)
Lactobacillus casei spp.	*Penicillium* sp., *Aspergillus* sp.	3-phenyllactic acid	MRS medium	Cortés-Zavaleta et al. (2014)
	Geotrichum sp., *Alternaria* spp., *Fusarium* sp., *Aspergillus* sp.	LAB cell-free supernatant	MRS medium	Lipińska et al. (2016)

Lactobacillus acidophilus spp.	*Penicillium* sp., *Aspergillus* sp.	3-phenyllactic acid	MRS medium	Cortés-Zavaleta et al. (2014)
	Geotrichum sp., *Alternaria* spp., *Fusarium* sp., *Aspergillus* sp.	LAB cell-free supernatant	MRS medium	Lipińska et al. (2016)
Lactobacillus rhamnosus sp.	*Penicillium* sp., *Aspergillus* sp.	3-phenyllactic acid	MRS medium	Cortés-Zavaleta et al. (2014)
Lactobacillus sakei sp.	*Penicillium* sp., *Aspergillus* sp.	3-phenyllactic acid	MRS medium	Cortés-Zavaleta et al. (2014)
Lactobacillus paracasei sp.	Various food spoilage bacteria and fungi	Antimicrobial peptide	MRS medium	Miao et al. (2014)
	Aspergillus niger, *Endomyces fibuliger*, *Penicillium roqueforti*	3-phenyllactic acid, polyporic acid	MRS medium	Valerio et al. (2016)
	Aspergillus oryzae, *Aspergillus niger*	LAB cell-free supernatant	bread	Muhialdin et al. (2011)
	Geotrichum sp., *Alternaria* spp., *Fusarium* sp., *Aspergillus* sp.	LAB cell-free supernatant	MRS medium	Lipińska et al. (2016)
Lactococcus lactis sp.	*Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*	LAB cell-free supernatant	Ornithine decarboxylase broth	Ozogul et al. (2015)
Streptococcus thermophiles sp.	*Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*	LAB cell-free supernatant	Ornithine decarboxylase broth	Ozogul et al. (2015)
Lactobacillus pentosus sp.	*Aspergillus oryzae*, *Aspergillus niger*	LAB cell-free supernatant	bread	Muhialdin et al. (2011)
Lactobacillus coryniformis sp.	natural spoilage bacteria	reuterin	silage	Tanaka et al. (2009)
Pediococcus pentosateus spp.	Environmental fungi	Protective culture	Wheat kernels	Suproniene et al. (2015)
	Aspergillus oryzae, *Aspergillus niger*	LAB cell-free supernatant	bread	Muhialdin et al. (2011)

Pediococcus acidilactici	*Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*	LAB cell-free supernatant	Ornithine decarboxylase broth	Ozogul et al. (2015)

3.5.1 Protective cultures

Apart from the low consumer acceptance, the application of pure bacteriocins (synthesised or isolated) involves certain difficulties. The most significant is the interaction or binding with food constituents, such as proteins or fats (Settanni and Corsetti, 2008), resulting in reduced efficiency and unwanted residues in the food matrix. On the other hand, the use of microorganisms as bio-preservatives has a number of advantages. Firstly, they produce a mixture of several antimicrobial-active compounds, including peptides, organic acids, fatty acids, ethanol and hydrogen peroxide amongst others (Crowley, Mahony and Van Sinderen, 2013; Oliveira, Zannini and Arendt, 2014). An overview of antimicrobial compounds produced by LAB is shown in Figure 5. The synergistic effects of this mixture provide substantial microbial inhibition at very low concentrations of the active compounds. Furthermore, competition for nutrients with spoilage organisms supports the inhibition further. Finally, it is noteworthy that the colonisation of grains with protective cultures can add functional properties to the product and so contribute to flavour, texture or nutritional value of the product, in addition to allowing a clean label (Oliveira, Zannini and Arendt, 2014).

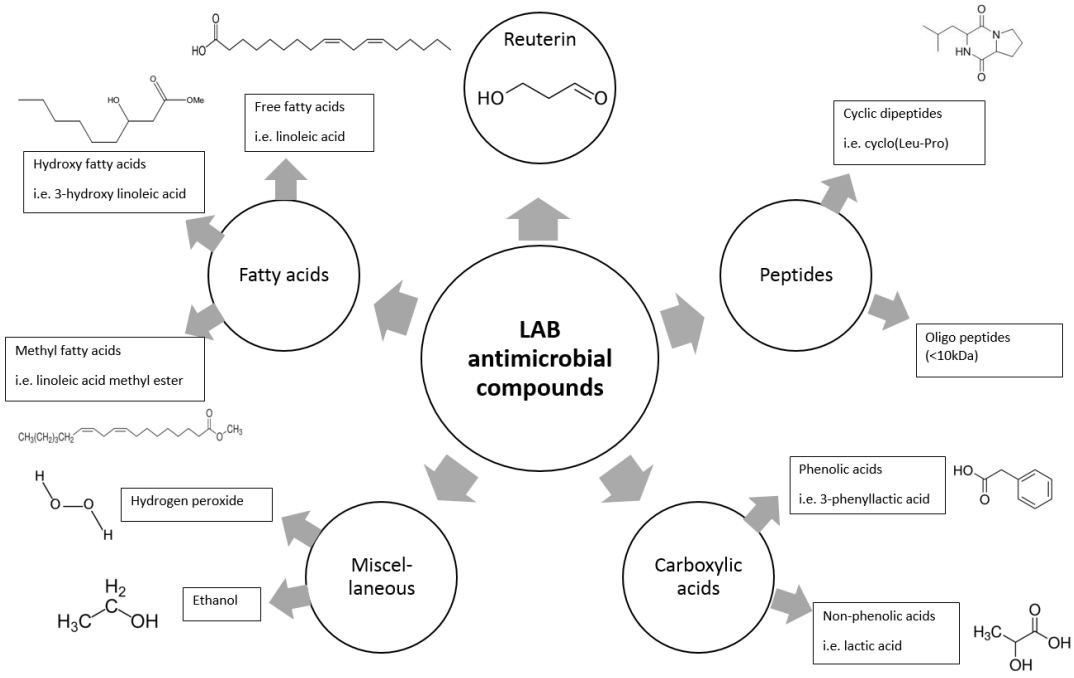


Figure 5: Antimicrobial compounds produced by lactic acid bacteria (LAB), adapted from Crowley et al. (2013).

As outlined above, due to their high antimicrobial activity and natural occurrence in fermented foods, LAB represent the microbial group most commonly used as protective cultures for food commodities. In addition, the use of LAB in foods is not regulated by harmonised legislation at EU level (except in Denmark and France) with regards to their use as starter cultures, protective cultures or food supplements (Gaggia et al., 2011). Consequently, LAB-protective cultures have been exploited in the production of several foods, including fruits and vegetables, rice cakes, beer and cheese (Baek et al., 2012; Cheong et al., 2014; Crowley et al., 2013; Gerez et al., 2010; Oliveira et al., 2015a; Oliveira et al., 2015b; Sathe et al., 2007), amongst others. In addition, strains of *Lactobacillus plantarum* were reported to protect grass and cereal silage from microbial deterioration during storage (Broberg et al., 2007). Also use of cultures as bio-preservatives for maize kernels intended for animal feed has been reported (Melin et al., 2007).

Numerous LAB isolates are known to be suitable as protective cultures in a cereal matrix. Such strains have been primarily investigated for their use as starter cultures and bio-protectants for sourdough in the baking industry. Several studies have reported an extended microbial shelf-life for the resulting products due to the use of specific strains as sourdough starters (Axel et al., 2016; Le Lay et al., 2016a; Le Lay et al., 2016b; Ryan et al., 2008). LAB isolates belonging to the species of *L. reuteri*,

L. amylovorous and *L. plantarum* were most frequently reported to significantly delay the outgrowth of common environmental bread spoilage fungi and yeasts, compared to breads baked with sourdough made using non-antifungal cultures. The antifungal activity of a *Lactococcu*s sp. was demonstrated by Varsha et al. (2014), using the strain as sourdough starter culture. After fermentation for 36 h, the resulting sourdough was incorporated in the bread making procedure. Slices of the baked breads were artificially contaminated with 10^4 spores per slice of *Aspergillus niger*, *Fusarium oxysporum* and *F. moniliforme*, respectively. The authors reported a shelf-life extension from 3 up to 5 days due to the antifungal *Lactococcus* strain. However, it is noteworthy that this study did not take the pH reduction, compared to the control, into account. However, Axel et al. (2016) compared the microbial shelf-life of sourdough bread produced with antifungal LAB strains as starter cultures with a chemically acidified sourdough bread. The bacterial fermented sourdough resulted in a substantially longer bread shelf-life compared to the chemically acidified sourdough. Hence, this study demonstrates that the *in situ* antifungal activity of LAB is due to the metabolites produced during fermentation, as opposed to simply the pH-reduction alone. Similar results were reported by other researchers, using the same strain as a replacement for conventional preservatives, such as salt and calcium propionate (Belz et al., 2012; Ryan, Dal Bello and Arendt, 2008). In addition, various LAB isolates belonging to the species *L. plantarum* (Ryan et al., 2011) and *L. hammesii* (Black et al., 2013) were found to be suitable bio-preservatives for the baking industries. However, one significant drawback in the use of LAB protective cultures, is he major impact that the substrate can have on the production of antifungal compounds (Axel et al., 2015). Substantial differences regarding the production of antifungal compounds by *L. amylovorus* DSM 19280, depending on the substrate being fermented, were reported (Axel et al., 2015). Similarly, the antifungal activity of *Lactobacillus rhamnosus* against various spoilage and pathogenic moulds *in vitro* was found to depend primarily on the carbon source present (Toplaghaltsyan, Bazukyan and Trchounian, 2016). However, the antifungal activity of LAB protective cultures *in situ* is usually significantly lower compared to *in vitro* trials (Le Lay et al., 2016a). The authors attributed this primarily to the lower concentration of active compounds, compared to *in vitro* challenge tests. However, influential effects of the food matrix and constitution regarding the antifungal performance should be considered and investigated as well. Thus, further optimisation regarding the antifungal activity of LAB, in order to obtain better applicability as bio-preservatives, is required for future research.

The application of selected LAB isolates on naturally contaminated wheat kernels resulted in significant inhibition of fungal growth, in particular for *Fusarium* spp. (Suproniene et al., 2015). In addition, the authors investigated the influence of the LAB isolates on seedling diseases and germination capacity but reported only minor influences. In addition, the production and accumulation of mycotoxins, in particular trichothecenes and fumonisins in wheat grains was found to be significantly reduced by the use of antifungal LAB (Chulze et al., 2015). However, despite the great diversity of products employing LAB as bio-protective cultures to-date, no study has yet examined their application during storage of cereals intended for food use. Thus, future research should focus on hurdles such as the environmental and nutritional needs of the bacteria, in order to make LAB a feasible option as protective cultures for cereal crops.

The antibacterial properties of LAB have been investigated to a lesser extent than their antifungal activity. However, some studies report the successful inhibition of food spoilage bacteria, in particular *L. monocytogenes*, *in vitro* (Voulgari et al., 2010) and *in situ* (Tomé et al., 2008; Randazzo, Caggia and Neviani, 2009; Trias et al., 2008; Maragkoudakis et al., 2009). However, as none of the *in situ* studies have investigated these antibacterial properties in the cereal matrix, no conclusion for the suitability in such application is possible. However, it was reported that typically used sourdough starter cultures have just very limited production of bacteriocins like substances (Corsetti, Settanni and Van Sinderen, 2004). On the other hand, the study found that LAB cultures which produce bacteriocin like substances are also capable of producing them in a sourdough matrix. These findings make it likely that certain LAB strains could express antibacterial activity during cereal storage likewise. Depending on the amino acids available for the bacteria, antibacterial activity can be achieved *in vitro* or *in situ*. However, further research is required to obtain more detailed information regarding the suitability of LAB as antibacterial control agent.

Although LAB are the most commonly used microbes for bio-protection, other bacterial species, and yeasts, have also been investigated for their potential in terms of protection against spoilage and pathogenic micro-organisms. The inhibitory effect of 58 yeast species against *P. roqueforti* during long term storage of high moisture wheat grains under air tight conditions revealed that 11 species showed minor inhibition of the fungus (reduction from 10^5 to 10^4 cfu/g). Another 9 species showed high inhibition, equal to reduction of *P. roqueforti* by 2-log units or more during storage. The best inhibitory effect was found for the yeast *Pichia anomala*

J121, resulting in total fungal inhibition over 6 month of storage (Druvefors and Schnürer, 2005; Druvefors et al., 2002). Furthermore, due to the fact that 27 species exhibited comparable growth to *P. anomala* J121, while showing no inhibitory effect, it was proposed that the competition for nutrients and space had a minor impact on the antifungal performance (Druvefors and Schnürer, 2005). Thus, the main focus when choosing a protective culture must to be on the isolates range of and ability to produce antimicrobial metabolites, rather than its competitiveness.

Aureobasidium pullulans, an antagonistic yeast-like fungus was tested for its suitability as protective culture against wheat kernel colonisation by *F. culmorum* during storage. Thereby, *A. pullulans* was found to inhibit the growth of *F. culmorum* significantly during the 6 month storage period compared to the control (Wachowska, Tańska and Konopka, 2016). Furthermore, minor decreases in the amounts of valuable bioactive compounds, namely carotenoids, sterols, tocols and alkyl resorcinols were reported due to the protective culture. The amount of wheat gluten proteins remained unchanged, indicating a good bread making ability after the treatment. Consequently, the suitability of *A. pullulans* as protective culture for food commodities, including cereal crops, was demonstrated. These findings are also in agreement with the results of Ferreira-Pinto et al. (2006) who successfully applied *A. pullulans* as biocontrol agent in pears.

In conclusion, if applied appropriately, microbial protective cultures could serve as an effective tool for post-harvest shelf-life extension of cereals. As outlined in this section, several studies have demonstrated the efficiency of LAB as protective cultures in various food products, including cereal matrices. However, the application to cereal storage systems is only sparsely investigated and has inherent difficulties. In order to provide sufficient bio-protection, the environmental conditions, including oxygen content, temperature and nutritional requirements have to be suitable for the bacterial metabolism. Another limitation is the fact that LAB need a fermentable substrate in order to express their antifungal activity. Therefore, it remains unclear if LAB can serve as efficient preservatives during long-term storage. The cultures may consume all fermentable nutrients and subsequently die during storage. However, the few studies applying LAB as bio-preservatives showed promising results in terms of fungal growth inhibition and prevention of mycotoxin accumulation. A possible explanation for these findings could be in the lysis of yeast or bacterial cells, leading to the release of antifungal compounds from the cytoplasm (Peyer et al., 2016). Fermentation times of 120 h have been shown to yield high amounts of lactic and acetic acids due to cell lysis. Therefore, future research

should be conducted in order to quantify the antifungal performance of LAB and yeasts after cell-lysis. This would allow deeper insights to the suitability of microbial protective cultures for cereal storage. On the other hand, the possibility of metabolic conversion of antifungal compounds by the protective culture itself, yielding less or completely ineffective compounds should be taken into account

However, more research using different cereals and target spoilage organisms, as well as long storage periods are required to fully evaluate the potential of protective cultures before they become a feasible option for industrial application. Despite the fact that no negative effects related to grain quality were found due to the application of protective cultures, the presence of a high bacterial loads might be undesirable for further down-stream applications. Finally, the use of fungal antagonistic yeast cultures has received little research attention to-date, but have shown promising initial results. Consequently, future research should also examine the possible application of microbes other than LAB as protective cultures.

3.5.2 Antimicrobial compounds

As outlined in the previous section, the application of living organisms, such as LAB for microbial protection presents various challenges, in particular with regards to the environmental and nutritional requirements of the protective cultures. Therefore, it appears potentially easier and more reliable to produce the antagonistic compounds *in vitro* and apply the isolated substances. Possibilities and hurdles encountered with this approach are evaluated in this section.

Antimicrobial compounds, mainly produced by LAB, have been studied extensively for several years. Unfortunately, most studies apply the living bacteria directly and therefore only report their antifungal activity. Few studies investigating the metabolic compounds responsible for this activity are available. Figure 5 shows an overview of different LAB metabolites known to be involved in their antimicrobial performance. In addition, several microorganisms other than LAB have been studied for their suitability as bio-preservatives. However, secondary metabolites reported to be responsible for the antimicrobial activity are very similar for all microbes employed as potential bio-preservatives. Thus, the metabolites investigated in the following sections have primarily been investigated for LAB but likewise, apply for other potential bio-protective cultures.

3.5.2.1 Carboxylic and phenolic acids

Several studies reported the substantial influence of the medium pH on the antimicrobial performance of LAB-fermented substrates (Gerez et al., 2013; Gerez et al., 2010; Cortés-Zavaleta et al., 2014; Rodríguez et al., 2012; Vermeulen, Gánzle and Vogel, 2006). In all cases, an increased pH resulted in reduced antimicrobial activity. Thus, the authors concluded that the antimicrobial activity of LAB is primarily attributed to the various carboxylic and phenolic acids produced during carbohydrate metabolism. Lactic, acetic and propionic acids are found in highest quantities after LAB fermentation. The antimicrobial properties of these acids, due to the reduction of extra- and intra-cellular pH of the target organism, are well known and applied for industrial food preservation (Axel et al., 2016; Dalié, Deschamps and Richard-Forget, 2010; Varsha and Nampoothiri, 2016). As the acid has to be protonated to penetrate the target organism, weaker acids with higher pK_a-values display better inhibition. Inside the target cell, the acid de-protonates and thus lowers the intra-cellular pH, inducing cell death (Dalié, Deschamps and Richard-Forget, 2010; Crowley, Mahony and Van Sinderen, 2013).

However, recent research interest has primarily focused on a variety of phenolic acids, such as phenyllactic acid (PLA), reported to elicit antimicrobial properties at much lower concentrations (Crowley, Mahony and Van Sinderen, 2013). The activity of these acids was found to not be dependent on the pK_a-value exclusively. Sánchez-Maldonado et al. (2011) investigated the relationship between hydrophobicity of the aromatic ring substituent and the microbial inhibition efficiency of phenolic acids, using 6 hydroxybenzoic acid and 6 hydroxycinnamic acid derivates. Better microbial inhibition was found with increasing number of hydrophobic substituents on the phenolic ring. On the other hand, increasing numbers of hydroxyl groups reduced the antimicrobial efficiency (Sánchez-Maldonado, Schieber and Gänzle, 2011). The authors attributed this mainly to the better cell-membrane penetration of the hydrophobic molecules. The antifungal potential of 4 phenolic acids (caffeic, ferulic, p-coumaric and chlorogenic acid) against toxigenic *Fusarium* spp. cultivated on maize meal agar was demonstrated by Ferruz et al. (2016). Significant growth inhibition of all species was found for concentrations of 2.5 – 10 mM. Most LAB produce, depending on the growth conditions, a wide range of carboxylic and phenolic acids, including the above mentioned compounds. However, due to the relatively low concentrations, antimicrobial performance of the bacterial cfs is highly strain dependent (Peyer et

al., 2016; Axel et al., 2016). Thus, synergistic effects between the individual components are proposed to have a major influence on the inhibitory efficiency. To-date, these synergisms are under investigation but require further research in order to obtain a complete understanding of LAB antimicrobial activity. However, certain acids were identified to have a key role and therefore received higher attention than others. Promotion of specific metabolic pathways yielding these compounds have been investigated recently, to improve and understand the antimicrobial performance of LAB.

In particular 3-phenyllactic acid has, due to its broad spectrum of antimicrobial activity, gathered the interest of several researchers. The antifungal activity of 13 LAB isolates, belonging to the species of *L. casei*, *L. rhamnosus*, *L. fermentum*, *L. acidophilus*, *L. plantarum*, *L. sakei* and *L. reuteri* against *Penicillium* and *Aspergillus* spp. was found to be in direct correlation with the amount of PLA accumulated in the respective supernatants (Cortés-Zavaleta et al., 2014). Different strains of *L. plantarum* have been reported by several authors to be capable of high PLA production rates if the environmental conditions are suitable (Valerio et al., 2016; Rodríguez et al., 2012). The influence of various modifications of the growth medium on production and accumulation of PLA were investigated by Rodríguez et al. (2012). Thereby, increased PLA production of *Lactobacillus plantarum* CECT-221 was found under high initial phenylalanine (Phe) and low initial glucose concentrations. These conditions stimulate the bacteria to metabolise Phe as source of energy via the Ehrlich-pathway. Hence, the Phe first undergoes a transamination by transferring the amino group onto a keto-acid acceptor. The synthesised phenylpyruvic acid (PPA) is then reduced to PLA by a dehydrogenase (Vermeulen, Gánzle and Vogel, 2006).

Up to 4.25 mM of PLA were accumulated during fermentation for 48 h under optimised conditions (Rodríguez et al., 2012). This led to a 63% growth inhibition of pathogenic *Salmonella* spp. over 24 h of incubation. Similar results were reported by Zhang et al. (2014) who increased the PLA accumulation by supplementing the growth medium of *L. plantarum* spp. with both Phe and phenylpyruvic acid (PPA). As a result, the spore germination inhibition of all the strains cfs against *P. roqueforti* improved substantially compared to the respective control cfs (not supplemented, fermented medium). Also LAB strains other than *L. plantarum* were found to be capable of high PLA production if supplemented with PPA (Valerio et al., 2016; Song et al., 2015). These include isolates of *L. paracasei*, *L. brevis* and *L. fermentum*, amongst others. For all of these strains, addition of PPA to the growth

medium led to increased accumulation of PLA, ultimately resulting in better growth inhibition of *A. niger* and *P. roqueforti* (Valerio et al., 2016). Song et al. (2015) reported similar results for the inhibitory capacity of LAB cfs against the pathogenic yeast *Endomyces fibuliger*.

However, Cortés-Zavaleta et al. (2014) reported that even the highest amounts produced by LAB are approximately 10 times lower than to the MIC of commercially available PLA (2.5 – 10 mg/mL) (Broberg et al., 2007; Ryan et al., 2011). Consequently, despite having an important role for the antifungal performance of LAB cfs, the involvement of other antagonistic metabolites is evident but not yet fully understood. A recent study showed the synergy between PLA and hydroferulic acid in terms of spore germination inhibition of *F. culmorum* compared to equal amounts of the individual acids (Peyer et al., 2016). Furthermore, Valerio et al. (2016) published the first detection of small amounts of polyporic acid (produced by condensation of 2 molecules of PPA) in LAB fermented media which were supplemented with PPA. The authors reported the best antifungal activity for bacterial cfs containing high amounts of both, polyporic and phenyllactic acid, thus demonstrating a synergistic effect between the two acids.

In conclusion, although carboxylic and phenolic acids are known to be essential for the antifungal activity of LAB, their exact mechanisms and synergies are still not fully understood. Furthermore, it is likely that not all compounds contributing to the antifungal performance have been identified. To-date, it is not possible to imitate the bio-preservative effect of LAB using synthetic mixtures of the bacterial compounds known to express antimicrobial activity. Hence, no *in situ* studies investigating the suitability of a phenolic acid mixture for the microbial preservation of cereals crops post-harvest have been performed. Therefore, more fundamental research is required before the use of microbial produced phenolic acids as bio-preservatives becomes a viable option.

3.5.2.2 Reuterin

Another very potent, broad spectrum antimicrobial compound that has gathered many researchers interest in recent years is reuterin, also known as 3-hydroxy propionaldehyde (3-HPA). It is understood that reuterin is released upon enzymatic dehydration of glycerol. However, in presence of sufficient amounts of fermentable

carbohydrates, in particular glucose, reuterin is reduced to 1,3-propanediol which has no antimicrobial activity (Gänzle, 2015). Reuterin was first discovered in substrates fermented by *Lactobacillus reuteri*. However, nowadays more species of LAB have been found to possess the ability to convert glycerol to reuterin. To accumulate this intermediate compound, the environmental and nutritional conditions during fermentation have to be chosen carefully. The most important factors are excess amounts of glycerol and limited glucose availability for the bacteria (Gänzle, 2015; Dishisha et al., 2014). The antimicrobial activity of reuterin is predominantly based on the reaction of the aldehyde group with the thiol group of proteins and small molecules, inducing an oxidative stress reaction in the target organism (Schaefer et al., 2010). Thus, 3-HPA shows a broad spectrum of antimicrobial activity against pathogenic and food spoilage organisms, but due to the unspecific mode of action, limited stability *in situ*. The bio-production of reuterin was mainly investigated to obtain a pre-cursor for the industrial production of acrolein. Only in recent years have researchers started to explore the possibilities of reuterin as food bio-preservative.

A study conducted by Ávila et al. (2014) demonstrated the antibacterial efficiency of reuterin against common food spoilage strains of *Clostridium* spp. *in vitro* and *in sit*u (milk). For the application of reuterin in milk, an additional synergy with different LAB-produced bacteriocins, such as nisin, against common food spoilage bacteria including *Listeria* spp., *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) was reported (Arqués et al., 2011). Furthermore, the application of reuterin-producing LAB starter cultures in cheese making, supplemented with glycerol, was reported to reduce the late blowing defect due to inhibition of spoilage bacteria (Gomez-Torres et al., 2014). Determination of the reuterin content in whey and cheese at different time points revealed substantial accumulation of reuterin, explaining the reduced spoilage activity. Thus, Gomez-Torres et al. (2014) reported the first successful application of *in situ* produced reuterin as food preservative. In addition, smoked salmon treated with microbial reuterin produced *in vitro* showed up to 2-log units reduced *L. monocytogenes* counts after 15 days of storage, compared to the untreated control (Montiel et al., 2014). The authors also succeeded in producing a purified aqueous reuterin stock solution with a concentration of 1.3 M. Such a purified and concentrated solution could potentially be applied as bio-preservative in several food products. It also could serve as sanitizing solution, i.e. for cereal crops prior to storage. However, more research is needed to explore the possibilities and limitations of reuterin before conclusions

regarding the suitability for cereal preservation can be drawn. It is noteworthy that in an early study conducted by Tanaka et al. (2009), the *in situ* production of 3-HPA by *Lactobacillus coryniformis* sp. in silage was reported. Due to the addition of glycerol to the fermentation mixture, significant amounts of reuterin accumulated and successfully supressed the growth of naturally occurring anaerobic spoilage bacteria.

To-date, few studies investigating the antifungal activity of reuterin, in particular with potential food application, are available. Increased microbial shelf life of milk bread rolls and pound cake was reported due to the application of *L. reuteri* spp. cfs during dough preparation and after baking, respectively. Here, bacterial cfs obtained from wheat flour hydrolysate (WFH) medium, supplemented with 150 mM glycerol resulted in significantly better microbial shelf-life compared to regular WFH (Le Lay et al., 2016a). The authors attributed this to the production of reuterin. However, the study did not include the determination of reuterin contents in dough or baked products. Thus, no existing study has directly investigated the suitability of reuterin as antifungal agent in food matrices.

In conclusion, reuterin appears to be a very promising novel approach for food preservation; in particular, if the substance can be produced *in vitro* and, after isolation and purification, applied as concentrated solution. This would avoid the difficult adjustment of bacterial growth conditions necessary for its accumulation in a food matrix. However, due to its high reactivity with low specificity, the applications might be limited to relatively simple food systems. Therefore, future research should pay more attention to the accumulation and application of reuterin as food preservative, in order to fully explore its possibilities and limitations.

3.5.2.3 Fatty acids

Another group of metabolites involved in the antimicrobial performance of LAB and with potential for application as bio-preservatives are free fatty acids (FAs) and derivates, mainly hydroxylated and methylated forms. Fatty acids are a class of organic acids characterised by the presence of a carboxyl-group on one end and a methyl chain on the other (Pohl, Kock and Thibane, 2011). The FAs discussed in this section of the article are substances that naturally and ubiquitously occur in the environment, usually incorporated in fats. After release and possible metabolic

conversion by microorganisms the products are reported to express high antimicrobial activity. In contrast to the carboxylic and phenolic acids, the mode of action of FAs and derivates is not based on the reduction of the intra-cellular pH. Instead, these compounds primarily target the cell membranes, as well as specific enzymes and metabolic pathways (Pohl, Kock and Thibane, 2011).

The most straight forward approach is the degradation of fats to glycerol and free FAs, which then express the antimicrobial activity. Therefore, Liu et al. (2008) investigated the *in vitro* antifungal activity of seven saturated (butyric acid, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid and palmitic acid) and two unsaturated (oleic acid and linoleic acid) FAs against three common pathogenic fungi, namely, *Alternaria solani*, *Colletotrichum lagenarium* and *Fusarium oxysporum* (2 strains). In general, saturated FAs were fund to have higher antifungal activity with regards to inhibition of spore germination and mycelial growth, compared to the unsaturated acids. Apart from oleic acid, all FAs resulted in efficient inhibition of at least one fungus tested. The authors further reported substantial differences in antifungal activity between the FAs and that the fungal sensitivity towards them was greatly dependent on the strain tested. The biggest spectrum of mycelial growth inhibition was found for butyric (C4:0) and caprylic (C8:0) acids, respectively. Both resulted in significant inhibition of all fungi tested when applied with concentrations ≥1 mM. The other saturated FAs tested were found to have more specific inhibition profiles. For instance, capric acid (C10:0) resulted in no substantial inhibition of *F. oxysporum* for concentrations of up to 2 mM, while leading to significant inhibition of *Alternaria solani* at only 0.1 mM (Liu et al., 2008). In contrast, Pohl et al. (2011) reported the broadest spectrum of antifungal activity for capric (C10:0) and lauric (C12:0) acids, as these compounds showed substantial inhibition against the largest variety of pathogenic and spoilage fungi, including *Fusarium*, *Aspergillus*, *Penicillium* and *Candida* spp., amongst others. Furthermore, the addition of isolated FAs to soil was found to enhance the growth of cucumbers and tomatoes, respectively (Liu et al., 2008). Thus, the investigation of the possibility for microbial protection of cereal crops combined with improved seed germination capacity would be of further interest.

In general, unsaturated FAs such as linoleic and oleic acid show less antifungal activity than saturated FAs (Liu et al., 2008; Pohl, Kock and Thibane, 2011). Linoleic and oleic acid applied at concentrations of up to 2 mM showed no significant effect on fungal spore germination *in vitro* (Liu et al., 2008). However, LAB also possess the ability to metabolise these unsaturated FAs into hydroxy FAs or methyl FAs.

These derivatives recently gathered many researchers interest as potential natural antimicrobial agents (Black et al., 2013; Crowley, Mahony and Van Sinderen, 2013; Dalié, Deschamps and Richard-Forget, 2010). Black et al. (2013) demonstrated the possible bio-conversion of linoleic acid into monohydroxy linoleic acid *in vitro* and *in situ*, using *Lactobacillus hammesii*. Furthermore, the addition of linoleic acid to the fermentation medium (bread sourdough) led to substantially prolonged microbial shelf-life of the resulting breads when challenged with environmental moulds. Similarly, Le Lay et al. (2016a) reported increased antifungal activity of *L. brevis* cfs, applied in milk bread rolls and pound cake, due to the addition of olive oil during fermentation. The authors attributed this improved preservation to the metabolism of linoleic acid by the bacteria but did not determine the content of linoleic acid or its derivatives.

An early study also investigating the FA derivatives responsible for the antifungal activity of the LAB cfs was conducted by Sjögren et al. (2003), using *Lactobacilllus plantarum* MiLAB 14. The authors isolated several hydroxyl-FAs from the bacterial cfs, namely 3-hydroxydecanoic acid, 3-hydroxy-5-cis-dodecanoic acid, 3-hydroxydodecanoic acid and 3-hydroxytetradecanoic acid and demonstrated their antifungal activity. MICs of racemic mixtures of the individual compounds against common food spoilage fungi, including *Penicillium*, *Aspergillus* and *Fusarium* spp. ranged between 10 and 100 µg/mL. Interestingly, the concentrations in the supernatants required for equal fungal growth inhibition were much lower compared to the MICs determined. Thus it can be assumed that other, yet unknown compounds contribute to the antifungal performance also. A later study examined the antifungal activity of different *Lactobacillus plantarum* strains *in vitro* against filamentous fungi, including *Aspergillus* and *Penicillium* spp., and also attributed it to FA metabolites (Ryu et al., 2014). The compounds isolated from the fermented medium and shown to have high antifungal activity were 5-oxododecanoic acid, 3-hydroxy decanoic acid and 3-hydroxy-5-dodecenoic acid (Ryu et al., 2014). To-date, this is the only report of 5-oxododecanoic acid production by LAB (Ryu et al., 2014).

Few studies have investigated the antibacterial properties of hydroxy FAs. However, Mundt et al. (2003) reported the antibacterial activity of coriolic acid, α-dimorphecolic acid and linoleic acid (all produced by the cyanobacterium *Oscillatoria radekai* HUB 051). The inhibitory effect was tested *in vitro* against 7 isolates of common spoilage bacteria, including *S. aureus*, *Micrococcus* spp. and *Bacillus subtilis*. Interestingly, linoleic acid was found to be the most efficient acid, as concentrations of 100 µg/mL were sufficient for substantial inhibition of 6 out of 7

strains tested. On the other hand, in terms of antifungal activity, linoleic acid was found to be very inefficient, in particular compared to hydroxy-FAs. This result can be attributed to the differences in the composition of fungal and bacterial cell membranes. It further strengthens the hypothesis that no single component alone can serve as sufficient preservative against various microbial threats. Similar results were reported by Shin et al. (2004), who even reported a reduced antibacterial activity for linoleic acid after bio-conversion by *Psuedomonas aeruginosa* PR3. The authors further reported strong activity of bio-converted ricinoleic and eicosadienoic acids against Gram-positive bacteria and less activity against Gram-negative bacteria.

Much less investigated than hydroxy-FAs are the antimicrobial properties of methyl FAs. However, a recent study attributed the antifungal activity of *Lactobacillus plantarum* cfs against mycotoxigenic *Fusarium* spp. primarily to different free and methyl FAs produced by the bacteria (Deepthi et al., 2016). The major antifungal compounds were identified as 10-octadecenoic acid methyl ester, palmitic acid methyl ester, heptadecanoic acid 16-methyl ester, as well as stearic and lauric acids. Furthermore, the antifungal activity of these compounds *in vitro* was successfully applied to the microbial preservation of stored maize kernels. These findings further demonstrate the potential of FAs and bio-converted FAs for microbial preservation of cereal crops.

Overall, fatty acids and their derivatives appear as very promising candidates for post-harvest microbial protection of cereals. Using the bacterial metabolism, they are easy and inexpensive to produce and originate from naturally occurring fats. However, future research has to explore the exact interactions with the targets cell membrane and metabolic pathways in more detail to apply the specific substances needed. In addition, investigations regarding their impact on stored products are necessary before industrial application becomes feasible.

3.5.2.4 Antifungal peptides and proteinaceous compounds

Compared to the compounds described in the previous sections, few studies have investigated the contribution of proteins and peptides to the antimicrobial performance of LAB. Moreover, even less research has been conducted to characterise these compounds, although early studies have demonstrated the great

potential of microbially produced bacteriocins. One bacteriocin, fully characterised, produced by LAB and already well-established in the food industry is nisin. However, this section is focused on more recent reports of newly reported and not yet established compounds, and their potential for application as bio-preservatives.

Amongst the smallest compounds reported to have antifungal activity are cyclic dipeptides. The first study reporting the production of cyclic dipeptides by LAB was conducted by Ström et al. (2002). The authors demonstrated the antifungal activity of cyclo(L-Phe– L-Pro) and cyclo(L-Phe–trans-4-OH-L-Pro) *in vitro* against spores of *P. roqueforti* and *A. fumigatus*. However, little research interest has been focused on the quantification and characterisation of cyclic dipeptides by various LAB. Axel et al. (2014) developed a method for quantification of cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo (Met-Pro) and cyclo (Phe-Pro) in MRS and wort, using stable isotope dilution. The authors also reported that the formation of cyclo(Leu-Pro), cyclo(Phe-Pro) and cyclo(Pro-Pro) was clearly related to the bacterial metabolism of *L. brevis* R2Δ during sourdough fermentation. Furthermore, Kwak et al. (2014) showed the involvement of cis-cyclo(L-Val-L-Pro) and cis-cyclo(L-Phe-L-Pro) in the antifungal activity of *L. plantarum* against *Candida albicans*. Nevertheless, the antimicrobial properties of cyclic dipeptides produced by bio-protective cultures is only sparsely investigated. In order to determine the full potential of these compounds, further research into their production, mode of action and side effects is needed.

More research has been conducted for larger peptides (<10 kDa) with antimicrobial activity that are produced by LAB. A novel peptide with antifungal properties against different toxigenic *Aspergillus* spp. was isolated form *Lactobacillus plantarum* IS10 grown in MRS broth (Muhialdin et al., 2016). The peptide was reported to have a net charge of +1, a hydrophobicity ratio of 58% and a molecular weight of 1253 Da. After isolation and purification, this peptide displayed good antifungal activity *in vitro* when applied at 5 mg/mL. Lower concentrations were reported to be inefficient. Another broad spectrum antimicrobial peptide (2.1 kDa) was recently isolated from *Lactobacillus paracasei* subsp. *tolerans* (Miao et al., 2014). Due to the peptides stability against heat and changes in pH, it appears to have the potential for various applications. Even treatment with proteolytic enzymes, such as pepsin and trypsin resulted only in partial loss of the antimicrobial activity. Furthermore, it is the first peptide isolated from this LAB species reported to express antifungal and antibacterial activity. Gerez et al. (2013) attributed the *in vitro* antifungal performance of *Lactobacillus fermentum* spp. to a heat stable peptide of <10 kDa. This conclusion was primarily based on the fact that the cfs` antifungal activity was

not compromised by the pH neutralisation of the medium. Antifungal activity was lost due to treatment with trypsin but was not affected by incubation with proteinase K, pepsin A or catalase. Good antifungal performance of this peptide was reported against several food spoilage fungi, including *Aspergillus niger*, *Fusarium graminearum* and different *Penicillium* spp. (Gerez et al., 2013). Finally, the *in situ* application of the partially purified antimicrobial peptide, LR14, produced by *L. plantarum* LR/14 was successfully used to protect whole wheat grains from fungal spoilage. During 2.5 years of storage under laboratory conditions, growth of *A. niger*, *R. stolonifer*, *M. racemosus* and *P. chrysogenum* was fully inhibited (Gupta and Srivastava, 2014).

In conclusion, for most LAB isolates, proteinaceous compounds appear to play a minor role in their antimicrobial performance. Other compounds such as phenolic acids, play a larger role. Therefore proteinaceous compounds have, to-date, appeared less for bio-preservation and therefore received little research attention. In addition, the isolation and purification of peptides and proteins presents numerous hurdles which can be avoided with the use of other metabolites. On the other hand, antimicrobial peptides would have a very specific mode of action preventing undesirable side effects on the preserved food. Thus, if future research can identify a broad spectrum antimicrobial peptide, produced in sufficient quantities, a purified solution of this peptide may serve as a highly efficient preservative.

3.5.2.5 Mixtures of active compounds (bacterial cell-free supernatants)

The antimicrobial activity of LAB is proposed to be based primarily on complex synergistic effects between a large variety of metabolites including organic acids, peptides and fatty acids, amongst others (Reis et al., 2012; Schnürer and Magnusson, 2005; Axel et al., 2016). A recent study used electron microscopy to visualize the interaction of LAB antifungal metabolites with different parts of the mycelial and spore cell membranes, proving the synergistic mechanisms of several active compounds (Sangmanee and Hongpattarakere, 2014). In general, the compounds responsible for the fungal inhibition are secondary metabolites of LAB, with the highest accumulation after 48 h of fermentation (Oliveira et al., 2015b; Rouse et al., 2008). The antimicrobial activity is further supported by the reduction in the pH of the fermented medium to <4.0, due to organic acids production (Schnürer and Magnusson, 2005). However, due to the broad chemical and

structural variety of the active compounds, extraction and quantification procedures are challenging (Crowley, Mahony and Van Sinderen, 2013; Dalié, Deschamps and Richard-Forget, 2010). Thus, application of the entire cfs from a food-grade fermentation medium appears to be the most straight forward and efficient option. Hence, this section will discuss the potential of *in vitro* produced antimicrobial supernatant for application as a bio-preservative.

The application of LAB cfs against common food spoilage bacteria, including *S. aureus*, *E. coli* and *L. monocytogenes* to prevent the accumulation of biogenic amines was investigated *in vitro*, using ornithine decarboxylase broth (Ozogul et al., 2015). Furthermore, the *in vitro* application of antifungal LAB cfs (grown in commercial MRS broth) was reported to be efficient in terms of growth inhibition against conidia (Gerez et al., 2013) and spore (Axel et al., 2016; Le Lay et al., 2016a; Lynch et al., 2016; Oliveira et al., 2015b; Peyer et al., 2016) suspensions of various food spoilage fungi, including *Zymoseptoria*, *Fusarium*, *Aspergillus* and *Penicillium* spp. In addition, the use of LAB cfs (grown in wheat flour hydrolysate (WFH) agar) obtained from antifungal isolates of strains belonging to the species *L. reuteri*, *L. brevis*, *L. citrei*, *L. sakei*, *L. plantarum* and *L. spicheri* were found to increase the microbial shelf life of bakery products significantly (Le Lay et al., 2016a). Here, the authors replaced the water in the recipe with the respective bacterial cfs. However, the same study reported that the antifungal performance *in situ* was substantially lower compared to *in vitro* trials (Le Lay et al., 2016a) and attributed this to the “dilution” with other dough constituents. Similar results were reported previously by Muhialdin et al. (2011) against *A. oryzae* and *A. niger*.

The inoculation of *Fusarium* spp.-contaminated maize–based poultry feed with the cfs obtained from *L. plantarum* MYS6 grown in MRS-broth was reported to reduce the fungal growth and mycotoxin production substantially (Deepthi et al., 2016). However, as demonstrated in previous studies, most *Fusarium* spp. are more sensitive to LAB antifungal metabolites compared to other spoilage fungi, such as *Aspergillus* or *Penicillium* spp. Hence, the production of a cfs expressing sufficiently high antimicrobial activity to inhibit spoilage fungi other than *Fusarium* spp. remains challenging. Therefore, the use of co-cultures of LAB strains was investigated. Interestingly, the antifungal activity decreased significantly in the co-culture system (Le Lay et al., 2016a).

Studies have recently been conducted to investigate the effect of varying environmental conditions on the accumulation of antimicrobial metabolites.

Enhanced antifungal activity for *Lactobacillus* isolates were reported in presence of different polyols, commonly used as sweeteners in low sugar products (Lipińska et al., 2016). However, to-date no studies investigating the mechanism of this synergy have been performed.

In addition, LAB cfs has been reported as an efficient tool for the production of silver nanoparticles from a silver-nitrate solution (Matei et al., 2015). These resulting particles were reported to express antifungal activity against various food spoilage fungi belonging to the genera *Fusarium*, *Aspergillus* and *Penicillium*. Therefore, the authors concluded that LAB cfs has the potential to become a more efficient and cost effective tool for the production of nanoparticles, with possible application in medicine and food preservation. Similar results were reported by Kumar and Poornachandra (2015).

To-date the synergistic mechanisms primarily responsible for the bio-preservative properties of LAB cfs are not fully understood. Thus, it remains difficult to design a growth medium for optimum antifungal and antibacterial activity against a broad spectrum of spoilage organisms. Future research in this direction, for the production highly efficient antimicrobial cfs, would allow the simple and fast production of a bio-preservative agent. Such a solution could be applied to a broad variety of matrices, including cereals, for microbial protection. Therefore, it appears essential that future research focuses on the optimisation of the antimicrobial activity of LAB, exploiting their metabolism to the fullest potential.

3.6 Microbial removal of mycotoxins

The best approach to ensure mycotoxin-free food is the prevention of fungal spoilage and mycotoxin production. However, as fungal spores are ubiquitous in the environment, field contamination and mycotoxin accumulation on cereal crops prior to the harvest cannot be ruled out. As demonstrated by Schmidt et al. (2016) the highest production rate of mycotoxins occurs during the initial infection, in order to overcome the grains defence mechanisms. Thus, it is possible to obtain a crop contaminated with mycotoxins but with very low amounts of fungal bio-mass. For such cases, the development of methods that do not only inhibit the fungal growth, but also remove or degrade previously accumulated toxins could reduce economic losses substantially. One approach that has gathered many researchers interest in recent years involves the use of microorganisms to remove mycotoxins (Zhu et al., 2015; Shetty and Jespersen, 2006; Alberts et al., 2009; Dalié, Deschamps and Richard-Forget, 2010; Ahlberg, Joutsjoki and Korhonen, 2015; Hathout and Aly, 2014). The two general approaches usually discussed are the degradation of toxins by microbial enzymes and the binding of toxins to the surface of microbes.

Despite numerous studies reporting the degradation of mycotoxins by antagonistic microbes, very little is known about the resulting degradation products. The decay of the parent toxin may result in compounds with unknown identity and toxicity (Shetty and Jespersen, 2006). Furthermore, toxins can be transformed into masked mycotoxins, which re-transform into the parent toxin during processing or in the human intestine (Berthiller et al., 2011). Thus, further research must elucidate the fate of enzymatically-degraded toxins, to evaluate the suitability of this approach for food and feed commodities. The topic of bio-transformation using microbial enzymes was recently reviewed by Hathout and Aly (2014) and is therefore not further evaluated here. Instead this section discusses the removal of mycotoxins by surface-binding to microbes.

Several microorganisms have been reported to bind mycotoxins. Due to their century long use for the production of fermented foods, *Saccharomyces cervisiae*, *Lactobacillus* spp. and *Propionibacterium* spp. are the most promising for potential food or feed applications (Shetty and Jespersen, 2006). Table 8 summarises previous reports regarding the microbial removal of mycotoxins due to surface adsorption. The most intensely investigated group of toxins are aflatoxins (AFs), in

particular aflatoxin B_1 (AFB_1), produced by various *Aspergillus* spp. and commonly found on cereal grains.

Table 8: Removal of mycotoxins by microbial surface binding.

Bacterial strain	Cell viability	Toxin	Matrix	Reference
Lactobacillus reuteri sp., *Lactobacillus casei* sp.	Viable and acid killed	Aflatoxin B_1	Phosphate buffered saline	Hernandez-Mendoza et al. (2009)
Lactobacillus rhamnosus sp., *Propionibacterium freudenreichii* sp.	viable	Aflatoxin B_1	Chicken duodenum	Gratz et al. (2005)
Lactobacillus plantarum sp.	viable	Aflatoxin B_1	olives	Kachouri et al. (2014)
Lactobacillus acidophilus spp.	viable	Aflatoxin M_1	milk	Adibpour et al. (2016)
Saccharomyces cerevisiae + *Lactobacillus rhamnosus* sp., *Lactobacillus delbrueckii* sp., *Bifidobacterium lactis* sp.	Heat killed	Aflatoxin M_1	milk	Corassin et al. (2013)
Saccharomyces cerevisiae + *Lactobacillus acidophilus* sp.	Viable, heat- and acid killed	Fumonisin B_1	Phosphate buffered saline	Pizzolitto et al. (2012)
Lactobacillus rhamnosus sp.	Viable and heat killed	Aflatoxins B_1, B_2, G_1, G_2	Wheat based bread dough	Elsanhoty et al. (2013)
Lactobacillus brevis spp., *Lactobacillus buchneri* sp., *Lactobacillus cellobiosus* sp., *Lactobacillus fermentum* spp.	viable	Aflatoxin B_1	Maize based bread dough	Roger et al. (2015)

Lactobacillus reuteri sp., *Lactobacillus casei* sp.	viable	Aflatoxins	Rats (oral treatment)	Hathout et al. (2011)
Lactobacillus plantarum spp.	Viable and heat killed	zearalenone	MRS medium, phosphate buffered saline	Zhao et al. (2015)
Lactobacillus pentosus spp.	viable	Zearalenone	Sodium acetate buffer	Sangsila et al. (2016)
Planocccus sp.	Viable, heat- and acid killed	Zearalenone	Ringers solution	Lu et al. (2011)
Lactobacillus plantarum spp.	Viable, heat- and acid killed	deoxynivalenol	Aqueous solution	Franco et al. (2011)
Lactobacillus rhamnosus spp., *Lactobacillus freudenreichii* sp.	Viable and heat killed	Deoxynivalenol, nivalenol, T-2 toxin, HT-2 toxin, fusarenon	Phosphate buffered saline	El-Nezami et al. (2002)

A screening of various bacterial strains for their ability to bind AFB_1 *in vitro* demonstrated the high efficiency of *Lactobacillus reuteri* NRRL14171 and *Lactobacillus casei* Shirota (Hernandez-Mendoza et al., 2009). Similarly, the partial removal of AFB_1 *in vitro* and *ex vivo* (chicken duodenum) combined with reduced bio-availability of the toxin was reported by Gratz et al. (2005) for various LAB species. Up to 66% of AFB_1 was removed *in vitro*, by applying a probiotic mixture of LAB and propionibacteria. The tissue uptake of toxin from the chicken duodenum was substantially reduced also. Removal of AFB_1 *in situ* from olives artificially infected with *Aspergillus flavus* was described for *Lactobacillus plantarum* (Kachouri, Ksontini and Hamdi, 2014). AFB_1 concentrations decreased over 4 days of storage, from initially 11.0 ppb to 5.9 ppb. AFM_1 was found to be successfully removed from yoghurt using *Lactobacillus acidophilus* spp. (Adibpour et al., 2016).

No influence due to the presence or absence of commercial yoghurt starter cultures was evident. Furthermore, synergistic effects between LAB and *S. cerevisiae* in terms of mycotoxin binding were reported for AFM_1 (Corassin et al., 2013) and fumonisin B_1 (Pizzolitto, Salvano and Dalcero, 2012), in milk and liquid medium *in vitro*, respectively.

The reduction of mycotoxins in cereal matrices, including wheat and maize based dough preparations, was demonstrated for various LAB isolates (Elsanhoty et al., 2013; Roger et al., 2015). Roger et al. (2015) reported up to a 64% reduction in AFB_1 during maize dough fermentation (120 h) using *L. buchneri* M11. Similarly, strain and toxin dependent removal of AFs during traditional baladi bread preparation (wheat based dough) was found for numerous LAB isolates (Elsanhoty et al., 2013). The additional use of commercial yeast for dough preparation had minor effects on the toxin binding. In particular, AFB_1 contents were found to decrease significantly due to the presence of LAB. Up to a 30% reduction was achieved using *L*. *rhamnosus* TISTR 511 (Elsanhoty et al., 2013). Furthermore, oral treatment of rats with *L. casei* and *L. reuteri* cell suspensions (10^{11} cfu/mL, giving 10 mL/kg body weight) for 4 weeks was found to reduce the toxic impact of AFs substantially (Hathout et al., 2011). As a result, the authors concluded that a reduced toxin bio-availability was due to binding with the bacterial cells. Furthermore, treatment with *L. reuteri* was reported to be more efficient than with *L. casei* (Hathout et al., 2011).

Mycotoxins other than AFs, frequently found in cereal crops are *Fusarium* toxins such as deoxynivalenol (DON) and zearalenone (ZEA). A study screening 27 *Lactobacillus plantarum* strains for their ability to remove ZEA from MRS and phosphate buffered saline (PBS) was conducted by Zhao et al. (2015). Similar to the binding of AFs, the ability to remove ZEA was found to be highly strain specific. Three of the tested strains resulted in significant ZEA removal rates of 38 – 48%, while the other isolates were inefficient. Comparable results were reported by Sangsila et al. (2016) and Lu et al. (2011), using *Lactobacillus pentosus* and *Planococcus* spp., respectively. Numerous LAB strains were found to successfully remove DON from aqueous solution (Franco et al., 2011). However, the efficiency of DON removal varied between different strains. Reduction rates ranged between 16.4% and 56.1% for *Lactobacillus plantarum* VII and *Lactobacillus plantarum* GTIII, respectively.

Despite promising results in terms of mycotoxin removal *in vitro* and *in situ*, all studies discussed above refer to the use of living bacteria and initial toxin binding. However, several studies propose a non-covalent, reversible binding to the bacterial cell wall. As a consequence, investigations into the mechanism of toxin removal are of further interest. In addition, for possible application in food or feed matrices the stability of the bacterium-toxin complex is essential to evaluate the suitability of microbial detoxification. Stability of the complex, in particular under conditions comparable to those found in mammalian intestines, would give valuable information for potential food applications. Otherwise, prior bound toxins could be released during passage through the intestine and pose a significant health hazard.

Increased efficacy of toxin binding, as a result of increasing cell density and toxin concentration was reported by numerous authors (Zhao et al., 2015; Sangsila et al., 2016; Adibpour et al., 2016; Franco et al., 2011; Pizzolitto, Salvano and Dalcero, 2012). Interestingly, the use of viable cells was found to be less efficient compared to dead cells. Regardless of toxin and organism applied, heat, acid or Triton-X killed cells resulted in higher reduction of AFs, DON and ZEA (El-Nezami et al., 2002; Franco et al., 2011; Hernandez-Mendoza et al., 2009; Lu et al., 2011; Pizzolitto et al., 2012). The toxins bound were not chemically modified (Franco et al., 2011; Pizzolitto, Salvano and Dalcero, 2012). This further strengthens the proposed mechanism of non-covalent surface binding to specific compartments of the bacterial cell wall. In particular treatments which induce a denaturation of proteins in the cell wall were found to improve the detoxification. Treatment of bacterial cells with different proteolytic enzymes resulted in significantly reduced toxin binding (Hernandez-Mendoza et al., 2009). Interestingly, the use of isolated and purified compartments of the cell wall was also found to be inefficient in terms of toxin removal, demonstrating the importance of the integrity of the cell (Hernandez-Mendoza et al., 2009). Furthermore, numerous environmental factors were identified as influencing the toxin binding kinetics. These factors include incubation time (Adibpour et al., 2016) and temperature (Sangsila et al., 2016; Zhao et al., 2015), as well as pH and ionic strength of the incubation medium (Franco et al., 2011). Considering the immense variety of mycotoxins commonly found in cereals and the fact that new toxins are continually discovered, the highly toxin-specific binding presents a significant drawback for this detoxification approach. Depending on the toxins present in the sample, the bacteria and environmental conditions applied would have to be carefully chosen in order to achieve sufficient detoxification.

Investigations regarding the complex stability have been conducted in order to estimate the suitability for food and feed applications. Partial removal of AFs from bacterial cells due to five consecutive washings with distilled water was reported by Elsanhoty et al. (2013). Similar results were reported for the removal of AFB_1 from *Lactobacillus* spp. (Hernandez-Mendoza et al., 2009). However, none of these washing procedures sufficiently mimicked the conditions in mammalian intestines, in particular regarding the pH and enzymatic activities. Thus, further research is required to establish the suitability of microbial mycotoxin removal for food and feed applications. It is noteworthy however, that possibilities to remove the bacterium-toxin complex from the matrix, without releasing the toxin, must be further explored. This could be particularly useful for the detoxification of cereal crops prior to storage. Washing with a bacterial cell suspension to bind the toxins, followed by the removal of the bacterium-toxin complex represents a promising approach. The main advantage is that toxins are not degraded and therefore no potentially toxic degradation or by-products remain in the sample. Considering that the toxicity of many mycotoxin derivates is still unclear, this approach could represent a safe and efficient method of detoxification.

However, further investigations regarding the exact binding sites of specific toxins and the binding kinetics are required to obtain sufficient process efficiency. At the current state of research, efficient mycotoxin removal by adsorption to a bacterial cell wall requires very high levels of toxin to be present and a high bacterial cell density. Also, the most efficient bacteria for each respective toxin and sample matrix are yet not identified. If researchers can find the optimum conditions, this approach shows potential to remove toxins from cereal crops that accumulate pre-harvest, without the need for chemicals or the risk of potentially toxic by-products.

3.7 Conclusions and future trends

The need for novel approaches in terms of microbial preservation of food commodities such as cereal crops is evident. This is particularly due the development of resistance amongst spoilage organisms and negative health effects caused by conventional disinfectants. All approaches discussed in this article have already demonstrated promising antimicrobial properties and potential. However, most of these findings were reported for *in vitro* trials or food matrices that do not resemble cereals. As a consequence, the suitability for application as post-harvest treatment to ensure the microbial stability during long-term storage of cereals remains unclear.

Cold plasma and electrolysed water technologies represent a new generation of chemical preservation. Both technologies generate the reactive species without the need for potentially hazardous chemicals. Furthermore, these active species have a very short life span, leading to rapid decomposition, without leaving residues on the samples. However, several hurdles, including antagonistic mechanisms with the sample matrix and the spectrum of activity are still present.

The use of bio-protective microbes to replace conventional chemical preservatives has achieved significant attention and has therefore been intensely investigated over the last decade. Very promising results in terms of bacterial and fungal inhibition have been achieved, in particular with LAB. However, the synergistic mechanisms and exact modes of action for many compounds are not yet fully understood. Recent research continues to report the discovery of new compounds showing antimicrobial activity, indicating that the entire spectrum of antimicrobial compounds remains unknown. As a consequence, the efficient and reliable application of LAB as industrial preservatives is still difficult. More research is required to fully understand the origin of LAB antimicrobial activity, which then would allow the most efficient application.

The use of microbes to remove mycotoxins *in vitro* and *in situ* has also been found to give promising results. However, many parameters, including the type of cells and the stability of the cell-toxin complex still require further investigation and optimisation. It also appears unlikely that one bacterial strain can serve to detoxify a crop and also inhibit further fungal growth. Instead, washing of contaminated grains with a bacterial suspension to remove accumulated toxins, followed by alternative treatments to suppress further fungal spoilage appears more promising.

It is also clear, however, that each approach discussed here has certain disadvantages. Thus, it is unlikely that one single treatment can serve the purpose of complete microbial decontamination and mycotoxin detoxification. The most efficient approach for future research would be a combination of different approaches, to uncover potential synergies between the treatments. This would minimise the shortcomings of each individual treatment and result in higher decontamination efficiency with little to no impact on the product quality. For instance, the use of cold plasma or electrolysed water to reduce the microbial load post-harvest could be combined with microbial removal of mycotoxins. The subsequent use of bio-preservative cultures or their isolated compounds could then serve to maintain the microbial stability of the crop during long-term storage. Such a combination of the approaches discussed in this review could potentially serve as efficient treatment to ensure high quality, yet safe, cereal products.

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Chapter 4: Impact of fungal contamination of wheat on grain quality criteria

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4.1 Abstract

The aim of this study was to investigate the spread of minimal, field born *Fusarium* infections in wheat during storage and the resulting impact on grain quality. Therefore, *F. culmorum* was chosen as the representative strain. Wheat grains were artificially infected and stored for 6 weeks in a model system. To estimate the fungal growth, the ergosterol content was determined as this correlates with the fungal biomass. Ergosterol levels revealed a rapid spread of the infection during storage conditions. Furthermore, analysis of nine mycotoxins showed that Deoxynivalenol and Zearalenone occurred in concentrations exceeding the maximum residue limits. Scanning electron microscopy illustrated the penetration of the fungus into the endosperm and showed the degradation of important seed constituents, such as starch and storage proteins. This is mainly due to the increased activity of proteases and amylases by the fungal metabolism. The results of this study show how small levels of field contamination can easily spread during storage and so lead to significant losses in grain quality and present a potential consumer health hazard. Thus, it demonstrates the need to develop efficient methods for crop protection during storage, without compromising the quality.

4.2 Introduction

Cereals have made an essential contribution to human nutrition for centuries. Wheat, in particular, is one of the most important cereals worldwide. Nowadays, with a production of 713 million tons per year wheat is, after maize and rice, the third most produced cereal worldwide (FAOSTAT, 2014). It is grown on an area of 216 million hectares globally. In the 2013/2014 season 697 million tons of wheat were consumed worldwide (USDA, 2014). Its high prominence is mainly because it is the basis for a large variety of products. By far the most important is its use in the baking industry, mainly for bread, but also for cakes, biscuits etc. In addition, breakfast cereals, pasta and alcoholic beverages like beer are often based on wheat and it is also used for animal feed (USDA, 2013).

However, crop losses due to fungal contamination represent a significant problem for many cereals globally. Especially for cereals like wheat, different toxigenic *Fusarium* spp. are frequently found as contaminants. A very common disease due to mould contamination in the field is *Fusarium* Head Blight, mainly caused by *Fusarium culmorum* (Parry, Jenkinson and McLeod, 1995). Grain colonization with these moulds causes significant quality losses due to the fungus exploiting the grains nutrient resources. Furthermore, from a consumer health safety point of view, the production of mycotoxins is a major problem. Mycotoxins frequently produced by *F. culmorum* are type B trichothecenes, such as deoxynivalenol (DON) and nivalenol (NIV), type A trichothecenes, like T-2, and zearalenone (ZEA) (Llorens et al., 2006, Wagacha and Muthami, 2007). The predominant mycotoxin is DON, as it was found in 90% of all cereal samples analysed, but it can also indicate the presence of other mycotoxins (Sobrova et al., 2010). Due to its heat stability and water solubility, it is easily transferable into processed food (Lancova et al., 2008). Consequently, mycotoxins are well known as a substantive health hazard in the baking and brewing industries (Scudamore et al., 2009).

However, due to the permanent and ubiquitous presence of microorganisms and fungal spores in the environment, it is not possible to avoid contamination completely. Consequently, methods for reduction and control of fungal contaminations are of major interest.

Although *Fusarium* spp. are field fungi, infection can also occur post-harvest if the conditions are favourable. In the USA alone the post-harvest economic loss on wheat due to fungal spoilage and mycotoxins exceeds $300 million annually (Pitt and Hocking, 2009). Furthermore, if wet grains are harvested and not dried

immediately *Fusarium* spp. can grow post-harvest (Champeil, Dore and Fourbet, 2004). Due to this fact it is likely that i.e. during storage, small amounts of *Fusarium* spp. infected grains can contaminate larger amounts of healthy grains. Consequently, the mycotoxins produced could cause a health hazard for the consumer. Additionally, the technological and nutritional quality of the grains would be reduced significantly, due to the fungal metabolism. Therefore, a small field infection has the potential to cause immense economic damage.

Thus, the aim of this study was to investigate the spread of a *Fusarium culmorum* infection, of minor degree of initial infection under storage conditions, generally suitable for fungal growth. Although this does not resemble industrial storage practices it allows the study of the interactions of the fungus with the grains. Therefore, the behaviour of the fungus was characterised, regarding the development of biomass and the production of mycotoxins. Analysis of the grains ultrastructure was used to visibly illustrate the changes occurring to all important components due to the infection. Further information about the pathway of fungal infection was obtained by determining selected enzymatic activities. Finally, the fungal impact on major grain quality parameters, such as starch content and storage proteins was evaluated to estimate the reduction of marketability, leading to economic losses. Hereby, the results of this study provide essential information for the understanding of fungal proliferation during storage, which is of great interest to cereal science and industry.

4.3 Materials and Methods

4.3.1 Materials

Commercial hard winter wheat (*Triticum aestivum*), harvested in 2013, was supplied by Doves Farm Foods Ltd. (Hungerford, UK). Wheat grains were stored in barrels under cool conditions (< 15°C) and were aerated regularly.

Fusarium culmorum TMW 4.2043 originally isolated from brewing barley was provided by the culture collection of Lehrstuhl für Technische Mikrobiologie, TU-München Weihenstephan.

All reagents used in the following analysis were at least analytical grade.

4.3.2 Grain surface disinfection

The grains were disinfected according to the method described by Oliveira et al. (2012). Briefly, grain portions of 600 g were disinfected in 4 L 10% (w/v) hydrogen peroxide (H_2O_2) solution for 10 min with continuous stirring. Subsequently, the grains were washed for 5 min in 4 L distilled water. This procedure was repeated once, but with only 5 min of disinfection. Immediately, the grains were moved to sterile plastic boxes and dried at room temperature for 24 h under vertical sterile laminar flow. Finally, the grains were exposed to ultraviolet light (10 min) and collected aseptically for further use.

4.3.3 Preparation of fungal spore suspension and grain infection

The spore solution of *Fusarium culmorum* was prepared according to the method described by Oliveira et al. (2012). Briefly, fungus was cultivated at 25°C for 5 days on potato-dextrose-agar (PDA) plates. After cultivation six small fragments of inoculated PDA were transferred to 800 mL synthetic nutrient-poor bouillon (Nierenberg, 1976). Fungal suspensions were kept at room temperature under continuous stirring to induce spore production and filtered through 30 µm filter paper. The concentration of spores was determined to be 10^5 spores/mL, using a haemocytometer.

Infected wheat grains were prepared using the following procedure. Disinfected wheat grains were mixed with 2% (v/w) sterile filtered spore suspension of *F. culmorum*. Subsequently, grains were incubated for 10 days at 25°C with 75% relative humidity to allow fungal growth. The infected grains produced were defined as 100% infected. The infected grains were homogenised and analysed for moisture. Uncontaminated wheat was incubated in the same way and used as a negative control.

4.3.4 Mixing and storage trials

Infected and disinfected grains were mixed to samples of 4.5 kg (dry matter) with specific infection levels of 0, 5, 10 and 20% and stored in model systems under conditions generally suitable for fungal growth. Each mixture and the control sample was divided into nine portions and filled into sterile plastic bags. Every bag was sealed and perforated by two pipette tips with barrier filter to allow gas exchange. The bags were stored for 6 weeks at room temperature. After 0, 3 and 6 weeks, 3 portions of each sample were taken, milled to a whole grain flour (particle size < 0.5 mm), homogenised and stored at -20°C until further use.

4.3.5 Determination of ergosterol

Ergosterol content (free and ester bound) was determined using the method of Jedličkova et al. (2008) with the following modifications. The RPHPLC column used was a Nova-Pak C_{18} (300 x 3.9mm, 4µm). Peak-identity was verified using the UV-spectra recorded by the DAD. The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the signal/noise (s/n) ratio. The LOD was set for s/n of 3:1 and the LOQ was set for s/n of 10:1. For calibration, ergosterol standards between 1.0 and 200 µg/mL in methanol were prepared and analysed. The recovery was determined by spiking an ergosterol free sample with a standard solution and found to be 95 ± 1%.

4.3.6 Mycotoxin analysis

The analysis of mycotoxins was carried out by UHPLC-MS/MS according to the method of De Colli, Elliott and Danaher (2014). In brief, 2 g milled, homogenised

sample was extracted by shaking by hand in the presence of 10 mL acetic acid (0.1%, v/v) for 1 min. Subsequently, 10 mL of acetonitrile was added and again shaken for 1 min. Magnesium sulphate (4 g) and sodium chloride (1 g) were subsequently added to the tubes and they were shaken for 1 min. Samples were centrifuged (3,500 rpm, 10 min) and 2.5 mL of the resulting supernatants was transferred into 15 mL polypropylene centrifuge tubes, which were evaporated to dryness under nitrogen at 40°C. The residues were resuspended in 0.2 mL of a water/methanol mixture (90/10, v/v), and filtered through a 0.2 µm filter, before 2.5 µL was injected into the UHPLC-MS/MS system. The separation was carried out with a Waters Binary Acquity™ UHPLC System, using an Acquity BEH® C_{18} (100 x 2.1mm, 1.7µm) column at 40°C. The mobile phase consisted of A) 5 mM ammonium acetate in water/methanol (90:10, v/v) and B) 5 mM ammonium acetate + 0.1% acetic acid in methanol. The gradient used was 0 – 4 min, 100% A; 4 – 10 min 10% A, 90% B; 10 – 12 min 100% A. The flow rate was 0.4 mL/min and the run time 12 min. Detection was carried out using a Water Quattro Premier XE™ with ESI interface. The LOD and LOQ were determined from the s/n ratios. The LOD was set for s/n of 3:1 and the LOQ was set for s/n of 10:1. Standard curves for each mycotoxin analysed were obtained by matrix calibration. The samples were measured as single determinations.

4.3.7 Grain ultrastructure

Examination of the grain ultrastructure was carried out using a JEOL scanning electron microscope type 5510 (JEOL, Tokyo, Japan) as described by Oliveira et al. (2012). Briefly, freeze-dried grains were manually cut in several cross sections (n > 15). These fragments were mounted onto aluminium stubs with carbon double surface adhesive. After covering the samples with a 7 nm gold layer, using a Gold sputter coater (BIO-RAD Polaron Division, SEM coating system, England), samples were placed in the microscope and examined under constantly accelerating voltage of 5 kV.

4.3.8 Grain quality parameters

The stored grains were characterised by determining the moisture content and the pH-value, using the standard AACC methods 44-15A (moisture) and 02-52.01 (pH).

Furthermore, the water activity on the surface of the grains was determined with the HygroLabC1 water activity meter (Rotronic Messgeräte, Ettlingen, Germany), according to the instructions given in the manual (Rotronic, 2014). Changes in enzymatic activities due to the fungal infection were evaluated by determination of α-amylase activity, β-glucanase activity, endo-1,4-β-xylanase activity (Megazyme Int., Ireland), total protease activity, using a haemoglobin standard (Brijs et al., 2002), lipase activity by the copper soap assay (Rose and Pike, 2006) and β-amylase activity using the method described by Hyun and Zeikus (1985).

Proteins were sequentially extracted, following the method described by Oliveira, Waters and Arendt (2013). Freeze dried grains were milled to a fine flour. Each extraction was carried out in duplicate. Protein concentrations of the joined extracts were determined by the protein dye binding method of Bradford (Bradford, 1976), using bovine serum albumin as a standard.

The extracts obtained from the Osborne fractionation were analysed further by SDS gel electrophoresis, using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA), according to the instructions given in the manual (Agilent, 2014) for the Protein80+ chip. The analysis was carried out in a molecular weight range of 4.5 – 95kDa. The results were summarised in an electrophoretogram (graphic representation of protein peaks and intensities), where the peak heights equal the intensity of bands in the gel.

The total amount of starch was determined by the amyloglucosidase / α-amylase method (Megazyme Int, Ireland). Selected saccharides were determined as free sugars according to the method described by Wolter et al. (2014), using HPLC system with refractive index detector. Appropriate sugar standards, representing degradation of starch and fibres, were used to identify and quantify the concentration in the samples in correlation to the standard peak area.

4.3.9 Statistical analyses

Samples were stored in triplicate and after sampling homogenised. All analyses were run in triplicate, unless otherwise stated. Statistical analysis was performed using Minitab 17 software. Data were checked for outliers (Grubb's test) and evaluation of significant differences was performed using one-way analysis of variances (ANOVA). All differences were considered significant at $P < 0.05$. Where F-values were significant, pairwise comparisons were carried out with the help of

Tuckey Post Hoc test to describe the statistical significance between the infected and uninfected grains over time of storage.

4.4 Results and Discussion

For this study *Fusarium culmorum* was chosen as an indicator strain, as it is the main reason of grain spoilage, particularly in Central and Western Europe (Parry, Jenkinson and McLeod, 1995).

Because of its oxidising power, H_2O_2 is known to have bactericidal and bacteriostatic activity. Furthermore it has the GRAS (Generally Regarded As Safe) status (Juven and Piersen, 1996). Due to the short contact time with the grains and the immediate washing after disinfection it has just minor effects on the wheat quality in terms of germinating ability and technological performance. Furthermore, hydrogen peroxide breaks into water and oxygen, leaving no residue.

After the artificial infection, all samples showed a water activity of > 0.80 and a pH between 6.5 and 6.6 (data not shown). Therefore the storage conditions were found to be generally suitable for fungal growth and allowed the investigation of the spread of *F. culmorum* in wheat during the storage period. Consequently, it was possible to study the fungal impact on important grain quality criteria.

4.4.1 Fungal growth

To evaluate the spread of the infection during storage, the fungal biomass was determined by quantification of the total ergosterol content. The results of this determination are shown in Table 9.

For all natural and 0% infected samples (week 0, 3 and 6) no ergosterol could be determined, as the signals obtained were below the LOD. Consequently, no measurable fungal growth occurred in these samples during storage.

In all the infected samples a significant increase in ergosterol contents was found during storage. At least a six fold increase (20% infected sample) was observed within the 6 week storage time. The highest rise was found for the 5% infected sample with levels ranging from <LOD (<0.75 mg/kg) up to 44.2±1.3 mg/kg. This shows how fast even a minor level of initial infection can turn into a substantial contamination. Furthermore, after just 3 weeks of storage no significant difference between the three infection levels was found anymore ($p<0.05$). After 6 weeks, the 5% and 10% infected samples showed significantly higher ergosterol contents than the 20% infected sample ($p<0.05$). This result can be explained by the competition of the fungus for nutrients, which was at the beginning of the storage in the 20%

infected sample the highest. Furthermore, this sample had the highest moisture content. Consequently, the air-filled porosity of the grains was different. Therefore, it is possible that a “water-logged” situation appeared. This leads to reduced oxygen availability and increased CO_2 contents, inhibiting the growth of the fungus (Atalla et al., 2003).

Table 9: Ergosterol and mycotoxin contents of wheat samples[#].

sample / weeks of storage	ergosterol mg/kg	DON µg/kg	DON3G µg/kg	3-ADON µg/kg	ZEA µg/kg
natural / 0	<LOD	<LOD	<LOD	<LOD	<LOD
natural / 3	<LOD	<LOD	<LOD	<LOD	<LOD
natural / 6	<LOD	<LOD	<LOD	<LOD	<LOD
0% infected / 0	<LOD	<LOD	<LOD	<LOD	<LOD
0% infected / 3	<LOD	<LOD	<LOD	<LOD	<LOD
0% infected / 6	<LOD	<LOD	<LOD	<LOD	<LOD
5% infected / 0	<LOD	719	119	15.1	33.3
5% infected / 3	8.6 ± 0.8[a]	510	147	<LOD	<LOD
5% infected / 6	44.2 ± 1.3[b]	446	73	<LOD	<LOD
10% infected / 0	2.9 ± 0.1[c]	1271	417	19.7	62
10% infected / 3	10.8 ± 0.7[d]	912	231	<LOD	58
10% infected / 6	40.4 ± 1.3[e]	953	160	<LOD	70
20% infected / 0	5.2 ± 0.2[f]	1739	779	19.6	70
20% infected / 3	10.0 ± 0.7[d]	961	408	12.7	25.0
20% infected / 6	30.1 ± 1.2[g]	937	210	<LOD	78
100% infected / 0	15.8 ± 1.1[h]	8998	5501	39.0	411

[#] **Results shown are mean values ± confidence interval. Values in one column followed by the same lower case letter are not significantly different (p < 0.05).**

4.4.2 Mycotoxin analysis

The production of mycotoxins is related to the fungal growth (Oliveira et al., 2012). Accordingly, qualitative and quantitative analysis of characteristic *Fusarium* toxins

was carried out. Table 9 shows that the mycotoxins DON, DON3G and 3-ADON were found in all - and ZEA in some of the infected samples. However, no detectable amounts of T-2, HT-2, DAS, FUS-X or NIV were found in any of the samples analysed (data not shown). Regarding DON and its metabolites the mycotoxin contents at the start of the storage strongly correlated with the degree of infection. The 5% infected sample showed the lowest and the 20% infected sample, the highest amounts of DON, DON3G and 3ADON. However, in contrast to the ergosterol concentrations, the levels of DON and its metabolites were strongly decreasing during storage. The 20% infected sample presented the highest decrease within the 6 weeks (50%). For the DON3G an even higher loss of up to 75% was detected (20% infected sample). The ZEA concentrations presented only minor changes during storage and were found to be widely independent from the infection level.

This result indicates that mycotoxins, especially DON, DON3G and 3ADON, but also ZEA are mainly produced during the initial phase of colonisation, but not in the on-going spread of the infection. The reasoning behind this result is the key role, which DON and its metabolites play for the initial infection by supressing the pathogen-defence mechanisms of the grain (Hestbjerg, Felding and Elmholt, 2002). However, later on when the fungus had established itself after the initial infection, it was no longer forced to produce mycotoxins. This correlates well with previous studies which showed that *Fusarium* spp. during malting produce the most mycotoxins during the kilning stage, when the fungus was particularly exposed to environmental stress (Oliveira et al., 2012). Since this investigation applied no antifungal agents and the storage conditions were chosen to be very suitable for fungal growth. Hence, the environmental stress forcing *F. culmorum* to produce mycotoxins was missing. Thus, the energy was used to produce bio-mass instead, as analysed by the increasing ergosterol contents. However, one might expect that due to the lack of mycotoxins production, the amounts determined would not change during storage. But in fact, the amounts of DON, DON3G and 3ADON decreased considerably. This could be due to various reasons. Atalla et al. (2003) reported decreasing DON contents (produced by *Aspergillus* spp.*)* in wheat during storage and concluded that reabsorption by the fungus had occurred. Another explanation would be that the DON was metabolised by the grain or the fungus itself. Furthermore, DON3G and 3ADON are known to be highly unstable at room temperature. Consequently, a decay of these compounds was possible.

After at least 3 weeks of storage, all samples were below the highest permitted amounts for DON (1250 µg/kg) and ZEA (100 µg/kg) for unprocessed cereals (EFSA, 2006). However, the use of such samples in this study to produce a wholemeal flour, could still cause a health hazard. The USDA (2014) recommends a daily intake of wholegrain for women of at least 6 ounce equivalents, which corresponds to 170 g. The infected sample with the lowest amount of DON detected was the 5% infected sample after 6 weeks. It was found to contain 446 µg/kg DON. For the recommended daily consumption of wholegrain wheat this means a DON intake of 76 µg. The EFSA (2003) released a tolerable daily intake (TDI) for different mycotoxins such as DON (1.0 µg/kg body weight) and ZEA (0.2 µg/kg body weight, provisional). Even the sample with the lowest amount of DON analysed in this study would cause a daily intake of 75.8 µg for women, which is very likely already enough to exceed the TDI. The same "worst case" calculation for the ZEA intake leads to a similar result. Considering that this example applies to the sample with the lowest amount of mycotoxins detected, the other samples analysed would constitute an even bigger health hazard. Furthermore, it is noteworthy that wholegrain products are not the only source of mycotoxins in human nutrition and that the samples analysed in this study were artificially infected and intentionally stored under poor conditions. Therefore, they are likely to present higher mycotoxin contents than natural samples would do.

Finally, it can be concluded that even without an accumulation of mycotoxins during storage a serious health hazard could be caused by the wholegrain flours obtained from any of the infected samples presented here.

4.4.3 Grain ultrastructure

In order to obtain information about the fungal impact on the grain ultrastructure and to illustrate the pathway of fungal infection, scanning electron microscopy (SEM) was performed. As the surface disinfection and the level of initial infection had no visible impact on the grains structural characteristics, only images of the natural and 20% infected samples are shown in Figure 6.

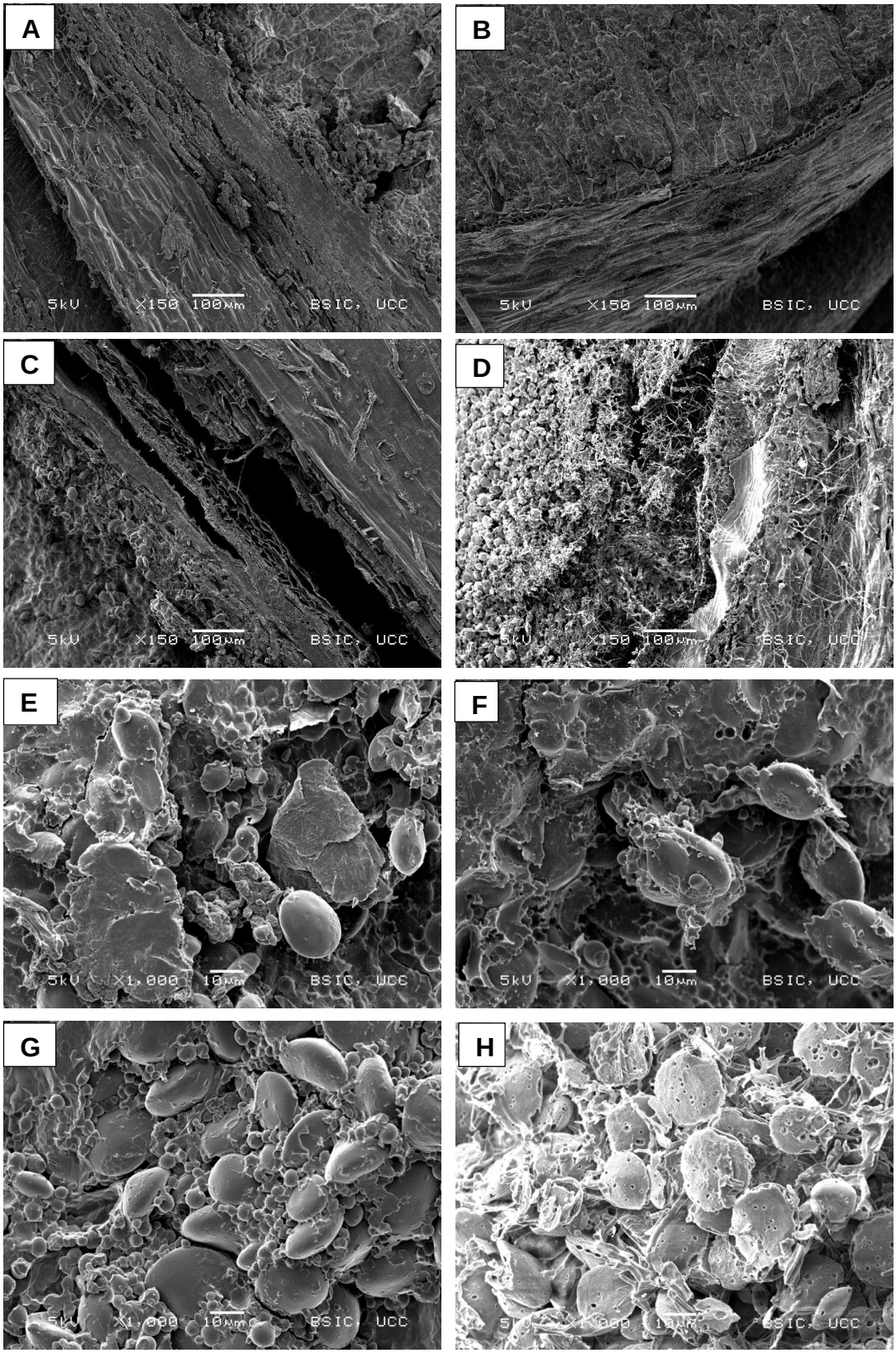

Figure 6: SEM micrographs of A) natural sample week 0 overview; B) natural sample week 6 overview; C) 20% infected sample week 0 overview; D) 20% infected sample week 6 overview; E) natural sample week 0 endosperm; F) natural sample week 6 endosperm; G) 20% infected sample week 0 endosperm; H) 20% infected sample week 6 endosperm.

Figures 6A and 6B show the natural sample, week 0 and 6, respectively. The different husk layers are well visible in both samples. However, on the outer layers of the sample in image 6A some small fungal hyphae can be seen. Since this sample was neither infected nor disinfected, these hyphae are most likely due to natural field contamination. However, as no substantial amounts of ergosterol (indicating fungal growth) and mycotoxins were found in this sample, the occurrence of these few hyphae is not representative, and likely represents a low level of natural contamination which would present negligible effects on grain quality and consumer health. Following the husk, the endosperm of the grain occurs. In the endosperm of both samples many starch granules are visible, embedded in a dense protein network. The 20% infected sample after week 0 and 6 is shown in Figures 6C and 6D, respectively. In 6C, the different husk layers are well visible. Since there are no fungal hyphae present, the damage of the husk (in form of cracks) is due to the manual cutting of the kernel. This is in contrast with sample 6D. The outside of this sample is visibly overgrown with fungal hyphae. The husk appears to be penetrated and severely damaged by the fungus. In addition, it is clearly deformed and no individual layers can be differentiated anymore. Furthermore, fungal hyphae were also found in the starchy endosperm. While the endosperm in 6C looks quite similar to the natural sample, in 6D even in the areas without fungal hyphae, it shows a much less dense protein network, indicating proteolytic activity. The results obtained in this study correlate well with the findings of Tucker and Talbot (2001) where the fungal infection starts with adhesion of macro conidia on the grain surface. This is followed by emergence of the germ tube. Immediately, release of various plant pathogen signals, enzymes and hormones follows. Directly afterwards, the hyphal network develops and host penetration occurs. Furthermore, the testa was found to be a strong barrier, which the fungus penetrated only after 3 weeks of storage. These outcomes are in good correlation with the findings of Oliveira et al. (2012) on barley, infected with *F. culmorum.* They concluded that this is due to the high concentration of phenolic acids in the testa, which can act as antifungal agents.

The endosperm of the natural sample after week 0 and 6 is shown in Figures 6E and 6F, respectively. Both images show small and big starch granules embedded in a dense protein matrix. The starch granules appear in a smooth and regular shape and no signs of degradation are visible. Furthermore, no fungal hyphae are noticeable. The endosperm of the 20% infected samples after week 0 and 6 is shown in Figures 6G and 6H, respectively. In Figure 6G the protein matrix appears to be much less dense, indicating degradation and leading to very exposed starch

granules. However, no fungal hyphae were found in this sample and the small and big starch granules show no signs of degradation. In contrast to this, there are hardly any small starch granules visible in image 6H. Also the big starch granules present obvious signs of degradation in the form of holes. Furthermore, the protein matrix appears to be widely degraded and replaced by fungal hyphae. These images show that just 6 weeks of storage were enough to cause severe damage in the infected samples. This also correlates well with the results reported by Jackowiak et al. (2005). Furthermore, these results indicate the production, or at least activation, of hydrolytic enzymes by the fungus, leading to the degradation of starch and proteins. Consequently, it can be expected that the grains infected with *F. culmorum* show a significant quality loss. To analyse this further, an investigation into the fungal effects on the grain carbohydrates and proteins was performed.

4.4.4 Fungal impact on carbohydrates

The visible degradation of the starch granules during the storage due to the fungal infection was also quantified, as this parameter has a major impact on the wheat quality, in terms of technological applications. Accordingly, starch and selected free saccharides were quantified. The results are summarised in Table 10.

The starch content of the uninfected samples was found to be between 63±1 and 65±1%, which is within the normal range for wheat of 63% - 72% (Borght et al., 2005). Furthermore, no significant changes occurred during the storage. Also the infected samples week 0 were found to contain 63±1% – 65±1% starch. However, in contrast to the healthy grains they presented a substantial loss of 10% to 12% starch during storage. Consequently, the starch contents of these samples after 6 weeks were between 52±1% and 54±1%, which means a substantial loss in grain quality. Furthermore, it has to be mentioned that at the end of the storage no noteworthy differences between the starch contents of the three initial infection levels were found, indicating that the starch degradation occurs widely independent from the level of initial infection.

Table 10: Quantification of selected carbohydrates[#].

Sam-ple	total starch	Malto-triose	Raffi-nose	Maltose	Sucrose	Glucose	Fruc-tose
nat/0	65±1^{ab}	< LOD	5.2±0.3^{a}	< LOD	12.8±0.3^{a}	<LOD	< LOD
Nat/3	64±1^{abg}	< LOD	8.4±0.1^{b}	< LOD	15.3±0.5^{b}	<LOD	< LOD
nat/6	64±1^{abdg}	< LOD	7.0±0.2^{c}	< LOD	16.0±0.3^{b}	<LOD	< LOD
0%/0	64±1^{abdg}	< LOD	6.6±0.1^{d}	< LOD	16.5±0.1^{c}	<LOD	< LOD
0%/3	61±1^{c}	< LOD	5.4±0.3^{a}	< LOD	11.2±0.4^{d}	<LOD	< LOD
0%/6	63±0^{dg}	< LOD	6.0±0.2	< LOD	12.9±0.6^{a}	<LOD	< LOD
5%/0	66±1^{a}	< LOD	6.4±0.1^{d}	< LOD	21.0±0.1^{e}	<LOD	< LOD
5%/3	60±1^{c}	2.9±0.2^{a}	2.1±0.1^{e}	6.0±0.3^{a}	< LOD	1.4±0.3^{a}	1.8±0.2^{a}
5%/6	54±1^{ef}	1.9±0.2^{b}	< LOD	3.8±0.4^{b}	< LOD	7.1±0.4^{b}	5.1±0.3^{b}
10%/0	65±1^{a}	< LOD	4.9±0.1^{a}	< LOD	19.5±0.3^{f}	< LOD	< LOD
10%/3	63±1^{abdgh}	3.0±0.1^{a}	2.1±0.1^{e}	7.3±0.3^{c}	< LOD	1.9±0.2^{ad}	2.2±0.2^{a}
10%/6	54±0^{ef}	2.3±0.1^{c}	< LOD	5.1±0.3^{d}	< LOD	6.1±0.2^{c}	4.0±0.1^{c}
20%/0	63±0^{dg}	< LOD	4.2±0.1^{f}	0.5±0.1^{e}	14.0±0.1^{g}	< LOD	< LOD
20%/3	62±0^{h}	3.5±0.1^{d}	2.1±0.1^{e}	9.2±0.2^{f}	< LOD	2.1±0.1^{d}	2.1±0.1^{a}
20%/6	52±1^{e}	2.2±0.2^{bc}	< LOD	3.2±0.4^{b}	< LOD	5.2±0.4^{e}	3.5±0.2^{d}
100%	63±2^{abcdgh}	2.8±0.1^{a}	4.3±0.1^{f}	19.1±0.7^{g}	19.6±0.6^{f}	4.5±0.1^{f}	3.3±0.1^{d}

[#] **Results shown are average values ± confidence interval. Values in one column followed by the same lower case letter are not significantly different ($p<0.05$).**

In good correlation to this were the levels of free maltotriose, maltose and glucose measured. None of those saccharides were found in the uninfected samples or the infected samples at week 0. However, substantial amounts were measured in the infected samples after 3 and 6 weeks. Consequently, it can be concluded that these saccharides were released from starch and fibres due to their degradation by fungal enzymes. Additionally, it is evident that the highest amounts of maltotriose and maltose occurred after 3 weeks, whereas the highest amount of glucose was found after 6 weeks. This is caused by the further degradation of the oligosaccharides to glucose, which is used as a nutrient by *F. culmorum*. The analysis of free raffinose

and sucrose revealed that they occur naturally in wheat, as they were found in the uninfected samples. Although the infected samples also showed noteworthy amounts in week 0, after 3 (sucrose) and 6 (raffinose) weeks they could not be detected anymore. This, again, indicates degradation by fungal enzymes to monomeric glucose, fructose and galactose, which are consumed by the fungus. In correlation with this, free fructose was found in the infected samples after 3 and 6 weeks of storage. However, the amounts of monosaccharides present were relatively low compared to the level of degradation of the di- and trisaccharides apparent. This proves that these monosaccharides were consumed as nutrients by the fungus. Generally, the results of the carbohydrate quantification, with the reduction in poly- and oligosaccharides and the release of monosaccharides, shows a significant loss in grain quality, correlating well with the structural deterioration, visible on the SEM images.

4.4.5 Fungal impact on Protein fractions

Table 11: Quantification of protein fractions (obtained from Osborne fractionation)[#].

sample / weeks of storage	albumins [mg/g]	globulins [mg/g]	prolamins [mg/g]	glutelins [mg/g]
natural / 0	11.7 ± 0.1^{ab}	7.5 ± 0.6^{a}	13.4 ± 0.1^{acf}	6.3 ± 0.3^{abd}
natural / 3	13.1 ± 1.3^{ab}	8.0 ± 0.6^{a}	12.8 ± 0.7^{acf}	5.9 ± 0.4^{abcd}
natural / 6	12.0 ± 0.4^{ab}	8.0 ± 0.8^{a}	14.2 ± 0.5^{bcdef}	6.4 ± 0.3^{abcd}
0 % infected / 0	13.4 ± 0.4^{bc}	8.1 ± 0.3^{a}	13.5 ± 0.3^{abcf}	6.6 ± 0.4^{abcdg}
0 % infected / 3	14.4 ± 0.1^{bd}	7.8 ± 0.5^{a}	14.5 ± 0.1^{bde}	6.5 ± 0.4^{abcd}
0 % infected / 6	13.6 ± 0.8^{bcd}	7.6 ± 0.5^{a}	14.4 ± 0.5^{beg}	6.2 ± 0.1^{abcd}
5 % infected / 0	13.0 ± 0.8^{bc}	8.1 ± 0.4^{a}	13.0 ± 0.7^{abcf}	6.2 ± 0.3^{abcd}
5 % infected / 3	13.1 ± 0.3^{bc}	8.7 ± 0.8^{a}	13.9 ± 0.3^{bcef}	6.6 ± 0.5^{abcdg}
5 % infected / 6	16.4 ± 0.6^{e}	8.6 ± 1.0^{a}	15.5 ± 0.6^{eg}	7.0 ± 0.3^{bcdeh}
10 % infected / 0	13.6 ± 0.4^{bc}	8.2 ± 0.3^{a}	12.7 ± 0.6^{acf}	7.4 ± 0.0^{fh}
10 % infected / 3	13.7 ± 0.8^{bcd}	8.4 ± 0.2^{a}	13.3 ± 0.6^{abcef}	7.3 ± 0.3^{cdefgh}
10 % infected / 6	16.3 ± 0.5^{e}	8.2 ± 0.6^{a}	13.9 ± 1.1^{abcdefg}	7.4 ± 0.2^{efgh}
20 % infected / 0	14.4 ± 0.2^{bdg}	7.9 ± 0.4^{a}	14.1 ± 1.1^{abcdefg}	7.5 ± 0.3^{efgh}
20 % infected / 3	15.9 ± 0.4^{e}	7.9 ± 0.4^{a}	14.3 ± 1.1^{abcdefg}	7.9 ± 0.3^{gh}
20 % infected / 6	20.1 ± 0.2^{f}	8.1 ± 0.5^{a}	15.4 ± 0.6^{eg}	7.3 ± 0.3^{cdefgh}
100 % infected / 0	14.9 ± 0.3^{g}	7.6 ± 0.5^{a}	15.1 ± 1.2^{bdeg}	7.2 ± 0.2^{cdefh}

 # Results shown are average values ± confidence interval. Values in one column followed by the same lower case letter are not significantly different ($p<0.05$).

Aiming to characterise the changes occurring to the proteins, the Osborne fractions of all samples were quantified using the Bradford (1976) dye binding assay and the results are shown in Table 11. As expected, no substantial changes were detected in the uninfected samples during the storage. For the albumin fraction of the infected samples increasing amounts of extractable proteins were determined during the storage ($p<0.05$). Since this fraction mainly contains metabolically active proteins, the increase indicates a gain in enzymatic activities, as could be expected due to the degradation of carbohydrates, shown above. In contrast to this, the globulin, prolamin and glutelin fractions of the infected samples showed no substantial differences in soluble protein contents compared to the uninfected controls. However, the degradation of proteins also increases their solubility, leading to a better extractability and so masking the consumption of some degradation products by the fungus.

From the grain quality point of view the storage proteins are of major interest, as they contain the proteins which form the gluten network. Since gluten quality and quantity are the most important quality parameters for wheat flour, the prolamin and glutelin fractions were further characterised using gel electrophoresis. These results are shown in Figure 7. In both fractions none of the uninfected samples showed any significant differences during storage (data not shown). The electrophoretogram of the prolamin fraction (Figure 7A) shows 15 distinguishable peaks, excluding the internal markers at 1.6 and 95 kDa. A significant decrease was found for five of the protein peaks, visible in Figure 7A (1, 3, 9, 11, 12) due to the fungal infection. On the other hand, Figure 7A shows several peaks with an increased intensity in the infected sample at the end of the storage (2, 4, 5, 6, 7, 13, 15). Most likely these peaks represent the degradation products of prolamins of higher molecular weight. The prolamin fraction of wheat is essential for the formation of the gluten network. Consequently, its degradation by the fungus leads to a significant loss in grain quality. The electrophoretogram of the glutelin fraction (Figure 7B) contains, apart from the internal standards at 1.6, 3.5 and 95 kDa, 8 distinguishable peaks for the natural sample. The infected sample after 6 weeks shows a total breakdown of the proteins in the size range analysed, as there were no peaks distinguishable anymore. The contrast to the results obtained from the Bradford assay, where no changes in the glutelin content were found, is caused by the fact that the Bioanalyzer measured just between 4.5 and 95 kDa, whereas the dye binding assay detects all proteins that dissolve. Furthermore, degradation of the glutelins improves their solubility. In similarity to the prolamins breakdown, the degradation of the

glutelins causes a quality loss as they are essential for the gluten network as well. Generally, the electrophoretic results confirmed the visible degradation of the protein matrix in the endosperm, as seen on the SEM images.

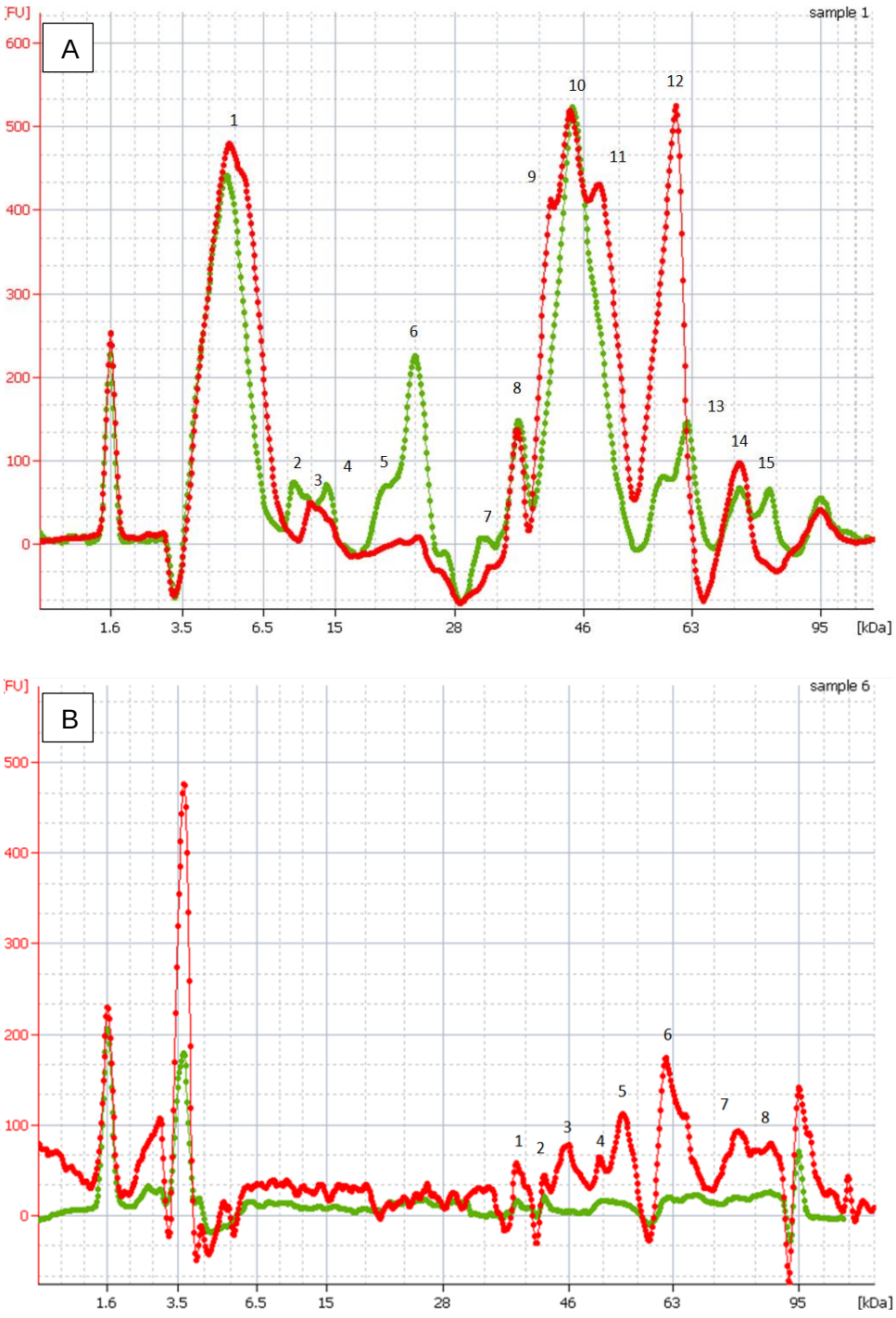

Figure 7: Prolamins (A) and glutelins (B) electrophoretogram profile for the natural sample week 0 (red) and the 20 % infected sample week 6 (green).

4.4.6 Fungal impact on enzymatic activities

In order to obtain further information about the pathway of fungal infection, regarding the penetration into the endosperm and hydrolysis of proteins and polysaccharides (as quantified and visible on the SEM images), the activity of selected enzymes was determined. The results of these enzymatic determinations are summarised in Table 12.

Lipases and xylanases are primarily tools of the fungus to overcome the physical barriers of the grain. The xylanase is, due to degradation of arabinoxylan, known as one of the major cell wall degrading enzymes for wheat. Lipases, on the other hand, are mainly released to degrade the cuticle, which is the outer protective barrier of wheat grains. Regarding the activity of lipases, the infected samples showed, particularly between weeks 3 and 6, a substantial increase. Values of the 10% and 20% infected samples were found with a 4 fold increase. While at the beginning of the storage no significant differences were detected between the three infection levels, after 6 weeks the lipase activity was found to be in direct correlation with the initial infection level. Thus, the expression of lipase, which degrades the cuticle, occurred basically during the second half of storage. Consequently, the increase in lipase activity can be seen as an indication for the on-going infection process. It also shows that a higher level of infection leads to a faster penetration into deeper grain layers. A similar development was found for the xylanase activity, except that the activity was widely independent from the level of initial infection, even after 6 weeks. Thus, increased xylanase activity also indicates the on-going infection due to penetration into the endosperm. In the uninfected controls, activities of both enzymes remained consistently low, without significant changes ($p<0.05$), during the whole storage period.

These results also correlate well with the observation from the SEM images, showing that the fungus first overgrows the surface, before penetrating into deeper layers. The penetration into the kernel is of major importance to the fungus and the on-going infection process, as it provides access to the endosperm, which is a source of nutrients. In addition, it is noteworthy that free arabinose was also detected (data not shown), which is a degradation product of the arabinoxylan of the cell walls. As substantial amounts of free arabinose were only found in the infected samples after 6 weeks, the result correlates with the xylanase activity, showing that the fungus penetrates into the endosperm after a few weeks of storage. Additionally, the degradation of the cell walls also leads to a loss in fibre. This reduces the

nutritional value of the whole meal flour obtained from these grains and also effects technological quality parameters such as water binding during dough preparation.

However, from the technological point of view the protein and starch degrading enzymes have an even higher impact on the grain quality. Analysis of these enzymes revealed an increase in total proteolytic, α-amylase and β-amylase activity for all three infection levels during the storage, but not for the uninfected samples. This is in good correlation with the degradation of starch and storage proteins, as seen on the SEM images and quantified above. Thus, it can be concluded that this increased activity is caused by *F. culmorum*, in order to obtain essential nutrients in form of amino acids and glucose. Also the β-glucanase activity was found to increase during the storage of the infected samples. The degradation of glucans, such as cellulose, provides glucose for the fungus. Consequently, the increase of activity in the infected but not in the uninfected samples correlates with the aim of the fungus to release nutrients, which provide energy for growth. It is noteworthy that no significant differences in the enzymatic activities between the different levels of initial infection could be found after 6 weeks. Accordingly, it shows once more that a 5% level of initial infection can cause similar damage as a 20% initial infection level. Since all these enzymes cause a quality loss due to degradation of either nutrients or cells, the grain quality is substantially reduced, widely independent of the initial infection level. However, the results cannot show if the enzymes are produced by the fungus or the grain and activated due to the stress of the fungal infection. In either case, their activity is related to the fungal infection.

Table 12: Enzymatic activities of wheat samples, with method of determination[#].

sample / weeks of storage	endo-β-1,4-xylanase [U/kg]	β-glucanase [U/kg]	lipase [U/g]	protease [U/g]	α-amylase [CU/g]	β-amylase [U/g]
Method	MEGAZYME © Azo-Wheat	MEGAZYME© Azo-Barley Glucan	Rose and Pike, 2006	Brijs et al., 2002	MEGAZYME© Ceralpha	Hyun et al., 1985
natural / 0	3.7 ± 0.3^{adef}	10 ± 0^{ab}	2.05 ± 0.07^{a}	0.50 ± 0.02^{a}	11 ± 0^{a}	2.4 ± 0.2^{a}
natural / 3	4.8 ± 0.1^{bfh}	11 ± 1^{ab}	2.53 ± 0.79^{a}	0.32 ± 0.03	12 ± 1^{abc}	2.2 ± 0.2^{a}
natural / 6	3.0 ± 0.1^{ce}	6 ± 1^{c}	1.82 ± 0.43^{a}	0.45 ± 0.04^{a}	10 ± 1^{ab}	2.2 ± 0.1^{a}
0% / 0	3.7 ± 0.1^{adef}	6 ± 0^{c}	1.22 ± 0.23^{b}	0.57 ± 0.03^{b}	10 ± 0^{b}	1.5 ± 0.3^{bc}
0% / 3	3.6 ± 0.5^{acdef}	10 ± 1^{ab}	0.45 ± 0.04	0.52 ± 0.04^{ab}	13 ± 1^{c}	1.6 ± 0.1^{b}
0% / 6	3.0 ± 0.4^{ace}	13 ± 1^{bf}	1.11 ± 0.58^{b}	0.57 ± 0.06^{ab}	10 ± 1^{ab}	1.9 ± 0.1^{bc}
5% / 0	4.3 ± 0.5^{befh}	7 ± 1^{c}	1.58 ± 0.22^{b}	0.48 ± 0.03^{a}	11 ± 1^{abc}	3.5 ± 0.4^{deg}
5% / 3	4.5 ± 0.4^{befh}	25 ± 0^{d}	2.44 ± 0.36^{a}	0.95 ± 0.03^{c}	39 ± 1	3.9 ± 0.2^{de}
5% / 6	15.6 ± 0.8^{g}	152 ± 10^{e}	7.34 ± 1.67	1.50 ± 0.02^{d}	163 ± 10	5.9 ± 0.3^{f}
10% / 0	4.6 ± 0.2^{bfh}	14 ± 0^{f}	2.39 ± 0.58^{a}	0.68 ± 0.06	16 ± 0	2.8 ± 0.1
10% / 3	3.5 ± 0.7^{acdef}	47 ± 1	3.12 ± 0.35^{c}	1.06 ± 0.04^{e}	56 ± 1	3.6 ± 0.1^{deg}
10%/ 6	12.3 ± 0.3	160 ± 3^{e}	13.27 ± 0.52	1.51 ± 0.00^{d}	219 ± 7	5.9 ± 0.3^{f}
20% / 0	4.6 ± 0.3^{bfh}	31 ± 2^{g}	3.41 ± 0.35^{c}	1.02 ± 0.03^{ef}	34 ± 1	3.2 ± 0.1^{d}
20% / 3	6.8 ± 0.3	36 ± 6^{g}	5.35 ± 0.58	0.93 ± 0.06^{cf}	81 ± 2	4.2 ± 0.1^{e}
20% / 6	16.5 ± 0.8^{g}	136 ± 5^{e}	23.36 ± 0.61	1.51 ± 0.02^{d}	245 ± 8	8.5 ± 0.2
100% / 0	4.7 ± 0.6^{befh}	70 ± 7	16.23 ± 0.22	1.15 ± 0.05^{e}	374 ± 15	6.4 ± 0.4^{f}

[#] Results shown are average values ± confidence interval. Values in one column followed by the same lower case letter are not significantly different (p<0.05).

4.5 Conclusions

The aim of this study was to investigate the spread of a *F. culmorum* infection in stored wheat and its impact on the grain quality. The increasing amounts of ergosterol detected during the storage indicates a rapid increase of fungal biomass under the chosen storage conditions. Firstly, a substantial quality loss of the grains was shown by the mycotoxins detected, which are likely to cause a health risk to a potential consumer. Furthermore, quality loss from the technological point of view was shown, mainly due to degradation of polysaccharides, such as starch, and storage proteins. After storage, none of the infected samples fulfilled the quality requirements for wheat flour. Thus, the need to develop efficient decontamination methods for cereals which can be applied during storage, without affecting the grain quality, has clearly been demonstrated. The contribution of enzymes, produced exogenously or induced in grains by fungal stress, was illustrated but warrants further study to fully understand the pathways of fungal infection. In addition, the fungal impact on gluten quality and the resulting dough and bread would be of further interest.

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Chapter 5: Impact of post-harvest degradation of wheat gluten proteins by Fusarium culmorum on the resulting bread quality

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5.1 Abstract

This study was conducted to investigate the impact of wheat gluten protein degradation during storage in a dough and bread system due to different levels of fungal contamination with *F. culmorum*. Therefore, artificially infected grains were stored in a model system and analysed for their gluten content and quality. This revealed a substantial loss in wet gluten content and a weakening of the gluten network in the infected samples during storage. Subsequently, the samples were used to bake wholegrain breads and the obtained dough and breads were analysed. Reduced quality as a consequence of storage was found for all samples, but in the infected samples to a greater extent than in the healthy grains. The breads presented a reduced quality, mainly due to their high bake loss, deformed loaf shape and open crumb structure. The reason for these results was mainly found to be due to the increased enzymatic activities originating from the fungal metabolism. Furthermore, the deterioration of the bread quality after storage was independent from the level of initial infection. Consequently, this study demonstrates the importance of optimal storage conditions and the development of efficient methods to supress fungal proliferation during storage, in order to reduce economic losses and ensure a consistent high quality of the resulting products.

5.2 Introduction

Over thousands of years cereals have played an essential role in human nutrition. Wheat in particular is one of the most produced and consumed cereals. Its high prominence is mainly due to the fact that wheat represents the main raw ingredient for a large variety of products. By far the most important of these are bakery products, mostly bread, but also cakes, biscuits and others (USDA, 2013). Bread, in all of its variations is an essential part of human nutrition all over the world and most commonly it is produced from wheat flour (McGee, 2004). Consequently, it is crucial to ensure a high and continuous grain quality, being essential for a product of the highest quality.

One of the main reasons for poor product quality of cereal based foods is fungal contamination and spoilage. A very common cereal disease is *Fusarium* head blight (FHB), caused by different *Fusarium* spp. (Clark et al., 2012). It is responsible for significant losses relating to the wheat quality and yield. Although fungal contamination usually occurs in the field, it was shown in a previous study (Schmidt et al., 2016) that even low levels of initial infection can spread during storage if conditions are suitable. Pitt and Hocking (2009) reported that the post-harvest economic losses of wheat due to fungal spoilage and mycotoxins exceed $300 million annually, just in the USA alone. In particular, less developed countries face even bigger damage due to post-harvest fungal spoilage (Pitt and Hocking, 2009). However, although this is such an important topic, both from safety and grain quality point of view, there are just very few publications investigating this topic.

Subsequently, this study investigated the impact of post-harvest fungal spoilage on wholemeal dough and bread quality. The impact of the enzymatic activities of infected wheat samples on storage proteins and polysaccharides, such as starch and different fibres, were evaluated previously (Schmidt et al., 2016). In this study the impact on the dough characteristics and final bread quality were examined. Therefore, different gluten quality parameters, in particular the development time and maximal network strength, were determined. Furthermore, important quality characteristics of the dough and baked breads, produced from the infected and stored samples, were analysed. Thus, the impact of fungal spoilage during storage on the final bread quality could be illustrated.

5.3 Materials and Methods

5.3.1 Materials

Commercial hard winter wheat (*Triticum aestivum*), harvested in 2013, was supplied by Doves Farm Foods Ltd. (Hungerford, UK). Wheat grains were stored in barrels at 20±2°C and were regularly aerated. *Fusarium culmorum* strain TMW 4.2043 was originally isolated from barley and provided by the Lehrstuhl für Technische Mikrobiologie, TU-München Weihenstephan.

For the baking trials, dry yeast was purchased from Puratos, Belgium; salt from Glacia British Salt Limited, UK; commercial vegetable oil and sugar from Nordzucker, Ireland. All reagents used were at least of analytical grade and sourced from Sigma Aldrich.

5.3.2 Preparation of fungal spore suspension and grain infection

The spore solution of *Fusarium culmorum* was prepared according to the method described by Oliveira et al. (2012). Briefly, fungus was cultivated on potato-dextrose-agar (PDA) plates. After 5 days at 25°C, six small fragments of inoculated PDA were transferred to 800 mL synthetic nutrient-poor bouillon. To induce spore production, fungal suspensions were kept at room temperature under continuous stirring. Prior to use, the suspensions were filtered through 30 µm filter paper. The concentration of spores was determined to be 10^5 spores/mL, using a haemocytometer.

Preceding the artificial fungal infection, grains were disinfected using hydrogen peroxide (10%, w/v) and ultra violet light as described by Oliveira et al. (2012). In brief, grains were disinfected by washing for 10 min in a 10% (w/v) hydrogen peroxide (H_2O_2) solution (using 4 L per 600 g of grains). After, rinsing the grains for 5 min in distilled water (4 L) the procedure was repeated once. Subsequently, the grains were transferred into sterile plastic boxes and dried under vertical sterile laminar flow for 24 h at room temperature. After drying, the grains were subjected to ultraviolet light (10 min) and collected aseptically for further use.

Infected wheat grains were prepared using the following procedure. Disinfected wheat grains were mixed with 2% (v/w) sterile filtered spore suspension of *F. culmorum*. Subsequently, grains were incubated for 10 days at 25°C with 75%

relative humidity to allow fungal proliferation. The infected grains produced were defined as 100% infected. After homogenisation the samples moisture content was determined.

5.3.3 Mixing and storage trials

Infected and disinfected grains were mixed together to a total sample size of 4.5 kg (dry matter) with specific infection levels of 0, 5, 10 and 20% and stored in lab-scale model systems under conditions generally suitable for fungal growth. This allowed to study the fungal impact in relation to the level of initial infection. Each mixture and the control sample was divided into nine portions and filled into sterile plastic bags. Every bag was sealed and perforated with two pipette tips with barrier filter to allow gas exchange. The bags were stored for 6 weeks at room temperature. After 0, 3 and 6 weeks, 3 portions of each sample were taken and milled (Laboratory Disk Mill DLFG, Bühler AG, Uzwil, Switzerland) to a whole grain flour (particle size < 0.5 mm). The flours obtained from the three portions of each sample were combined, homogenised and stored at -20°C until further use.

5.3.4 Gluten characterisation

Wheat flour samples were analysed using the Glutomatic (Perten Instruments GmbH, Hamburg, Germany) and the GlutoPeak (Brabender GmbH & Co. KG, Duisburg, Germany). The Glutomatic provides essential information regarding the amount of gluten (wet and dry gluten content) but also about the quality (water binding capacity and gluten index). The determination of wet and dry gluten content, water binding capacity and gluten index was carried out according to the AACC method 38-12.02 (1999) for wholemeal wheat flour.

For further characterisation of the gluten quality, in terms of its development time and maximum strength, the GlutoPeak was used. Therefore, the sample was mixed with water under high shear force until the gluten network formed and broke down again. The viscosity was recorded graphically as a function of time (Figure 8). Hence strong flours, as desired for bread making, developed fast, showing high peaks with short peak times (Brabender, 2016). The test was performed according to the manufacturer recommendations (Brabender, 2016). Briefly, 9 g of sample (14.0% moisture content) and 9 g of distilled water were weighed into the mixing

chamber and equilibrated. Sample and water were mixed with 2,500 rpm at 27°C. After the maximal mixing resistance was detected, or latest after 10 minutes, the measurement was stopped. Peak time and height were recorded to evaluate the gluten quality.

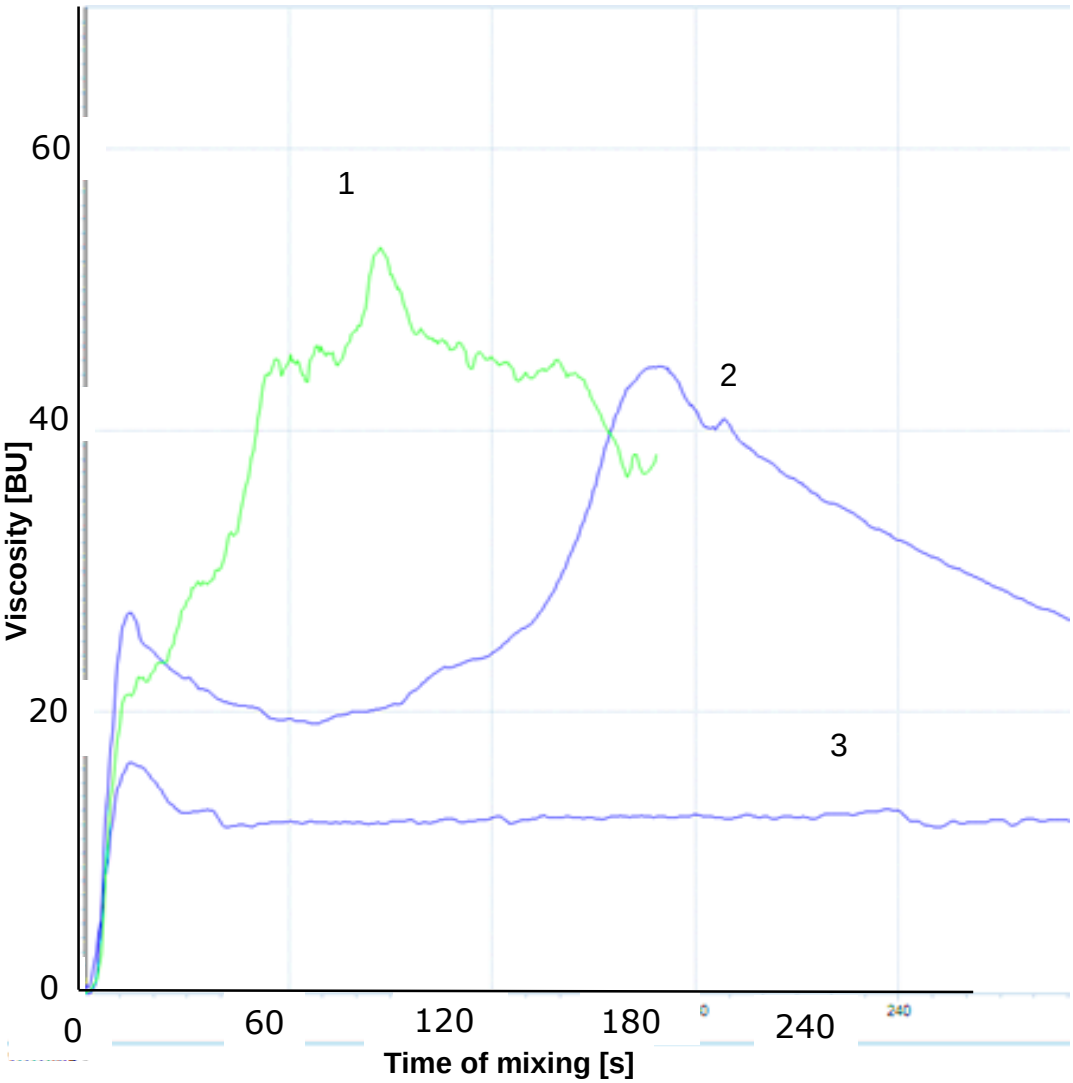

Figure 8: Comparison of GlutoPeak Graphs of the (1) natural sample, week 0 (strong flour), (2) 10% infected, week 3 (weak flour) and (3) 20% infected sample, week 6 (very weak flour).

5.3.4 Analysis of dough quality

Important quality characteristics of the bread dough were analysed using a TA-XT2i texture analyser (Stable Micro Systems, Surrey, UK), equipped with a 5 kg load cell. Firstly, the stickiness of the freshly prepared dough was measured using the SMS

Chen-Hoseney dough stickiness cell, equipped with a 25-mm Perspex spherical probe with a uniform adherence surface. The probe first compressed the dough (test speed 2.0 mm/s) until the test force (40 g) was reached. This force was maintained for 0.1 s until the probe moved upwards (100 mm at 10 mm/s). The maximum force measured was used to evaluate the stickiness.

In addition, the dough strength was determined for all samples, using the Kieffer cell dough extensibility rig (Stable Micro Systems, Surrey, UK). The freshly prepared dough was rolled by hand into a cylindrical shape, placed in the lubricated Teflon mould and compressed with the lubricated top Teflon plate. The sample was allowed to rest for 25, 50 and 75 min, in the proofer to investigate the development of the dough strength during proofing. Subsequently, by clamping the sample between the Kieffer rig plates, analysis was performed according to the method described by Dunnewind et al. (2004) and the maximum force in tension recorded. Five replicates of each dough formulation were measured.

5.3.5 Baking procedure

Baking trials were carried out with the whole grain flour obtained from the stored samples (particle size < 0.5 mm). The percentage of water (based on flour weight) required to yield a dough consistency of 500 Brabender units (BU) (determined by using a Brabender-farinograph according to the AACC method 54-21.02 (1999)) was equal to 83% for the natural sample at week 0. This amount of water was used for all the wholemeal wheat flour samples baked.

Additionally, 2% yeast, 3% oil, 3% sugar and 2% salt (each based on flour weight) were used in the bread recipe. Prior to dough preparation, yeast was reactivated, using the whole amount of water (30°C) of the recipe, and placed in a proofer (KOMA sunriser, Roemond, Netherlands) at 30°C and 80% relative humidity for 10 minutes. Afterwards, the yeast suspension and the dry ingredients were combined in a mixer (Kenwood Chef Classic KM336). Mixing was carried out with a dough hook at speed I for 1 minute, followed by scraping down the sides of the bowl and further mixing at speed II for 7 minutes. After mixing, the dough was divided into portions of 65 g, manually rounded, placed in non-stick baking tins (dimensions-top inside, 50 mm x 90 mm; bottom outside, 45 mm x 85 mm; inside depth, 30 mm; Sasa UK, Enfield Middlesex, UK) and proofed for 75 minutes (30°C, 85% RH). Subsequently, the tins were transferred to the oven (Belling, Prescott, UK) and

baked for 27 minutes at 175°C (top and bottom). Afterwards, the bread loaves were immediately removed from the tins and allowed to cool to room temperature for 120 min before further analysis.

5.3.6 Analysis of bread quality

After cooling to room temperature, the breads were weighed to determine the bake loss (weight reduction during baking). The specific volume was measured using a Volscan profiler (Stable Micro Systems, UK). After slicing the breads (25 mm width), the four slices from the centre of each loaf were used for further analysis. First, the crumb colour was analysed, using a chroma meter CR-400 (Konica Minolta Inc., Tokyo, Japan) with CIE standard illuminant D65. The L*-value, as calculated by the software, was used to evaluate the crumb lightness. The C-cell Bread Imaging System (Calibre Control International Ltd., UK) was used to characterise the crumb structure. The following parameters were evaluated: number of cells per slice, total area of cells as a percentage of the total slice area and average diameter of the cells.

The crumb texture was characterised by texture profile analysis (TPA), using a TA-XT2i texture analyser (Stable Micro Systems, Surrey, UK), equipped with a 25 kg load cell and a 20-mm aluminium cylindrical probe. A speed of 5 mm/s and a force of 0.98 N were applied to compress the middle of the crumb to 50% of its original height. 12 slices per batch were analysed on the baking day and the crumb hardness evaluated.

5.3.7 Statistical analysis

Samples were baked in two batch replicates. All analyses were run in triplicate, unless otherwise stated. Statistical analysis was performed using Minitab 17 software. Data was checked for outliers (Grubb's test) and evaluation of significant differences was performed using one-way analysis of variances (ANOVA). All differences were considered significant at $P<0.05$. Where F-values were significant, pairwise comparisons were carried out with the help of Tukey Post Hoc test to describe the statistical significance between the infected and uninfected samples over the time of storage.

5.4 Results and Discussion

The impact of a fungal contamination on wheat quality criteria was investigated in a previous study conducted by the authors (Schmidt et al., 2016). Thereby, grains infected with *F. culmorum* were stored in a model system to investigate the impact of post-harvest fungal spoilage. Subsequently, this study was carried out, to examine the impact of this infection on the grains' technological performance during bread making. The activities of different enzymes, in relation to the level of initial fungal infection and time of storage were determined and discussed in chapter 4 (Schmidt et al., 2016). As this study refers to the same samples, these results were used to understand how *F. culmorum* and its metabolism influenced the bread quality. A table summarising the enzymatic activities of all samples analysed is provided in chapter 4 (Table 12).

5.4.1 Gluten characterisation

Results of the gluten characterisation for the infected and stored wheat samples, as well as for the uninfected controls are summarised in Table 13. The wet and dry gluten content in natural and 0% infected grains did not change significantly during the 6 weeks of storage ($p<0.05$). The wet gluten contents of the uninfected samples ranged between 38.6±3.3 – 46.3±2.0 g/100g and the dry gluten contents between 14.2±1.6 – 18.1±0.4 g/100g. In contrast, the infected samples revealed a significant decrease in both wet and dry gluten content, over time of storage. In particular the wet gluten content was found to decrease rapidly, due to the fungal infection. A reduction of up to 30% in wet gluten content (20% infected sample) was measured during the 6 weeks. No substantial differences between the infected and uninfected samples were found at week 0, while after 6 weeks remarkably reduced values were determined for the infected grains ($p<0.05$). Consequently, the loss of wet gluten during storage is related to the fungal infection. However, also the reduction in dry gluten content was significant ($p<0.05$) but notably less severe. The 5% infected sample even showed no significant loss over time at all ($p<0.05$). Furthermore, after 6 weeks no substantial differences, regarding the dry gluten content, were found between the three levels of initial infection. Consequently, the water binding capacity, calculated from wet and dry gluten contents (Perten, 2016), also decreased over time in the infected grains, but not in the uninfected ones. A reduction of up to 50% (20% infected sample) was found during the storage. After 6

weeks, a clear indirect proportionality between water binding capacity and the level of initial infection was apparent, as the 20% and 0% infected sample showed the lowest and the highest water binding capacity, respectively. Since the binding of water is one of the main purposes of gluten during bread making, this finding resembles a quality loss in terms of technological performance (Kieffer et al., 1998).

Table 13: Mean results of gluten characterisation by Glutomatic and GlutoPeak with corresponding standard deviations for the stored wheat samples[#].

Sample / weeks of storage	Glutomatic				GlutoPeak	
	wet gluten content [%]	dry gluten content [%]	water binding capacity [g/100 g]	gluten index	peak time [sec]	peak height [BU]
nat / 0	40.0 ± 1.3^{a}	14.8 ± 1.2^{a}	25.2 ± 0.2^{abc}	97 ± 3^{abc}	87 ± 0^{a}	52 ± 2^{ab}
nat / 3	40.8 ± 0.7^{a}	15.2 ± 1.1^{a}	25.6 ± 0.4^{abc}	98 ± 0^{ab}	76 ± 12^{abc}	54 ± 1^{ab}
nat / 6	38.6 ± 3.3^{ab}	14.2 ± 1.6^{a}	24.5 ± 1.6^{abc}	99 ± 0^{ac}	68 ± 12^{abc}	49 ± 1^{ac}
0% / 0	44.0 ± 0.3^{c}	17.7 ± 0.1^{b}	26.3 ± 0.5^{bc}	99 ± 0^{ac}	107 ± 1de	48 ± 1^{c}
0% / 3	46.3 ± 2.0^{c}	18.1 ± 0.4^{b}	28.1 ± 2.4^{bc}	98 ± 0^{ab}	113 ± 11de	44 ± 0^{d}
0% / 6	41.9 ± 5.0^{a}	16.2 ± 2.4^{ab}	25.7 ± 2.6^{abc}	99 ± 0^{ac}	108 ± 1de	47 ± 2^{c}
5% / 0	40.8 ± 5.3^{a}	15.5 ± 1.8^{a}	25.2 ± 3.5^{abc}	99 ± 0^{ac}	54 ± 6^{c}	57 ± 2^{b}
5% / 3	39.3 ± 3.1^{ab}	16.2 ± 1.1^{a}	23.1 ± 1.9^{ac}	99 ± 1^{abc}	127 ± 11^{d}	43 ± 0^{e}
5% / 6	31.0 ± 0.3^{d}	13.3 ± 1.7^{a}	17.7 ± 1.3^{de}	64 ± 0^{d}	188 ± 2^{f}	44 ± 1de
10%/0	42.7 ± 4.5^{abc}	17.5 ± 0.7^{b}	25.2 ± 3.8^{abc}	94 ± 0^{a}	62 ± 1^{c}	56 ± 1^{b}
10%/3	33.2 ± 2.3^{be}	15.2 ± 0.4^{a}	18.0 ± 1.9^{de}	65 ± 2^{d}	151 ± 6	45 ± 2de
10%/6	28.2 ± 3.7^{def}	14.0 ± 1.7^{a}	14.2 ± 2.0^{e}	47.8 ± 6.2^{f}	200 ± 16^{f}	41 ± 3de
20%/0	36.9 ± 2.6^{b}	16.2 ± 0.3^{a}	20.7 ± 2.9^{cde}	82 ± 0	63 ± 1^{bc}	56 ± 1^{b}
20%/3	32.6 ± 0.9^{e}	15.5 ± 1.5^{a}	17.1 ± 0.7^{de}	75 ± 0	116 ± 4^{d}	47 ± 4^{cde}
20%/6	24.1 ± 2.2^{f}	14.1 ± 1.1^{a}	10.0 ± 1.1^{f}	49.5 ± 3.2^{f}	-	-
100%	33.4 ± 2.3^{e}	14.1 ± 0.9^{a}	19.3 ± 1.5^{de}	62 ± 5^{d}	75 ± 6^{bc}	50 ± 1^{ac}

[#] Results shown are mean values ± standard deviation. Values in one column followed by the same lower case letter are not significantly different ($p<0.05$).

The gluten index (GI) measures the strength of the gluten network and therefore represents a key parameter for the bread making performance of a flour (Perten, 2016). In this regard, the uninfected samples revealed no significant changes during storage ($p<0.05$). Values of 97±3 to 99±0 were determined, indicating a very strong

flour (Perten, 2016). On the contrary, the infected samples showed a significantly reduced GI value, indicating an evident loss of gluten network strength due to the fungal infection. While at week 0 the lowest GI value was found for the highest infection level, after 6 weeks no noteworthy differences between the 10 and 20% infected samples could be detected anymore ($p<0.05$). The biggest reduction (from 94±0 to 48±6) was determined for the 10% infected sample, indicating that even small contamination levels can result in remarkable damage.

Table 13 further shows the results for peak time and height obtained from the GlutoPeak. Both values were used to evaluate the gluten development time and network strength. Thereby, strong flours present short peak times with high intensities. Weak flours show longer peak times with lower intensities. In extreme cases no peak is detected at all (Figure 8). The results obtained for peak time and height correlate well with each other. Samples with long development times also present low peak maxima, both indicative of a weak gluten network. Furthermore, the samples found to be the weakest by the GlutoPeak also presented the lowest GI values. The uninfected samples were found to be strong flours without significant changes in peak height or development time throughout storage ($p<0.05$). In contrast, the infected ones showed a decrease in gluten network strength over time of storage.

After 3 and 6 weeks, the 5 and 10% infected samples presented more than a 3-fold prolonged peak development time compared to week 0. Consequently, the peak intensities were substantially decreased also. The biggest decrease was found for the 20% infected sample which, after 6 weeks, produced no detectable peak anymore (Figure 8). Consequently, the fungal damage in this sample was so severe that no gluten network could form. This correlates well with the electrophoretic analysis of the storage proteins (Schmidt et al., 2016), where the 20% infected sample after 6 weeks presented a total degradation of the gliadin fraction.

This loss of gluten quality is most likely due to the substantially increased proteolytic activity in the infected samples during storage (Table 12). According to Wang et al. (2005), fungal proteases primarily attack the storage protein fractions. Furthermore, this degradation of the glutenins and gliadins also improves their solubility due to the reduced molecular weight. Consequently, the water binding capacity, as determined by the Glutomatic, decreases similarly. The proteolytic activity of the infected samples in week 6 revealed no substantial differences between the infection levels. In contrast, the gluten quality parameters were affected more by the

higher contamination levels. The reason behind is in the increased lipase and xylanase activities after 0 and 3 weeks. Both enzymes are released to overcome some of the grains physical protection barriers, namely the cuticle (lipase) and cell walls (xylanases). Thus, increased activities of these enzymes indicate a faster penetration of the fungus into the endosperm due to higher initial infection. Consequently, this resulted in more damage after 6 weeks. Thus, the highest loss in gluten strength occurred in the 20% infected sample (shown by the GI and GlutoPeak), while no noteworthy changes occurred to the gluten network of the uninfected ones.

These results show that even minor levels of *F. culmorum* contamination (5%) during storage, if conditions are suitable, result in a substantially reduced quality of the gluten network.

5.4.2 Dough characterisation

To characterise the dough obtained from the stored samples, the stickiness of the freshly prepared formulation and its strength after 25, 50 and 75 minutes of proofing were determined. These analyses assessed the impact of fungal contamination and storage time on the most important dough characteristics. The results are summarised in Table 14.

Regarding dough stickiness, the samples from week 0 show the expected trend, in the natural and the three infected samples the stickiness increased with the initial level of fungal contamination. The significantly higher stickiness of the disinfected sample ($p<0.05$) is potentially due to denaturation of the proteins during the initial sanitation, which led to a decreased water binding ability. After 3 weeks of storage a substantially increased stickiness was found for all samples, including the uninfected ones. In fact, the biggest increase was found for the natural sample (from 20.1±5.5 to 51.1±3.8 N/cm^2), whereas the 20% infected showed just a minor increase from 43.3±4.9 to 53.8±5.5 N/cm^2. From week 3 to 6 however, the stickiness of all samples increased only slightly, which was not found to be significant ($p<0.05$). Finally, after 6 weeks of storage no substantial differences between the infected and uninfected samples could be determined anymore. However, the outcome for the dough stickiness analysis was generally in good correlation with the results of the gluten characterisation, as samples shown to contain a poor quality gluten network also resulted in more sticky dough. As shown

above (section 5.4.1), the fungal infection reduced the water binding capacity of gluten during storage significantly. Thus, the water binding ability of the dough is also lower, leading to an increased dough stickiness for the respective samples. In addition, the increased enzymatic activities of amylases (α and β), xylanase and glucanase in the infected samples (Schmidt et al., 2016) (Table 12) promote the degradation of various polysaccharides, which would otherwise also absorb water (Biliaderis, Izydorczyk and Rattan, 1995). Thus, the amount of free water in the dough was further increased. The higher amylase activity also caused a delay in starch gelatinisation, which reduced the water binding capacity of the dough even further (Tester, Qi and Karkalas, 2006).

Interestingly, the stickiness of the dough produced from uninfected grains was not constant during the storage period either. A potential conclusion would be that the stickiness primarily depends on the time of storage, not the rate of fungal infection. On the other hand, for the week 0 samples a direct correlation between dough stickiness and the level of infection was visible. This also confirms the results of Dexter, Clear and Preston (1996), who reported a more sticky dough as a result of *Fusarium* damaged kernels. It also has to be mentioned that after 3 and 6 weeks the infected dough samples produced a batter, rather than a proper dough. Thus, differences in viscosity were more evident than in stickiness. This caused certain difficulties during the measurement, which are likely to have influenced the results of the analysis. Consequently, differences between the samples could not be determined properly. However, a comparable study of Nightingale et al. (1999) used the endosperm flour of 20% *F. graminearum* infected wheat kernels without storage. The authors reported that the degree of fungal infection had just little impact on the water absorption ability of the dough. In addition, the present study used wholemeal flour, containing a high amount of fibre from the husk layers. The water absorption of these fibres in the dough served to mask the effects of the gluten degradation further. Thus, just minor differences in terms of dough stickiness were observed.

Table 14: Mean results of dough strength and stickiness with corresponding standard deviation of the stored samples[#].

Sample / weeks of storage	Dough strength [g]			Dough stickiness [g]
	25min	50min	75min	
natural / 0	14.6 ± 1.6^{a}	11.3 ± 2.0^{a}	11.4 ± 1.1^{a}	20.1 ± 5.5^{a}
natural / 3	10.5 ± 1.4^{b}	7.1 ± 0.7^{b}	7.2 ± 1.0^{b}	47.6 ± 5.5^{bc}
natural / 6	8.3 ± 0.7^{c}	5.5 ± 0.5^{cd}	5.7 ± 0.5^{bc}	51.2 ± 3.8^{bc}
0% infected / 0	11.7 ± 1.3^{ab}	10.5 ± 0.7^{a}	10.2 ± 1.0^{a}	32.7 ± 6.7
0% infected /3	9.6 ± 1.3^{bc}	6.0 ± 0.4^{bc}	6.5 ± 0.4^{bc}	38.5 ± 11.8^{bc}
0% infected / 6	5.4 ± 0.5^{d}	4.7 ± 0.4^{c}	4.8 ± 0.2^{d}	48.6 ± 3.7^{bc}
5% infected / 0	10.0 ± 1.0^{bc}	10.1 ± 0.7^{a}	10.3 ± 0.7^{a}	23.4 ± 6.5^{a}
5% infected /3	6.3 ± 1.6c^{de}	4.1 ± 1.1^{cde}	4.5 ± 1.4^{cd}	44.2 ± 5.9^{bc}
5% infected / 6	2.9 ± 0.5^{f}	2.6 ± 0.5^{def}	2.7 ± 0.1^{e}	45.9 ± 3.2^{bc}
10% infected / 0	8.7 ± 0.7^{bc}	8.5 ± 0.7^{b}	7.8 ± 0.9^{b}	27.8 ± 3.7^{a}
10% infected /3	4.0 ± 0.4^{g}	3.4 ± 0.8^{d}	3.3 ± 0.3^{f}	44.8 ± 4.8^{bc}
10% infected / 6	2.6 ± 0.4^{f}	2.3 ± 0.4^{ef}	2.1 ± 0.4^{g}	49.9 ± 4.8^{bc}
20% infected / 0	7.5 ± 0.4^{c}	7.0 ± 0.4^{b}	6.2 ± 0.5^{bc}	43.3 ± 4.9^{bc}
20% infected /3	4.2 ± 0.6^{eg}	3.5 ± 0.2^{de}	3.8 ± 0.4^{f}	38.9 ± 5.2^{b}
20% infected / 6	2.6 ± 0.4^{f}	2.5 ± 0.3^{ef}	2.5 ± 0.1^{eg}	53.8 ± 5.2^{bc}

[#] Results shown are mean values ± standard deviation. Values in one column followed by the same lower case letter are not significantly different ($p<0.05$).

The dough strength was analysed using the Kieffer cell dough extensibility rig. A decreasing trend was found for the strength of all samples with increasing time of proofing and storage (Table 14). A weakening of the dough during proofing is normal behaviour, as the wheat enzymes become activated and degrade proteins and polysaccharides (Koehler et al., 2007). For all samples, independent from the fungal infection, the main weakening was found within the first 50 minutes of proofing. Afterwards the strength remained on a constant level. However, the

doughs obtained from the infected samples were significantly weaker than the corresponding uninfected ones ($p<0.05$). This is in good correlation with the weakening of the gluten network as a result of fungal proteolytic activity, as discussed in section 5.4.1. Furthermore, the strength of the dough in the week 0 sample correlates well with the initial level of infection. Meyer, Weipert and Mielke (1986) previously reported an inverse relationship between the level of *Fusarium* infection and dough strength. However, after 6 weeks no substantial differences between the three infection levels could be determined anymore. But, as already mentioned the proteolytic activity after 6 weeks was found to be equal in all the infected samples also. Nonetheless, compared to the natural and 0% infected samples, the infected ones produced a significantly weaker dough. These results correlate well with the determined loss in gluten network strength. It is also striking that the infected samples after 6 weeks did not get significantly weaker during proofing. This becomes evident by the consistent dough strength determined, independent from the duration of proofing. This is mainly because most of the gluten in the infected grains was already degraded and during proofing the dough could not get much weaker. A reduced resistance to extension due to the fungal infection was also reported by Nightingale et al (1999).

Overall, the impact of *F. culmorum* on the dough properties resulted in a reduced dough quality, ultimately leading to a decreased marketability. In particular from the technological point of view, a stickier and less elastic dough is more difficult to handle. Furthermore, the high amounts of free water and weakness of the dough would be expected to cause problems regarding the final product quality, such as high bake loss and poor crumb structure. In order to investigate this topic further, baking trials were performed using the dough samples discussed in this paragraph.

5.4.3 Bread characterisation

The wholegrain breads produced from the grain samples were analysed for the following quality parameters: bake loss, specific loaf volume, crumb lightness, crumb structure and physical crumb texture. This allowed essential information to be obtained in order to evaluate the fungal impact on the final product quality. Images of the breads produced from the natural, 5% infected and 20% infected samples, after 0 and 6 weeks of storage, are shown in Figure 9.

Figure 9: Images of natural and infected samples breads. A) natural week 0; B) natural week 6; C) 5 % infected week 0; D) 5 % infected week 6; E) 20 % infected week 0; F) 20 % infected week 6.

The natural sample after week 0 and 6 is shown in Figures 9A and 9B, respectively. Although the sample in 9B displays a more open crumb structure, overall it maintained a good bread making quality during storage. However, the breads obtained from the infected flours after storage were found to have a much wider and irregular crumb structure, indicating a more substantial quality deterioration due to the fungal infection. In particular, the 5% infected one shows significant differences between week 0 (9C) and 6 (9D). While at week 0, the crumb presented a closed and dense structure with pale colour, after 6 weeks it appeared to be very open with big holes and a much darker, brown colour. Furthermore, the shape of the breads after 6 weeks resembled the shape of the tins used, indicating a weak gluten structure. In contrast to this, the loaf in 9C had an oval shape, due to the strong gluten network retaining the produced gas and maintaining the original loaf shape. In addition, some of the slices in 9D indicate that the breads slightly collapsed, visible by the raised edges. Images 9E and 9F show the 20% infected sample after 0 and 6 weeks, respectively. These breads had an irregular shape even in week 0. Regarding the crumb structure and colour no significant differences to the uninfected ones are visible. In contrast, the stored samples led to breads with a dark

brown colour, huge holes and a very irregular loaf shape. The reason for this is the degradation of gluten and polysaccharides by fungal enzymes, such as proteases, amylases and xylanases (Schmidt et al., 2016) (Table 12). This breakdown weakened the dough so much, that it could not hold its shape and the expanding gas during baking. The release of reducing sugars and amino acids as enzymatic degradation products caused the dark crumb colour, due to Maillard reaction during baking (Mei et al., 2010).

Selected important bread quality parameters were quantified and the results are summarised in Table 15. Firstly, the bake loss was found to be significantly higher for the infected samples, compared to the healthy ones. This reduced the quality but also the marketability as bread is sold by weight. However, none of the samples revealed a substantial change in bake loss during storage ($p<0.05$). Furthermore, no noteworthy differences were found between the three initial infection levels. These findings propose that the bake loss increased due to the fungal infection but widely independent of the degree of infection. This correlates just partially with the results obtained for the gluten characterisation. One would expect the bake loss to be in direct correlation with the reduction in water binding capacity, which decreased with time of storage. However, the bake loss was found to be independent from the time of storage for all samples. That is primarily because wholegrain flour was used for baking. Thus, it is not exclusively the gluten that is binding water but also the polysaccharides, reducing the influence of the gluten. The infected samples provided more free water for the remaining starch, enhancing its gelatinisation and so partly reducing the bake loss further (Biliaderis, Izydorczyk and Rattan, 1995). Besides, the increased lipase activity due to the infection improves the starch gelatinisation by degrading starch lipids (Koehler and Wieser, 2013). The complex interactions of all these factors determine the final bake loss measured.

Table 15: Mean results of loaf and crumb characteristics with the corresponding standard deviations for the breads from the stored wheat samples[#].

sample / weeks of storage	bake loss [%]	volume [ml/g]	crumb lightness	crumb hardness day 0 [N]	no of cells / slice area	area of cells [%]	cell diameter [mm]
natural / 0	19.3 ± 0.6^{a}	1.9 ± 0.1^{a}	54.1 ± 2.2^{a}	28.5 ± 5.6^{a}	1.01 ± 0.13^{ac}	46.7 ± 1.3^{a}	1.2 ± 0.1^{a}
natural / 3	21.0 ± 1.5^{ab}	2.2 ± 0.2^{ab}	49.0 ± 1.8^{b}	14.1 ± 2.0^{bc}	0.65 ± 0.02	50.0 ± 0.4^{b}	1.9 ± 0.1^{bc}
natural / 6	19.4 ± 0.9^{ac}	1.6 ± 0.1^{c}	48.3 ± 1.9^{bc}	12.5 ± 2.1^{bcd}	0.57 ± 0.03^{bd}	51.8 ± 0.8^{cd}	2.1 ± 0.1^{bce}
0% infected / 0	20.6 ± 0.4^{bc}	1.7 ± 0.1^{c}	52.6 ± 2.2^{a}	21.6 ± 3.9^{a}	0.84 ± 0.04^{ac}	48.6 ± 0.4^{a}	1.5 ± 0.1^{d}
0% infected / 3	22.4 ± 2.0^{b}	2.2 ± 0.2^{ab}	49.0 ± 2.4^{b}	14.3 ± 2.5^{bc}	0.55 ± 0.03^{bd}	52.5 ± 0.9^{cd}	2.4 ± 0.2^{ce}
0% infected / 6	21.8 ± 1.8^{bc}	2.3 ± 0.1^{b}	48.3 ± 2.1^{bc}	19.3 ± 2.9^{c}	0.58 ± 0.03^{bd}	51.7 ± 0.9^{cd}	2.2 ± 0.3^{bcef}
5% infected / 0	25.5 ± 0.6^{d}	2.5 ± 0.0^{d}	54.4 ± 1.6^{a}	32.1 ± 7.3^{a}	1.03 ± 0.09^{a}	47.3 ± 0.6^{a}	1.3 ± 0.1^{ad}
5% infected / 3	24.9 ± 1.0^{d}	2.6 ± 0.1^{de}	47.7 ± 2.9^{bc}	10.2 ± 2.3^{bcd}	0.56 ± 0.05^{bd}	53.0 ± 1.1^{cd}	2.6 ± 0.4^{cef}
5% infected / 6	27.2 ± 1.3^{de}	2.9 ± 0.1^{f}	44.8 ± 2.1^{c}	10.1 ± 2.1^{bcd}	0.51 ± 0.03^{bd}	55.1 ± 1.6^{de}	3.3 ± 0.9^{f}
10% infected / 0	25.4 ± 0.6^{d}	2.7 ± 0.1^{ef}	53.2 ± 1.4^{a}	30.8 ± 6.8^{a}	0.97 ± 0.07^{a}	47.5 ± 0.8^{a}	1.3 ± 0.1^{ad}
10% infected / 3	23.2 ± 1.8^{bd}	2.4 ± 0.1^{bd}	47.4 ± 2.5^{bc}	8.9 ± 2.1^{d}	0.51 ± 0.02^{d}	54.8 ± 1.1^{de}	3.1 ± 0.4^{f}
10% infected / 6	25.5 ± 1.9^{de}	2.3 ± 0.1^{b}	44.1 ± 2.2^{c}	9.1 ± 3.0^{bcd}	0.47 ± 0.06^{d}	56.4 ± 1.8^{e}	3.7 ± 0.4^{f}
20% infected / 0	25.0 ± 1.0^{d}	2.9 ± 0.1^{f}	50.5 ± 2.1^{a}	15.9 ± 3.0^{b}	0.75 ± 0.02	50.3 ± 0.6^{b}	1.7 ± 0.1^{bd}
20% infected / 3	28.4 ± 1.1^{e}	2.9 ± 0.1^{f}	48.8 ± 2.4^{bc}	10.7 ± 3.6^{bd}	0.53 ± 0.03^{bd}	53.6 ± 1.3^{ode}	2.7 ± 0.5^{cef}
20% infected / 6	25.4 ± 0.9^{d}	2.8 ± 0.1^{ef}	44.3 ± 2.4^{c}	8.2 ± 1.3^{d}	0.49 ± 0.03^{d}	54.9 ± 1.0^{de}	3.1 ± 0.4^{f}

[#] Results shown are mean values ± standard deviation. Values in one column followed by the same lower case letter are not significantly different ($p<0.05$).

At the same time, the specific loaf volumes were found to increase due to the fungal infection. Although the volumes of all breads were significantly increasing due to the storage, the infected samples resulted in noteworthy higher volumes after 6 weeks, independent from the degree of initial infection. The increased volume is most likely due to the gluten proteolysis, leading to higher amounts of free water and a reduced network strength. This resulted in an increased gas pressure of the expanding water combined with a reduced ability of the gluten to restrain this expansion and maintain the shape. In addition, the higher bake loss in the infected samples reduced the loaf weight while the total volume remained constant, hence leading to a bigger specific volume. Furthermore, higher amounts of free sugars compared to the uninfected samples (Schmidt et al., 2016) were available for the yeast during proofing. In consequence, the gas production increased and led to a bigger rise of the dough. Nonetheless, this rise of the dough requires a certain gas holding capacity, provided through the improved gelatinisation of the remaining starch. Thus, the weakening of gluten and gelatinisation of starch created a balance, leading to the increased volume in the infected samples. Nightingale et al. (1999) reported decreasing bread volume due to fungal proteases when using the endosperm flour. This further supports the assumption that in the present study primarily starch and fibres are responsible for the water and gas holding in the dough.

To evaluate the fungal impact on the bread crumb characteristics, first the crumb lightness was determined. As the results in Table 15 show, there were no significant differences between the samples of week 0 ($p<0.05$). After 3 and 6 weeks, all five samples presented a significantly reduced lightness but the reduction in the three infected samples was notably bigger compared to the uninfected ones. Similar to the loaf characteristics, no substantial differences were found between the three initial infection levels. The darkening indicates higher amounts of free amino acids and reducing sugars, leading to a higher rate of Maillard reaction during baking and thus the darker crumb colour. This also correlates with the increased protease and amylase activities in the infected samples after the storage period (Schmidt et al., 2016) (chapter 4, Table 12). Likewise, the uninfected samples showed lower but still quantifiable enzymatic activities, explaining the slight darkening of the breads obtained from the stored grains. In addition, the proteolytic activities and free sugar contents of the infected samples were found to be widely independent from the level of infection (Schmidt et al., 2016). Consequently, the rate of Maillard reaction was also independent from the contamination level. This increase in Maillard reaction due to *F. culmorum* can cause a further loss in bread quality. As shown by Bråthen

and Knutsen (2005), increased levels of free amino acids (e.g. asparagine) in a bread system are likely to increase the formation of acrylamide, a neurotoxic by-product of the Maillard reaction (Mei et al., 2010).

The crumb structure was analysed using the C-cell bread imaging system. The results of selected parameters are given in Table 15. In week 0, no significant difference between the disinfected and natural sample regarding the number of cells per slice area was evident. In contrast, the infected ones presented a decreasing number of cells with increasing level of infection. During storage all five samples revealed a substantial decline of cells per slice area ($p<0.05$). However, the reduction in the infected samples, due to the fungus, is notably bigger than in the controls. In addition, after 6 weeks the number of cells per slice on the infected breads was found to be independent of the initial level of infection. In order to fully understand this parameter, it has to be evaluated in connection with the average area of cells and the average cell diameter (Table 15). While the number of cells was found to decrease in the uninfected samples, the percentile area of cells increased significantly over time of storage. Consequently, the cells on these slices got fewer but bigger. Likewise, the area of cells per slice in the infected samples was found to decrease substantially during storage, while the percentile area of cells increased. Nonetheless, the percentile area of cells after storage was noteworthy bigger, compared to the uninfected flours. Likewise, the cell diameters showed a notable increase due to the fungus during the 6 weeks. While no significant differences between the cell diameters of the five samples were found in week 0, after 6 weeks the infected samples led to substantially bigger cells than the uninfected ones.

Since a fine structure with many small cells is desirable for most types of commercial bread, the *F. culmorum* infection results in reduced bread quality and marketability. In particular, the increased cell-size of the infected samples equates to a substantial quality loss, which is also visible in the figures 9D and 9F, respectively. These breads were found to have a visibly more open and irregular crumb structure. This correlates well to the increased activity of hydrolytic enzymes and the weakening of the gluten network, as discussed above. Due to the higher moisture content and yeast activity the infected dough contained more gas, expanding while baking and the weakened gluten was unable to retain the gas in small cells. Furthermore, the degradation of polysaccharides by fungal enzymes is known to cause poor crumb structure (Wang et al., 2005). Similar to other quality

parameters, the damage after 6 weeks was found to be independent from the degree of initial infection.

Finally, the physical crumb texture of the breads was analysed by TPA and the results are summarised in Table 15. The results reveal that no significant differences regarding the crumb hardness were found between the samples of week 0, except for the 20% infected sample which had a significantly softer crumb than the other breads. The storage period resulted in a substantially decreased crumb hardness for all samples ($p<0.05$), excluding the disinfected one. However, in correlation to other quality parameters, the decrease was noteworthy bigger in the infected samples, compared to the uninfected ones. In addition, the softening of the infected breads appeared widely independent from the level of initial infection. This is yet another consequence of the improved starch gelatinisation in the infected samples, as discussed above. Although a soft crumb is generally desired, the crumbs of the infected samples were too soft for easy handling, such as for buttering a slice of the bread. Consequently, the softening of the crumb has to be considered as another point representing reduced quality and marketability due to the fungal infection of the grains.

5.5 Conclusions

In conclusion, this study clearly demonstrates that using wheat grains, infected with *F. culmorum*, has a major impact on the final bread quality. During the six weeks of storage the fungus substantially reduced both, gluten quantity and network strength. This resulted in a substantial loss in dough and bread quality, primarily expressed by the high dough stickiness, bake loss, very open crumb structure and a softer bread crumb. Furthermore, the quality deterioration was found in similar extent for all three infection levels (5%, 10% and 20%) and thus was concluded to be widely independent from the initial degree of contamination. This shows how even minimal field contamination can, due to poor storage practices, lead to substantial post-harvest economic losses. Furthermore, it was shown previously that the infected samples used for baking contained substantial amounts of mycotoxins (Schmidt et al., 2016). Hence, in combination with the quality deterioration, the breads are also likely to impose a potential consumer health hazard. Although the model system investigated here applied a relatively high initial fungal contamination and very poor storage conditions, the time of storage (6 weeks) was very short. Therefore, it is likely that under better conditions but with a more realistic storage time similar results would be achieved. Thus, the results illustrate the importance of good storage practices and grain decontamination strategies to prevent post-harvest fungal spoilage, to reduce economic losses and ensure a consistently high product quality.

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Chapter 6: Fundamental study on the improvement of the antifungal activity of Lactobacillus reuteri R29 through increased production of phenyllactic acid and reuterin

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6.1 Abstract

Lactic acid bacteria have shown great potential as bio-preservative agents to maintain high food quality and safety. The strain *Lactobacillus reuteri* R29 is reported to have a broad spectrum of antifungal activity and suitability for application in food systems. Its antifungal activity is predominantly based on 3-phenyllactic acid (PLA). Furthermore, it is potentially a producer of reuterin, a potent antimicrobial agent. This study focused on increasing the antifungal activity *in vitro* by supplementation of the growth medium with phenylalanine and glycerol to increase the yield of PLA and reuterin, respectively. For PLA, the addition of 1.5% phenylalanine (w/v) to MRS, resulted in significantly increased accumulation of PLA and antifungal performance against *Fusarium culmorum*. Supplementation of MRS with 500 mM glycerol combined with a reduced glucose content (1.5%) showed the highest reuterin accumulation combined with fungal inhibition. To investigate the antifungal performance in situ, these cell-free supernatants (cfs) were applied in a bread system. The application of PLA-enriched cfs resulted in significantly extended (4 days) microbial shelf life compared to the control. The reuterin-enriched medium did not lead to significant shelf life extension. In conclusion, *Lactobacillus reuteri* R29 and its PLA-enriched cfs were found to be very promising alternatives for food bio-preservation.

6.2 Introduction

Lactic acid bacteria (LAB) are key microorganisms used for the production of various bakery products. They substantially improve nutritional and technological properties, as well as the flavour. However, current research is predominantly focused on their antimicrobial properties and potential applications as food bio-preservatives (Dalié, Deschamps and Richard-Forget, 2010). The use of such LAB has the potential to replace chemical preservatives, such as calcium propionate, which would allow a “clean label” and hence lead to higher consumer acceptance (Pawlowska et al., 2012). In recent years, several strains of different genera have been found to express antimicrobial activity *in vitro* and *in situ* against food spoilage fungi (Axel et al., 2016; Crowley, Mahony and Van Sinderen, 2013).

The antifungal activity of LAB is predominantly based on complex synergistic effects between several compounds. The production of these compounds is highly dependent on the strain and growth substrate. The exact interactions between the bacterial metabolites which create this synergistic effect are not yet fully understood. However, several metabolites that contribute to the antifungal activity have been identified and characterised. These metabolites, amongst others, include carboxylic acids (Ryan, Dal Bello and Arendt, 2008) and 3-hydroxypropionaldehyde (3-HPA) also known as reuterin (Lüthi-Peng, Dileme and Puhan, 2002).

Recently, the production and activity of 3-phenyllactic acid (PLA) has received great attention (Mu et al., 2012). This broad-spectrum antimicrobial compound originates in LAB fermented products from the catabolism of phenylalanine (Phe). Thereby, the Phe first undergoes a transamination by transferring the amino group onto a keto-acid acceptor. The synthesised phenylpyruvic acid is then reduced to PLA by a dehydrogenase (Vermeulen, Gánzle and Vogel, 2006). The antifungal activity of PLA is also dependent on a synergistic mechanism with other bacterial metabolites. Nevertheless, if the metabolic pathway yielding PLA is promoted, the antifungal activity is likely to increase. This could expand the field of application for LAB as bio-preservatives.

A very promising antimicrobial agent is the multicomponent system called reuterin, which results from the conversion of glycerol to 3-HPA (Engels et al., 2016). The antimicrobial activity of reuterin is, according to Schaefer et al. (2010), mainly based on its reactivity with free thiol groups of proteins, inducing oxidative stress in the target cells. It is understood that 3-HPA is released upon enzymatic dehydration of glycerol. Recent research conducted by Engels et al. (2016) suggests a rapid *in situ*

conversion to acrolein, which is mainly responsible for the antimicrobial activity of the reuterin system. However, if sufficient amounts of carbohydrates, in particular glucose, are available, 3-HPA is further reduced to 1,3-propanediol which has no antimicrobial activity (Gänzle, 2015). Unfortunately, the research on reuterin so far has mainly focused on its industrial use as precursor for the synthesis of acrolein, not on food applications. To-date, there are only two studies conducted by Gomez-Torres et al. (2014) and Ortiz-Rivera et al. (2017) available, investigating a possible application of reuterin as an antibacterial agent in a food system. As Gänzle (2015) observed, the applicability of reuterin, particularly in heat treated food systems, remains unclear. Further research is required, in particular to elucidate the effect of glycerol on bacterial growth and reuterin production.

A strain previously reported as demonstrating strong antifungal activity and suitability for food applications is *Lactobacillus reuteri* R29 (Axel et al., 2016; Oliveira et al., 2015). Oliveira et al. (2015) demonstrated that the cell-free supernatant (cfs) of medium fermented for 48 h with this strain showed antifungal activity against *F. culmorum*, with PLA being the predominant antimicrobial compound produced. As it belongs to the species *L. reuteri*, the strain is likely to be a reuterin producer. To-date there have been no studies carried out investigating the ability of *L. reuteri* R29 to produce and accumulate reuterin. Thus, the enhancement of the production and accumulation of antifungal compounds, namely reuterin and PLA, in the bacterial cfs represents a promising opportunity in particular, if this antifungal activity is applicable in food systems.

Therefore, the aim of this study was to increase the *in vitro* antifungal activity of *L. reuteri* R29 cfs. Different variations of the fermentation medium composition were investigated to increase the accumulation of PLA and reuterin. *F. culmorum*, *A. niger* and *P. expansum* were used as indicator moulds to determine the antifungal activity *in vitro*. Secondly, characterisations regarding the heat stability of the most efficient cfs were evaluated. Finally, antifungal cfs delivering the best results were applied in the bread system to obtain information regarding suitability for application in a food matrix. This study provides important information regarding the potential of *L. reuteri* R29 cfs as antifungal agent and its application as a bio-preservative agent, in the bread system.

6.3 Materials and Methods

6.3.1 Materials

Ingredients for microbiological media, chemicals and analytical standards used in this study were at least analytical grade and obtained from Sigma-Aldrich (Dublin, Ireland), unless otherwise stated. The analytical standards had a purity of ≥95%.

Wheat flour (bakers flour, Odlums, Dublin, Ireland), dry yeast (Puratos, Groot-Bijgaarden, Belgium), salt (Glacia British Salt Limited, Cheshire, UK), commercially available sunflower oil, sodium stearoyl lactylate (SSL) (Danisco, Denmark) and ascorbic acid (Storefast solutions, UK) were used for the baking trials.

6.3.2 Cultures, media and growth conditions

Fusarium culmorum TMW4.2043, *Aspergillus niger* FST4.21, *Penicillium expansum* FST 4.22 originated from the culture collection of School of Food and Nutritional Sciences, University College Cork (Cork, Ireland). Fungal spore suspensions were prepared according to the method described by Oliveira et al. (2012).

Lactobacillus reuteri R29, originally isolated from human intestine, was obtained from the UCC culture collection (School of Food and Nutritional Sciences, University College Cork, Cork, Ireland). For long term storage, the culture was kept in commercial de Man Rogosa and Sharpe (MRS) broth containing 40% glycerol at -80°C. The culture was routinely refreshed on MRS agar plates (Fulka Chemie AG, Buchs, Switzerland) under anaerobic conditions at 37°C for 48 h.

6.3.3 Production of LAB cell-free supernatants

For production of 1-step fermented LAB cfs, a single colony, grown as described in 6.3.2, was inoculated in 5 mL MRS broth for 24 h at 37°C. Subsequently, a 1% inoculum into fresh fermentation medium (Table 16 and 17) was prepared and incubated at 37°C for the designated time. After fermentation, cell cultures were centrifuged (5,000 g, 15 min, 4°C) and the supernatant was sterile filtered through a 0.22 µm filter. The cfs obtained was stored at -20°C until further use.

The 2-step fermentation process was based on the method described by Doleyres et al. (2005) with the following modifications. After the first fermentation step, cells were washed and resuspended in sterile distilled water, containing 400 mM glycerol, at a final concentration of 10^9 CFU/mL. The suspension was incubated for 2 h at 30°C and the cfs collected as described above.

Table 16: Concentration (ppm) of phenyllactic acid (PLA) and antifungal activity over 120 h (% of growth inhibition) of cell-free supernatants of *Lactobacillus reuteri* R29 grown in MRS for 0, 24 or 48 h at 37°C in presence of various amounts of phenylalanine[#].

Medium	fermentation time [h]	PLA [ppm]	antifungal activity after 120 hours of incubation [% growth inhibition against 10^7 spores/mL]		
			P. expansum	*A. niger*	*F. culmorum*
MRS	0	-[a]	-[a]	-[a]	-[a]
	24	38.4±1.3[b]	1.2±0.2[b]	0.2±0.1[a]	1.5±0.2
	48	85.3 ±1.1	3.1±0.4[c]	2.2±0.4[b]	18.4±1.4[c]
MRS + 0.5% Phe	0	-[a]	-[a]	-[a]	-[a]
	24	41.6±1.8[b]	1.4±0.1[b]	3.2±0.5[bc]	3.4±2.2[bc]
	48	103.2±2.3	3.6±0.3[c]	3.7±1.4[c]	14.1±2.8[c]
MRS + 1.0% Phe	0	-[a]	-[a]	-[a]	-[a]
	24	57.5±1.5	2.2±0.3	2.8±1.4[b]	6.1±2.5[c]
	48	237.1±1.4	16.5±1.3	24.7±3.7	50.3±3.2
MRS + 1.5% Phe	0	-[a]	-[a]	-[a]	-[a]
	24	116.4±1.3[c]	8.3±0.8	11.0±0.5	25.4±3.1[d]
	48	361.2±2.4[d]	32.7±0.2[d]	63.5±2.1[d]	84.1±3.2[e]
MRS + 2.0% Phe	0	-[a]	-[a]	-[a]	-[a]
	24	113,2±1.8[c]	14.1±2.5	16.0±1.1	27.1±2.1[d]
	48	363.8±2.0[d]	33.1±0.6[d]	62.9±1.6[d]	83.5±2.2[e]

[#] Results shown are mean values ± confidence interval. Values in one column followed by the same lower case letter are not significantly different, values without letter of significance are significantly different from all other values in the same column ($p<0.05$).

Table 17: Concentration of reuterin (mM) and antifungal activity (% growth inhibition) against *Penicillium expansum*, *Aspergillus niger* and *Fusarium culmorum* of cell-free supernatants of *Lactobacillus reuteri* R29 grown in various MRS formulations, containing 1.0 – 2.0% of glucose and 250 – 1000 mM of glycerol, for 0, 24 and 48 h (1-step fermentation process) and for the 2-step fermentation process (2 h in water/glycerol)[#].

Medium fermented by *L. reuteri* R29	Fermentation time [h]	reuterin [mM]	antifungal activity [% growth inhibition against 10^7 *F. culmorum* spores/mL]		
			P. expansum	*A. niger*	*F. culmorum*
Control (MRS)	0	-[a]	-[a]	-[a]	-[a]
	24	-[a]	0.2±0.1	0.5±0.1[b]	0.3±0.2[a]
	48	-[a]	3.1±0.7[b]	8.2±0.4[c]	18.4±1.4[b]
MRS + 250mM glycerol; 1.0% glucose	0	-[a]	-[a]	-[a]	-[a]
	24	12.5±0.1	18.3±1.7[d]	22.5±0.2[d]	39.5±2.7
	48	-[a]	2.1±0.4[b]	8.9±0.6[c]	8.4±1.2[c]
MRS + 250mM glycerol; 1.5% glucose	0	-[a]	-[a]	-[a]	-[a]
	24	20.5±1.1[b]	17.0±1.2[d]	24.5±1.3[d]	55.8±3.1[d]
	48	0.4±0.1	3.5±0.4[b]	3.5±0.6[e]	8.7±2.1[c]
MRS + 250mM glycerol; 2% glucose	0	-[a]	-[a]	-[a]	-[a]
	24	0.9±0.2	4.5±0.2[e]	11.1±1.9[f]	15.7±2.6[e]
	48	-[a]	2.2±0.3[b]	1.8±0.5	14.6±0.1[e]
MRS + 500mM glycerol; 1.0% glucose	0	-[a]	-[a]	-[a]	-[a]
	24	18.6±0.3	35.6±1.6	21.8±0.1[d]	58.7±2.3[d]
	48	0.2±0.1[a]	2.4±0.2[b]	3.3±0.3[e]	8.8±0.6[c]
MRS + 500mM glycerol; 1.5% glucose	0	-[a]	-[a]	-[a]	-[a]
	24	27.5±0.6[c]	55.0±2.1	64.9±1.8	69.1±0.4
	48	1.2±0.3[d]	3.1±1.1[b]	4.5±0.4[e]	17.9±2.1[be]
MRS + 500mM glycerol; 2% glucose	0	-[a]	-[a]	-[a]	-[a]
	24	1.3±0.2[d]	8.4±0.5	7.6±0.9	19.1±1.5[e]
	48	-[a]	2.8±0.7[b]	2.2±0.5[g]	16.3±0.4[e]
MRS + 1000mM glycerol; 1.0% glucose	0	-[a]	-[a]	-[a]	-[a]
	24	21.5±1.4[b]	14.7±1.0	13.4±0.2[f]	57.1±0.5[d]
	48	0.3±0.1[a]	-[a]	0.2±0.1[b]	-[a]

MRS + 1000mM glycerol; 1.5% glucose	0	-[a]	-[a]	-[a]	-[a]
	24	26.6±0.3[c]	26.5±1.1	28.5±1.5[d]	52.9±2.4[d]
	48	0.5±0.4[a]	1.0±0.1[c]	1.4±0.2[h]	18.6±1.9[be]
MRS + 1000mM glycerol; 2% glucose	0	-[a]	-[a]	-[a]	-[a]
	24	1.4±0.1[d]	3.6±0.7[e]	2.6±0.1[g]	18.1±1.6[be]
	48	-[a]	0.8±0.2[c]	1.2±0.2[h]	17.5±1.2[be]

Medium (2-step fermentation process)	Fermentation time [h]	reuterin [mM]	antifungal activity [% growth inhibition against 10^7 *F. culmorum* spores/mL]		
			P. expansum	*A. niger*	*F. culmorum*
distilled water + 400mM glycerol	0 (control)	-[a]	-[a]	-[a]	-[a]
	2 (sample)	93.1±2.0	100±0	100±0	100±0

[#] **Results shown are mean values ± confidence interval. Values in one column followed by the same lower case letter are not significantly different, values without letter of significance are significantly different from all other values in the same column ($p<0.05$).**

6.3.4 Evaluation of *in vitro* antifungal activity and determination of minimal inhibitory concentration (MIC_{90})

Pre-trials were carried out to determine the ideal fungal spore concentration to investigate the increase of antifungal performance in the respective cell-free supernatants. Thereby, a concentration of 10^6 spores/mL as applied in the assay described were chosen for the subsequent experiments (data not shown). To determine the *in vitro* antifungal activity, 1 mL of *Fusarium culmorum*, *Aspergillus niger* or *Penicillium expansum* spore solution (containing approximately 10^6 spores) was transferred into a 2 mL microcentrifuge tube and centrifuged at 3,000 g (10 min, 4°C). The supernatant was discarded and the spore pellet resuspended in 1.0 mL of diluted LAB cfs (10%, v/v, in unfermented MRS). Subsequently, 200 µL aliquots were pipetted into a 96-well microplate (Sarstedt AG and Co, Nurembrecht, Germany). To control for the effect of condensation, each supernatant was also inoculated with 1 mL of sterile synthetic nutrient-poor bouillon, instead of fungal spore solution (blank). The microplate was sealed with an optically clear seal for Q-PCR (Thermo Scientific, Waltham, USA) and incubated in a Multiskan FC microplate-reader (Thermo Scientific, Waltham, USA) for 5 days at 25°C. The optical density was recorded at 620 nm (OD620) every 2 h, with agitation in 4 s

intervals. Values of each cfs inoculated with spore solution were corrected by the respective mediums blank value. Antifungal activity was calculated as the percentage of OD reduction compared to the respective 0 h fermented medium and expressed as “percentage of growth inhibition”.

To determine the MIC_{90} of PLA the substance was added to unfermented MRS-broth to a final concentration of 20,000 ppm and the pH adjusted to match the fermented medium. Due to the lack of a commercially available standard for reuterin, a serial dilution of the 2-step fermented supernatant was used to determine the MIC_{90}. The MIC_{90} was defined as the minimal concentration of the respective antifungal compound required to achieve at least 90% reduction of fungal growth after 120 h under the assay conditions compared to a control medium without the antifungal compound.

6.3.5 Determination of antifungal fermentation products

6.3.5.1 Determination of carboxylic antifungal compounds

An Agilent 1260 high-performance liquid chromatography system equipped with an ultraviolet-diode array detector (UV/DAD) was used to quantify antifungal carboxylic compounds. Standard calibration curves for lactic and acetic acid (2 – 32 mM), as well as 13 phenolic compounds (catechol, hydroxyphenyllactic acid, 4-hydroxybenzoic acid, hydrocaffeic acid, caffeic acid, phloretic acid, hydroferulic acid, p-coumaric acid, ferulic acid, benzoic acid, salicylic acid, hydrocinnamic acid, methylcinnamic acid, vanillic acid and 3-phenyllactic acid) were prepared, using five different concentrations in duplicate. Calibration curves showed good linearity with correlation coefficients of ≥0.999 for all compounds. Extraction of antifungal organic acids (acetic acid and lactic acid) was carried out as described by Axel et al. (2016). For separation of the acids a Hi-Plex H Column (300×7.7 mm, 8 µm; Agilent, Cork, Ireland), equipped with a guard column (50×7.7 mm, 8 µm; Agilent, Cork, Ireland) was used. Setting the UV/DAD at 210 nm, lactic and acetic acids in the cfs were determined after elution with 0.004 M sulphuric acid at 65 °C with a flow of 0.5 mL/min. Injection volume used was 20 µL.

Antifungal phenolic compounds were extracted, using a QuEChERS approach, as described by Peyer et al. (2016). Separation was carried out using a Gemini C_{18}

column (150 x 2.0 mm, 5 µm; Phenomenex, Macclesfield, UK), equipped with a guard column (AF0-8497; Phenomenex, Macclesfield, UK). The mobile phase consisted of A) H_2O with 0.1% formic acid and B) acetonitril with 0.1% formic acid. To ensure separation from other compounds, a gradient flow was performed (0 – 5 min – 90% A, 30 min – 80% A, 35 min – 60% A, 45 min – 5% A, 45 – 70 min – 90% A), using a flow rate of 0.2 mL/min at a temperature of 30°C. The volume injected was 10 µL and detection carried out at a wavelength of 210 nm.

If necessary, samples were diluted prior to extraction to ensure peak areas were within the calibration range. Identity of the peaks evaluated in the cfs samples was confirmed as PLA by comparison of the UV/VIS spectrum with the standard solution. The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the signal/ noise (s/n) ratio. The LOD was set for s/n of 3:1 and the LOQ was set for s/n of 10:1. Recovery rates of the phenolic compounds were done with 3.0 mg/L of each analyte added in MRS and in chemically acidified MRS (pH 3 with 0.1 N HCl), and ranged from 89.1% (hydrocinnamic acid) to 118.1% (vanillic acid) of the total spiked amount.

6.3.5.2 Colorimetric assay for reuterin quantification

The concentration of reuterin in the cfs of the 1- and 2-step fermentations was determined using the colorimetric assay described by Lüthi-Peng, Dileme and Puhan (2002). Calibration was carried out, using acrolein as standard in distilled water (0.1 – 6.0 mM) and MRS broth (0.2 – 10 mM). Both calibration curves showed good linearity with correlation coefficients of >0.999.

6.3.6 Heat stability of antifungal fermentation products

Heat stability of the antifungal supernatants was determined by subjecting the cfs to 100°C for 1 h. Subsequently, samples were cooled immediately and kept in the fridge until further use. Control samples (not heat treated) were kept in the fridge at all times. Analysis of *in vitro* antifungal activity and quantification of antifungal compounds was carried out as described in sections 6.3.4 and 6.3.5, respectively but only *F. culmorum* was used as indicator mould.

6.3.7 Application of cfs in bread system and shelf-life analysis

For baking trials, water in the recipe was replaced by the respective LAB cell-free supernatant. For preparation of breads 63% cfs, 3% yeast, 3% oil, 2% salt, 0.5% SSL and 0.1% ascorbic acid (each based on flour weight) were used. Reactivation of the yeast and dough preparation was carried out according to Heitmann, Zannini and Arendt (2015). The dough was then divided into 65 g portions, placed in non-stick baking tins (dimensions: top inside, 50 mm x 90 mm; bottom outside, 45 mm x 85 mm; inside depth, 30 mm; Sasa UK, Enfield Middlesex, UK) and proofed for 75 minutes (30°C, 85% RH). Doughs were transferred into the oven (MIWE, condo, Arnstein, Germany) and baked for 14 minutes at 210°C (top and bottom). Following baking the bread loaves were removed from the tins immediately and cooled to room temperature for at least 120 min before further use.

The microbial shelf life test was carried out according to Heitmann, Zannini and Arendt (2015). The amount of fungal spoilage was visually recorded over 13 days for each slice, using a calliper to determine the fungal colony size. Based on the percentage of the total surface area of the slice where fungal outgrowth occurred, each slice was categorised every day as A) mould-free, B) <10% covered with mould, C) 11 – 25% covered with mould, D) 26 – 50% covered with mould or E) >50% covered with mould.

6.3.8 Statistical analysis

Baking trials and all analyses were carried out from three independent fermentations, analysing each sample in duplicate. Statistical analysis was performed using Minitab 17 software. Data points were checked for outliers (Grubb's test) and evaluation of significant differences was performed using one-way analysis of variances (ANOVA). All differences were considered significant at $P < 0.05$. Where F-values were significant, pairwise comparisons were carried out with the help of Tuckey Post Hoc test to describe the statistical significance between the different fermentation media and times.

6.4 Results and Discussion

6.4.1 Effect of phenylalanine on antifungal activity and production of phenyllactic acid

Fungal isolates of *Fusarium culmorum*, *Aspergillus niger* and *Penicillium expansum* were used as indicator moulds to evaluate the impact of Phe supplementation on the antifungal activity *in vitro* and PLA production. The results obtained are summarised in Table 16. The sole addition of up to 2% Phe (w/v) to MRS broth had no noteworthy effect on the fungal growth in the unfermented medium (0 h fermentation). The PLA quantities for all 0 h fermented samples were below the limit of detection. Thus, all media used for inoculation of the different fungal spores were generally suitable for their growth and any inhibition obtained in the fermented samples was due to the antifungal metabolites produced by *L. reuteri* R29. Concentrations of antifungal carboxylic and phenolic acids were determined for all samples analysed. However, no significant differences ($p < 0.05$), apart from the PLA concentration, were found between any of the samples fermented for equal amounts of time (data not shown).

After 24 h of fermentation, all samples were found to contain substantial amounts of PLA. In addition, the PLA quantities were in direct correlation to the amount of Phe initially added. This confirms the findings of Rodríguez et al. (2012), that more PLA is produced when more Phe is available during fermentation. The PLA level of the unsupplemented MRS at this point (24 h) was 38.4±1.3 ppm. When challenged with the fungal spores (10^7 spores/mL) the antifungal activity of the cfs was relatively poor. After 120 h no noteworthy inhibition was detected against either of the three fungal isolates. The cfs obtained through fermentation of MRS+1.5% Phe and MRS+2% Phe resulted after 120 h of incubation in significantly ($p < 0.05$) better inhibition of fungal spore germination, compared to cfs produced from unsupplemented MRS. But, it needs to be mentioned that the supernatants presented stronger inhibition against *F. culmorum* than against *A. niger* and *P. expansum*. Hence, it can be concluded that *F. culmorum* is the most sensitive of the three fungal species towards PLA. Similarly, the concentration of PLA in the cfs was found to be 3 times higher compared to the control. Addition of 0.5% or 1.0% Phe was much less efficient in terms of PLA accumulation and antifungal activity.

Finally, after 48 h of fermentation, all cfs showed the best spore germination inhibition for the respective medium. The fermentation of unsupplemented MRS

resulted in limited inhibition of fungal spore germination over 120 h of incubation. Compared to the unfermented medium, spore inhibition rates of 18.4±1.4%, 2.2±0.4% and 3.1±0.4% were found against *F. culmorum*, *A. niger* and *P. expansum*, respectively. In contrast, the addition of 1.0% Phe to the medium led to substantially improved antifungal activity (60.3±3.2%) over 120 h when challenged against *F. culmorum*. The most efficient medium composition, in terms of antifungal performance, was the addition of 1.5% Phe. This cfs resulted in 84.1±3.2% inhibition of spore germination against *F. culmorum* after 120 h. When challenged against the other fungal genera, spore germination inhibition was found to be less substantial, yet significantly increased compared to the unsupplemented medium. In contrast, the supplementation with higher amounts of Phe did not further improve the antifungal performance. The addition of less than 1.0% Phe showed no positive effect on the antifungal performance. In general, the quantities of PLA determined for each cfs correlate well with its respective antifungal activity. Thus, up to the addition of 1.5% Phe, the amounts of PLA increased in direct correlation with the level of Phe initially added. However, no further increase could be achieved by supplementation with higher quantities of Phe. Furthermore, the concentration of PLA after 48 h of fermentation was always the highest for the respective medium, compared with shorter fermentation times. This also correlates with the findings of Oliveira et al. (2015) who concluded that PLA is produced by resting cells. The reason behind is the promotion of amino acid metabolism under nitrogen poor conditions (Hazelwood et al., 2008), which yields the antifungal carboxylic acids, like PLA. Thus, after consuming the majority of nitrogen during the initial growth phase, the resting bacteria have to survive in a relatively nitrogen poor environment. This leads to increased catabolism of Phe and hence the high release of PLA.

Furthermore, it was evident that the antifungal activity of cfs obtained from *L. reuteri* R29 was clearly related to the amounts of PLA accumulated in the medium. For all supernatants tested, a significant increase in PLA yield also led to improved antifungal performance. These results are in correlation with previous findings of Oliveira et al. (2015), who reported the antimicrobial activity of PLA and its role as lead antifungal substance produced by *Lactobacillus reuteri* R29. Conversely, Axel et al. (2016) found that it is not necessarily the strain with the highest production of antifungal compounds that shows the best antifungal activity, demonstrating the key role of the type of the antifungal compounds and their synergistic effects. Nevertheless, the increased accumulation of antifungal active compounds by *L. reuteri* R29 improves the overall antifungal performance of this particular strain

(Valerio et al., 2016). Thus, the increased accumulation of PLA, as the lead antifungal compound of *L. reuteri* R29, led to improved antifungal activity of the cfs. This clearly demonstrates the great potential of microbial produced PLA as an antifungal substance.

It has to be mentioned however, that MIC_{90} values of synthetic PLA in acidified MRS against all three fungi tested are more than 10 times higher compared to the amounts detected in the bacterial supernatants showing fungal inhibition. MIC_{90} values determined are 6,000 ppm against *F. culmorum* and 15,000 ppm against *A. niger* and *P. expansum* (data not shown). Considering the high concentration of 10^7 fungal spores/mL these values are in good correlation with the findings of Lavermicocca, Valerio and Visconti (2003). After all, these findings highlight that PLA, although being of major importance for antifungal performance, is not the only component responsible. Instead the synergy of PLA with other bacterial metabolites resulting in antifungal performance is evident.

In conclusion, the addition of Phe to the bacterial growth medium resulted in a supernatant which exhibited improved antifungal activity *in vitro* against fungal spores belonging to the genera of *Fusarium*, *Aspergillus* and *Penicillium*. This effect was clearly related to the higher PLA accumulation during LAB fermentation. The capacity to transfer this functionality to a food system remains unclear and will be investigated further in sections 6.4.3 and 6.4.4. This would allow the exploitation of the antifungal nature of LAB, particularly in systems that are unsuitable for direct bacterial fermentation. Thus, these results highlight novel and promising perspectives for the use of LAB as bio-preservatives.

As the 48 h fermentation of MRS broth supplemented with 1.5% phenylalanine was found to be the best antifungal medium, it was carried forward for further investigation, as discussed in sections 6.4.3 and 6.4.4. The medium and supernatant will henceforth be referred to as PMRS and Pcfs, respectively.

6.4.2 Effect of glycerol and sugar content on antifungal activity and reuterin production

Another promising antimicrobial compound with possible application in food systems and produced by certain strains of *Lactobacillus reuteri*, is the multicomponent system reuterin, with 3-hydroxy propionaldehyde (3-HPA) being the main active component. However, to-date no study has demonstrated the ability of *Lactobacillus*

reuteri R29 to produce reuterin via the bioconversion of glycerol. Various combinations of glycerol and glucose in the growth medium were tested, in order to achieve the highest possible reuterin yield and the best antifungal activity *in vitro* against *F. culmorum*. Results for the different cfs, fermented for 0, 24 and 48 h, respectively are summarised in Table 17.

Firstly, the results show that all the unfermented media (0 h fermentation) provide a suitable substrate for spore germination of *F. culmorum*. The growth curve obtained for each respective medium was used as reference, in order to evaluate the antifungal activity of the respective fermentation cfs. Furthermore, concentrations of antifungal carboxylic and phenolic acids were determined for all samples, showing no substantial differences between any of the 24 and 48 h fermented samples were found.

After 24 h of fermentation, the cfs obtained from unmodified MRS contained no detectable amounts of reuterin, as MRS broth contains no glycerol. However, MRS supplemented with 250 – 1000 mM glycerol and a glucose content of 1.0 – 1.5% led to cfs containing various concentrations of reuterin. Values determined ranged between 12.5±0.1 mM (250 mM glycerol, 1.0% glucose) and 27.5±0.6 mM (500 mM glycerol, 1.5% glucose). Any further increase of glucose in the medium resulted in significantly lower reuterin concentrations, as the data for the media containing 2% glucose (Table 17) demonstrate. This suggests that 1.5% glucose in the initial medium is the ideal amount required for *L. reuteri* R29 to accumulate reuterin. At this level of glucose, the bacteria are able to grow to high cell density in the medium, resulting in a high glycerol conversion to reuterin. However, this concentration is low enough to avoid expression of the 3-HPA reductase responsible for the reductive effect on antifungal activity by removing reuterin from the system through its conversion to 1,3-propanediol. Comparison of reuterin contents for media containing similar amounts of glycerol but varying glucose contents suggests that in presence of sufficient amounts of glucose the 3-HPA reductase is expressed already during the first 24 h of fermentation, preventing the accumulation of reuterin. The ideal amount of added glycerol was found to be 500 mM, as it resulted in the highest reuterin concentrations for each respective level of glucose in the medium. The amount of reuterin produced by *L. reuteri* R29 correlates well with the *in vitro* antifungal activity against fungal spores of *F. culmorum*, *A. niger* and *P. expansum*. As mentioned in section 6.4.1 the cfs obtained from normal MRS broth showed, due to the high spore concentration, very low levels of inhibition against the three fungi after 120 h of incubation. In contrast,

the supernatants containing reuterin presented substantially better spore germination inhibition, demonstrating the antifungal potential of reuterin. The cfs with the highest reuterin concentration (500 mM glycerol, 1.5% glucose) showed total inhibition of *F. culmorum* against 10^7 spores/mL over 96 h of incubation (data not shown), and, after 120 h still had 69±2% fungal inhibition. Similarly to the Pcfs, inhibition of *Aspergillus* and *Penicillium* spores was found to be less substantial but still significant compared to the control (unsupplemented MRS). In conclusion, it is evident from these results that a higher reuterin concentration results in enhanced antifungal performance of the cfs.

In contrast, after 48 h of fermentation, the amounts of reuterin and the antifungal performance for each supernatant decreased substantially, compared to the respective 24 h fermented medium. This may be due to the extremely high reactivity of reuterin. As MRS broth is a complex medium, it provides plenty of potential reaction opportunities for reuterin. Furthermore, if the fermentation time is too long the reuterin acts as a self-inhibiting antimicrobial compound against the LAB in the medium (Lüthi-Peng, Dileme and Puhan, 2002). Although *L. reuteri* R29 was shown to be able to produce and accumulate reuterin, exact control of the fermentation parameters, such as time and medium composition is crucial. As a consequence, cfs obtained after 48 h of fermentation showed no significant difference in antifungal performance compared to the unsupplemented control.

Overall, the most efficient modification in terms of reuterin yield and *in vitro* antifungal activity against *F. culmorum* was obtained by 24 h fermentation of MRS supplemented with 500 mM glycerol and 1.5% glucose, instead of the conventional 2% glucose. This concentration appears to be the best compromise to allow sufficient bacterial growth and at the same time inhibit the expression of 3-HPA reductase. This correlates well with the findings of Lüthi-Peng, Dileme and Puhan (2002). Consequently, the modification of the bacterial growth medium to induce reuterin production and accumulation was shown to be a promising method of improving the suitability of *L. reuteri* R29 as a bio-preservative agent.

In addition, the possibility of a combined increase in PLA and reuterin accumulation during LAB fermentation was attempted. However, this resulted in a total loss of antifungal activity (data not shown) of the cfs, due to the high reactivity of reuterin with the free amino-group of the Phe, needed as pre-cursor for PLA (Hazelwood et al., 2008).

As reuterin has industrial relevance as pre-cursor for the production of acrolein, several studies have been conducted to increase the reuterin production by LAB. These studies solved the problem regarding the carbohydrate content by application of a 2-step fermentation process. Although this process leads to high reuterin accumulation, with the cfs of the first fermentation step, also antifungal compounds produced by *L. reuteri* R29 are removed. Thus, it is uncertain if this process ultimately leads to effective fungal inhibition when applied *in vitro*. Therefore, the 2-step fermentation method of Doleyres et al. (2005) was adopted and tested for both, yield of reuterin and *in vitro* antifungal activity. Here, a reuterin content of up to 93.1±2.0 mM was achieved. Although this is much lower than the previously reported 176 mM (Doleyres et al., 2005) it equates to a more than 3 fold increase compared to the yield in the 1 step fermentation procedure (27.5±0.6 mM). This relatively low level of reuterin, compared to Doleyres et al. (2005), is most likely due to the use of a different strain of *Lactobacillus reuteri*. When the cfs was applied *in vitro* in the antifungal assay, it resulted in a total inhibition of all fungi tested over 120 h of incubation. Consequently, the significantly increased yield of reuterin in the 2-step fermentation method fully compensated for the loss of carboxylic acids. In fact, the cfs produced by this method had the highest *in vitro* antifungal activity of all the treatments investigated in this study.

This 2-step fermented supernatant was further used to determine the MIC_{90} of reuterin, as no commercial reuterin standards are available. Values found were 4 mM against *F. culmorum* and 8 mM against *A. niger* and *P. expansum* (data not shown). These values are in good correlation with the findings of Chung et al. (1989). Furthermore, it highlights the great potential of reuterin as antimicrobial and antifungal agent.

In conclusion, *Lactobacillus reuteri* R29 was shown to have the ability to metabolise glycerol to reuterin. This can be achieved using a 1 or 2 step fermentation procedure. Both approaches resulted in very high rates of fungal inhibition, when challenged against 10^7 spores/mL over 120 h (Table 17). Thus, the modification of the fermentation process of *L. reuteri* R29 towards the production of reuterin shows the potential for bio-preservation. But the need to control parameters such as glucose and glycerol content, and fermentation time carefully, to allow accumulation of reuterin has to be kept in mind. For a successful application in a food system, the high reactivity of reuterin might impose further challenges. This applies in particular, if heat treatment is involved, as this would further increase the reactivity of reuterin.

Furthermore, there is the risk of acrolein formation due to thermal dehydration (Gänzle, 2015).

6.4.3 Heat stability of PLA and reuterin

In order to get a more detailed picture with respect to the possible applications of the various antifungal cell-free supernatants obtained in sections 6.4.1 and 6.4.2, three selected media were subjected to heat treatment. In particular for use in food systems, a certain heat resistance would broaden the range of possible applications immensely. Therefore, the impact of heat treatment (1 h at 100°C) on PLA and reuterin content, and on the antifungal performance *in vitro* was determined, using *F. culmorum* as indication strain.

For the Pcfs, no significant reduction of inhibitory capacity against *F. culmorum* was found. Even after 120 h the heat treated supernatant resulted in 80±3% of fungal growth inhibition, compared to 84±3% for the not heat treated cfs (Figure 10A). Chromatographic analysis of the heat treated supernatant further revealed no substantial loss in PLA, compared to the unheated one (data not shown). Consequently, this cfs can be considered as heat stable with regards to the antifungal performance and the concentration of its lead antifungal substance, PLA. This result is in good correlation with the findings of Cortés-Zavaleta et al. (2014). Thus, the fermentation of Phe enriched medium by *L. reuteri* R29 appears to be very promising for the production of antifungal cfs and use as a bio-preservative in food systems.

For the reuterin-containing supernatants different results were obtained, depending on the fermentation method. For the cfs produced with the 2-step fermentation procedure, no substantial reduction in antifungal performance (Figure 10B) or reuterin content (Figure 10C) was observed. However, heat treatment of the cfs produced by the 1-step fermentation procedure resulted in a complete loss of antifungal activity (Figure 10D). Likewise, the concentration of reuterin in the cfs decreased from 27.5±0.6 mM to 0.5±0.1 mM, due to the heating process (Figure 10C). The reason for these differences is in the constituents of the respective supernatant, as heating primarily increases the reactivity of reuterin (Vollenweider et al., 2010). The 2-step fermentation produces a cfs that consists only of water, glycerol and reuterin, and thus no adequate reactive partner is present to sequester reuterin. Hence, the antifungal activity of this cfs was not notably effected by the

heating process. On the other hand, the 1-step fermentation produces a supernatant, containing all the ingredients of MRS broth and several metabolites produced by the bacteria which are potential reactive partners for the reuterin. Consequently, the increase in reactivity due to heating resulted in a substantial decrease in reuterin content and hence severely reduced antifungal activity.

To examine this topic further and evaluate the antifungal activity *in situ*, the reuterin enriched (1- and 2-step fermentation process) and the Pcfs were applied into a model system for food production that involves heat treatment, the bread making process.

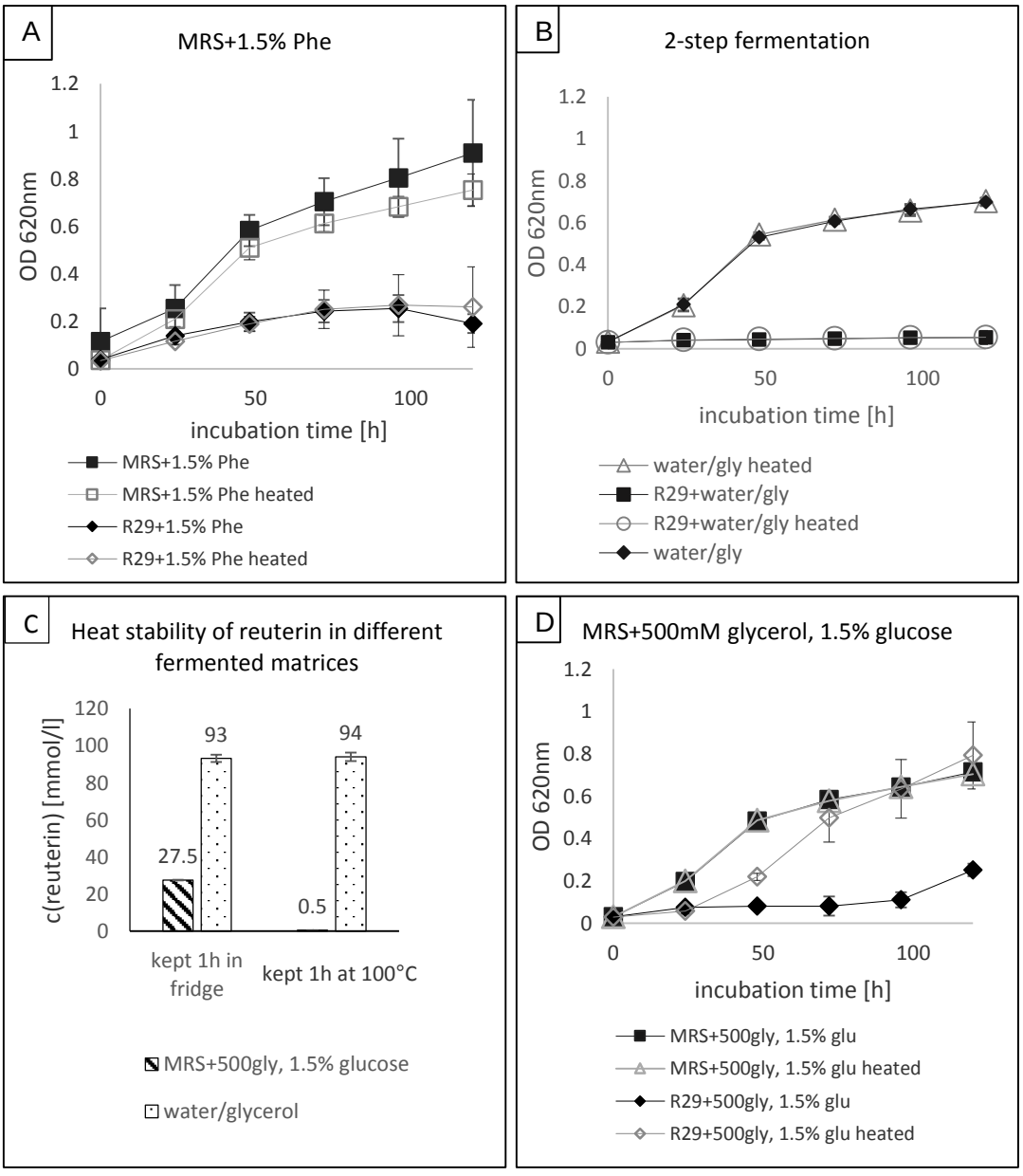

Figure 10: *In vitro* antifungal activity against *F. culmorum* in A) MRS + 1.5% Phe (48 h fermented), B) 2-step fermentation supernatant, C) impact of heat treatment on reuterin content of the cell-free supernatants and D) MRS + 500 mM glycerol; 1.5% glucose (24h fermented).

6.4.4 Effect of PLA and reuterin on bread microbial shelf-life

In order to investigate the antifungal activity *in situ*, it was attempted to transfer and exploit the increased antifungal activity (*in vitro*) in the food processing chain, using bread making as an example. Therefore, the water in the dough preparation was replaced with the cell-free supernatants and the effect on microbial-shelf life was tested by challenge against environmental fungi. Thus, use of the *in vitro* fermented cfs enabled comparison of the *in situ* antifungal performance of the different supernatants without the influence of other ingredients on the bacterial growth.

As demonstrated in previous studies the application of LAB fermented media into a cereal food matrix is possible (Le Lay et al., 2016; Peyer et al., 2016; Russo et al., 2017; Saladino et al., 2016). Furthermore, the routinely use of LAB as starter cultures for sourdough bread shows that the acidity has no substantial negative impact on the product quality and taste (Pawlowska et al., 2012). Hence, application of bacterial cfs, despite the strong acidic pH, as promising perspective for natural extension of microbial shelf-life.

As shown in Figure 11A, the use of cfs obtained by fermentation of normal MRS (24 h) resulted in a microbial shelf life of 5 days. After 13 days of storage 83% of the bread slices contained mould colonies (category B), whereas the remaining 17% were still completely mould free. A very similar result was achieved for the same medium, fermented for 48 h (Figure 11B). The microbial shelf life was determined to 4 days and after 13 days of storage 17% of the slices were still mould free. However, the mould was found to spread slightly faster, than with the 24 h fermented supernatant, as there were 6.5% of the slices in the “C” category (11 – 24% mouldy) and the rest in “B”. This outcome is interesting, as the antifungal activity *in vitro* was evident after 48 h of fermentation. However, as the breads were challenged against environmental fungi and not just one specific strain, variations in the antifungal performance are possible. In particular, as the antifungal performance is based on a synergistic mechanism, the amount of organic acids produced cannot serve as sole indicator for antifungal activity. Thus, it is possible that the mixture of antifungal compounds present after 24 h is more effective against certain environmental fungi than after 48 h. These samples, served as controls in order to evaluate if the increased antifungal activity as evident in *in vitro* trials could also be translated *in situ*.

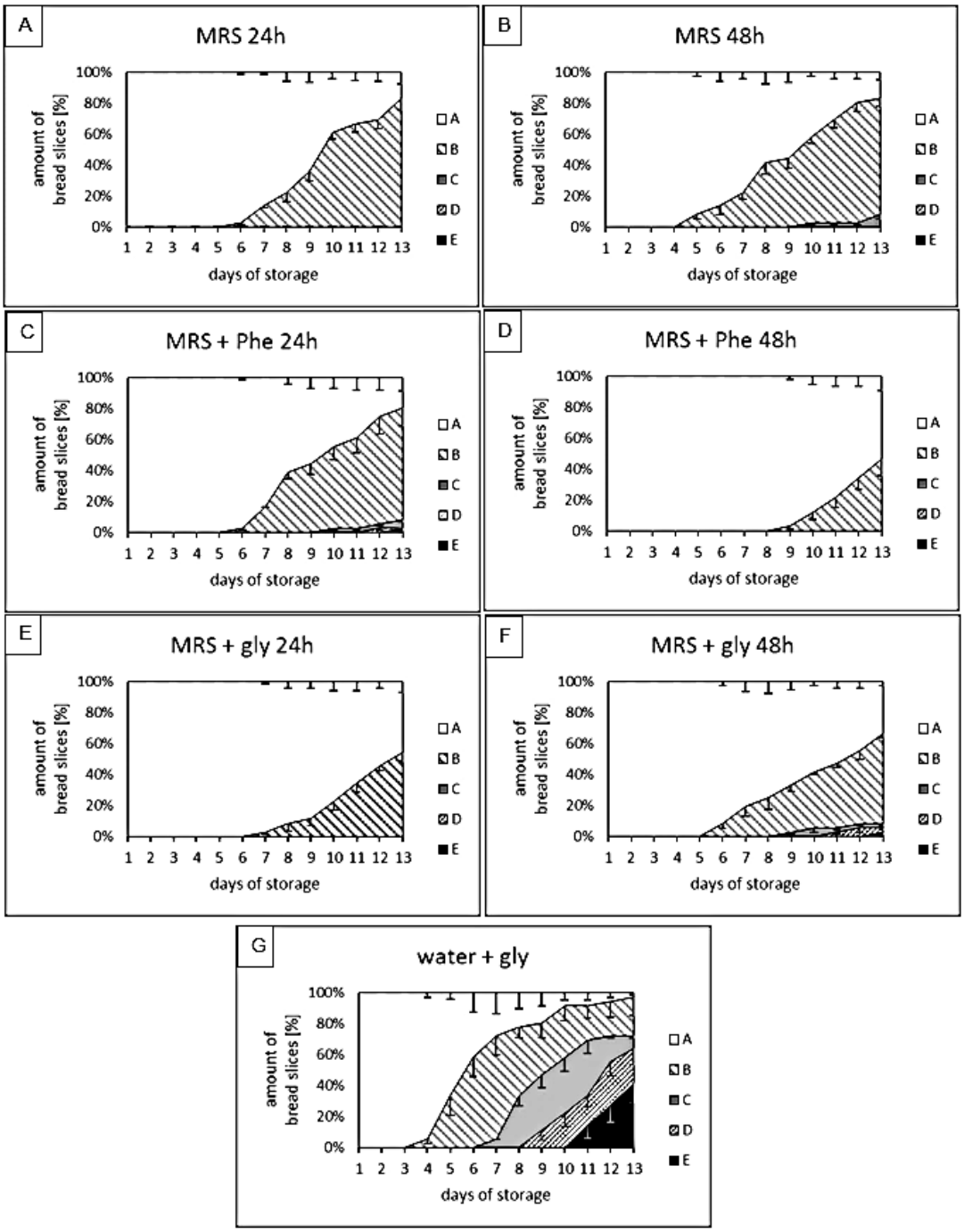

Figure 11: Shelf life of wheat bread against environmental mould during a 13-day storage period is indicated as the percentage of the total surface area of each slice, where fungal growth occurred. Mould-free slices (A, white), <10% mouldy (B, black stripes from left to right), 10-24% mouldy (C, gey), 25-49% mouldy (D, black stripes from right to left) and >50% mouldy (E, black). Mean values are shown (n=3); error bars indicate standard deviations.

In comparison, the Pcfs (24 h, Figure 11C) did not significantly increase the shelf-life, compared to the respective control (MRS / 24 h). The microbial shelf-life was determined to be 5 days and after 13 days, 19% of the slices were still mould free.

At the same time, 6.5% of the slices were mouldy to more than 10%. These differences to the 24 h fermented control are not statistically significant ($p<0.05$). Thus, the 24 h fermentation of Phe enriched MRS did not increase the antifungal performance *in situ*. In contrast, after 48 h of fermentation, the Pcfs led to substantially increased shelf-life (Figure 11D) when compared to the respective control. The shelf life increased from 4 to 8 days (100%), due to the use of Pcfs. In addition, the spread of fungal outgrowth was found to be notably retarded and the number of mouldy slices after 13 days of monitoring was, with 53%, significantly reduced also. This correlates well with the results of the *in vitro* assays for the Pcfs against *F. culmorum*. These results strengthen the conclusions previously made by Crowley, Mahony and Van Sinderen (2013), who reported that LAB are, due to their antimicrobial acids, very promising candidates as food bio-preservatives. In addition, this result demonstrates the broad antifungal activity of PLA against various spoilage organisms *in situ*. Thus, the results obtained in this study further prove the suitability of *L. reuteri* R29 as natural food preservative, using bread making as an example of such an application. The use of *L. reuteri* R29 as bio-preservative, in particular with emphasis on high PLA production appears to be a promising alternative to conventional preservatives.

Despite showing less promising results *in vitro* after heat treatment, the reuterin containing 1- and 2-step fermentations were also applied in the baking process. The respective 24 h and 48 h 1-step fermented supernatants (Figures 11E and F, respectively) were found to result in a shelf-life increase of 1 day, compared to their respective controls. Also the spread of the fungal outgrowth appeared to be slower. However, these improvements were not statistically significant ($p<0.05$). Due to the high reactivity of reuterin, in particular at high temperatures as shown in section 6.4.3, it is likely that the reuterin reacted with other dough constituents. Thus, when the microbial challenge test was carried out, no noteworthy levels of active reuterin were present. This indicates that the breads obtained from this supernatant should perform similar to the control in terms of microbial shelf-life, as the normally produced antifungal acids were present in both control and 1-step fermentation. Hence, the outcome of the shelf-life test, where no significant difference to the control was found, is in good correlation with the previous results, as discussed in section 6.4.3.

Finally, the 2-step fermented cfs showed the least antifungal activity of all samples, when applied to the baking process (Figure 11G). The shelf-life decreased substantially by 2 days, compared to the 24 h MRS control. The fungal spread was

also much faster than in any other sample, resulting in 42% of the bread slices being covered to more than 50% by fungi (category “E”), after 13 days of monitoring. Thus, the cfs obtained from the 2-step fermentation was found to be completely unsuitable for *in situ* microbial preservation in bread. The explanation for this, again, is related to the high reactivity of reuterin, in particular at high temperatures. Although this supernatant showed heat stability when tested *in vitro*, the dough matrix is very complex and thus provides plenty of reaction opportunities for the reuterin. In particular, free thiol groups which are the main target in the inactivated microbes present in the flour can interfere with the reuterins antimicrobial performance. Despite the fact that levels of free thiol groups in a dough formulation as it was used here are 10 – 100 times below the reuterin concentrations (Kohler, 2003; Reinbold et al., 2008) they are likely to compromise the antifungal activity significantly (Engels et al., 2016). Furthermore, the production process of this cfs eliminates the other metabolites produced by *L. reuteri* R29, such as antifungal acids. As a consequence, by losing the reuterin due to reactions with other dough constituents, no active antifungal compounds were present anymore. Hence, the outcome of an even further decreased shelf-life, compared to the control. The results of this study clearly demonstrate that reuterin is not suitable as food preservative if it is subjected to heating. However, it may still be suitable for unheated foods like salami or cheese, for which it has already been successfully applied (Gomez-Torres et al., 2014; Ortiz-Rivera et al., 2017).

6.5 Conclusions

In conclusion, this study demonstrates three possibilities for improving the efficiency of antifungal LAB *in vitro*, using *Lactobacillus reuteri* R29 as an example. Further understanding regarding production and stability of antifungal compounds was obtained. In particular, the key role of PLA for the antifungal performance of *L. reuteri* R29 became evident. However, from the MIC_{90} values of synthetic PLA it also became evident that microbial PLA just in synergy with other bacterial metabolites can serve as efficient antifungal agent. The results achieved *in vitro*, could only partly be transferred into the bread making process. Reuterin, due to its high reactivity, in particular at high temperatures (Vollenweider et al., 2010), did not lead to satisfactory results *in situ*. In contrast, the supplementation with Phe, to increase the production of PLA, was found to be very efficient in both *in vitro* and *in situ*. Hence, Phe supplemented fermentation media should be considered as promising options to improve the antimicrobial performance of LAB during production of food, such as bread or beverages. The proteolytic activity reported for *L. reuteri* R29 (Axel et al., 2016) also proposes the possibility to achieve this antifungal effect upon supplementation with Phe rich proteins. This work shows the potential for further exploitation of LAB as bio-preservatives, particularly in environments unsuitable for bacterial fermentation, for example, during grain storage. This demonstrates the potential to enlarge the field of application for the antimicrobial properties of LAB. Further research is required on the *in situ* production of PLA, including its stability and influence on sensory parameters. In addition, application of such methodologies investigated in this study in further food systems will serve to increase our knowledge in this increasingly pertinent area.

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Chapter 7: Screening of post-harvest decontamination methods for cereal grains and their impact on grain quality and technological performance

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7.1 Abstract

Cost effective approaches for microbial decontamination of cereals are of great industrial interest in order to reduce post-harvest crop losses and consumer health hazards. The objective of this study was to screen a range of 13 treatments, including physical (high hydrostatic pressure, ultrasound, vacuum packaging and microwaves with and without sodium hypochlorite), chemical (hydrogen peroxide, sodium hypochlorite, acetic acid, sorbate, propionate, quaternary ammonium compounds and cold plasma) and microbiological (lactic acid bacteria) decontamination, on artificially infected wheat prior to storage in a model system for six weeks. Each treatments success, compared to positive and negative controls, was evaluated based on fungal bio-mass and accumulation of 13 mycotoxins during storage. To estimate the treatments impact on grain quality and technological performance, a comprehensive flour analysis was carried out, including activities of important wheat enzymes, soluble protein distribution and gluten characterisation. The treated grains were also used to bake whole grain breads. The breads were analysed for quality parameters, such as specific loaf volume, crumb structure and physical crumb texture. Best results were found for vacuum packaging, high hydrostatic pressure, microwave, sorbate and propionate treatments, which fully inhibited fungal development and mycotoxin production. Quality deterioration was found due to the microwave treatment as a result of the internal heating. The comparison of traditional methods with the novel approaches revealed that the conventional methods remain superior to the novel approaches due to their simplicity and efficiency without negative impact on the grains.

7.2 Introduction

Cereals and cereal based products are amongst the most important staple foods worldwide and industries go to great lengths to ensure in-field protection of the crops. However, due to the permanent and ubiquitous presence of microorganisms and fungal spores in the environment, it is not possible to avoid contamination completely. As a consequence, grains carry a big microbial load when entering the stores (Magan and Aldred, 2006). Depending on field and climatic conditions this can include various filamentous fungi. The most dominant cereal spoilage fungi in Central and Western Europe are *Fusarium* spp. (Parry, Jenkinson and McLeod, 1995). It was shown previously how even small fungal contamination can spread rapidly during storage if the conditions are suitable (Schmidt et al., 2016). Furthermore, spoilage organisms can develop even during storage in ecological niches and result in substantial crop losses, as well as consumer health hazards due to mycotoxin contamination. Therefore, grains are usually stored at low moisture contents of 13 – 14%, to avoid microbial activity during storage. However, ineffective drying systems bear the danger to allow fungal activity and mycotoxin production. Unsuitable attempts of decontamination can even increase the mycotoxin hazard. Research proofs that mycotoxin production and accumulation is accelerated when the fungus is exposed to environmental stress (Kabak et al., 2006; Oliveira et al., 2012). Such stress factors also include sub-lethal doses of decontamination treatments.

Novel, efficient and cost effective approaches to prevent fungal development during storage are of major interest to the cereal industries. Developing countries in particular are struggling to prevent microbial spoilage and consumer health hazard, due to their limited financial and technological resources. In addition, also developed countries are searching for alternatives to conventional decontamination to increase consumer acceptance and safety. The methods applied should be simple, cost effective without compromising the final product quality. Hence this study investigated innovative approaches of microbial decontamination for their suitability in terms of fungal inhibition and effect on the grain quality and compares them to more traditionally applied methods. Therefore, a screening of various treatments, including microbiological, chemical and physical disinfection of fungal infected grains prior to storage were tested.

Lactic acid bacteria (LAB) have been studied extensively over the last decades as “green” alternatives to conventional chemical preservatives, in particular in cereal

matrices. Hence, LAB with antifungal activity were applied during grain storage by washing of the grains with LAB cell-free supernatant and cell suspension based on the method developed by Schmidt et al. (2018).

However, innovative approaches of physical decontamination are also rapidly gaining popularity. High hydrostatic pressure (HHP) is one of these emerging technologies. Already established applications of HHP include the preservation of meat products, oysters, fruit juices, and many ready-to-eat foods. HHP is reported to have the potential to inactivate vegetative microorganisms and fungal spores at relatively low temperatures without compromising sensory and nutritional properties (Heinz and Buckow, 2009; Polydera, Stoforos, and Taoukis, 2003). Microwave technology, another physical treatment, is widely used in the food industry and offers several advantages, including safety, high efficiency, and environmental protection, but often affects food quality (Aron Maftei et al., 2014). However, with the processing parameters carefully adjusted it is likely that microwave treatment can result in sufficient microbial decontamination without compromising the grain quality. Ultrasound is also considered as a promising, non-thermal approach for microbial disinfection of various surfaces and food matrices (Bilek and Turantaş, 2013) although findings for antimicrobial efficiency are controversial. Finally, as fungi require aerobic growth conditions (Magan and Lacey, 1984), grain storage under anaerobic conditions was investigated as well.

It further should be mentioned that research has discovered various innovative chemical alternatives to conventional decontamination methods. Due to recent advances in terms of microbial decontamination, cold atmospheric pressure plasma (CAPP) treatments find ever more applications in food industries. Plasma is referred to as the fourth state of matter. Overall the system is a neutral gas but contains several active, unstable species (Mir, Shah and Mir, 2016). These include electrons, ions, radicals, electronically excited species and vacuum ultraviolet radiation (Mir, Shah and Mir, 2016). These active species can interact even with the surrounding air and thus, after the treatment, leave no residues on the samples. The antibacterial effects of plasma are already routinely used for disinfection in areas, such as food contact materials. Only in recent years researchers started to focus on the direct decontamination of food commodities using CAPP (Niemira, 2012). Furthermore, acids commonly used as preservatives in cereal products, including sorbic and propionic acid, were also tested and compared to the more novel treatments. Despite the proven antimicrobial efficiency of sorbic and propionic acid the consumer acceptance for chemical additives is low (Razavi-Rohani and Griffiths,

1999). Quaternary ammonium compounds (QACs) are commonly used as disinfectants for food contact surfaces. However, the use for decontamination of food commodities is still unexplored. Industrial standard agents for microbial decontamination, such as solutions of hydrogen peroxide and sodium hypochlorite (Lukšiene et al., 2007) were also included and compared to the more unique and innovative approaches. Despite the proven efficiency of both agents in terms of microbial inhibition, concentrations greater than 6% (H_2O_2) and 1% (NaOCl) can reduce the seed germination rates (Lukšienė et al., 2007), causing the need for new methods.

In order to estimate the treatments impact on grain quality and technological performance, a comprehensive flour analysis was carried out, including activities of important wheat enzymes, soluble protein distribution, gluten characterisation and rheological measurements. In addition, the treated grains were used to bake whole grain breads. The breads were analysed for important quality parameters, such as specific loaf volume, crumb structure and physical crumb texture. As a result, this study provides important information for cereal industries to reduce crop losses due to post-harvest spoilage combined with increased consumer acceptance and cost efficiency.

7.3 Materials and Methods

7.3.1 Materials

Commercial hard winter wheat (*Triticum aestivum*), harvested in 2016, was supplied by Doves Farm Foods Ltd. (Hungerford, UK). The wheat grains were stored in barrels in a cool and dry place and were regularly aerated. *Fusarium culmorum* strain TMW 4.2043 was originally isolated from barley and provided by the Lehrstuhl für Technische Mikrobiologie, TU-München Weihenstephan. *Lactobacillus reuteri* R29, originally isolated from human intestine, was obtained from the UCC culture stock collection and routinely grown on MRS-agar plates under microaerophilic conditions at 37°C for 48 h. Long term storage was done in MRS-broth containing 40% glycerol at -80°C.

For the baking trials, dry yeast was purchased from Puratos, Belgium; salt from Glacia British Salt Limited, UK; ascorbic acid from Storefast Solutions, UK; Sodium Stearoyl Lactylate (SSL) from Danisco, Denmark; commercial vegetable oil and sugar from Nordzucker, Ireland.

All reagents used were at least of analytical grade and sourced from Sigma Aldrich.

7.3.2 Grain surface disinfection

Initial grain surface disinfection was carried out according to the method described by Schmidt et al. (2016). In brief, 600 g of grains were washed for 10 minutes in 4 L of 10% H_2O_2 solution. After rinsing with distilled water for 5 minutes the procedure was repeated but only with 5 minutes of washing in H_2O_2. After drying for 24 h under vertical sterile laminar flow, grains were exposed to UV-light for 10 minutes and collected aseptically.

7.3.3 Preparation of fungal spore suspension and grain infection

F. culmorum spore suspension and infected grains were prepared according to the method described by Schmidt et al. (2016). Briefly, *F. culmorum* was grown over 5 days at 25°C on potato dextrose agar (PDA) plates. Six small fragments were transferred into 800 mL of synthetic nutrient-poor bouillon (SNB) and continuously

stirred for 1 week at room temperature, filtered (30 µm pore seize) and adjusted to 10^5 spores/mL.

For preparation of infected kernels, disinfected wheat grains were mixed with 2% (v/w) sterile filtered spore suspension of *F. culmorum*. After incubation for 10 days at 25°C (75% relative humidity) fungal proliferation was visible and the grains were defined as 100% infected.

7.3.4 Mixing and storage

Infected and disinfected grains were mixed to samples of 180 g (dry matter), containing 5% infected kernels. Subsequently, sample treatments were carried out in triplicate, before the three replicates were combined and homogenised. The sample was then divided into 6 portions of approximately 100 g and filled into sterile plastic bags. These bags were sealed, perforated by two pipette tips with barrier filter to allow gas exchange, and stored at room temperature. After 0 and 6 weeks, three portions of each sample were collected, milled to a whole grain flour (particle size < 2mm), homogenised and stored at -20°C until further analysis.

Prior to storage, the surface water activity (a_w) of each sample was measured and if necessary adjusted to ≥0.8, using sterile distilled water, to provide suitable growth conditions for the fungus.

7.3.5 Production of LAB antifungal fermentation products

The procedure for the production of antifungal fermentation products was adapted from a previous study (Schmidt et al., 2018). Briefly, 1 single colony (grown as described in section 7.3.1) per 5 mL MRS broth was inoculated for 24 hours at 37°C. Subsequently, a 1% inoculum in fresh MRS broth, containing 1.5% Phe, was carried out and incubated for 48 h at 37°C. So obtained LAB fermentation products were immediately applied, with and without centrifugation (15 min, 10°C, 4000 rpm), as cell-free supernatant or cell suspension, respectively.

7.3.6 Chemical/ microbiological disinfection

For chemical disinfection, grain portions of approximately 200 g were washed in 2.5

L distilled water for 5 minutes under continuous stirring. Subsequently, the grains were sanitized for 15 minutes in 2.5 L of the respective disinfectant solution. Finally, grains were washed again in 2.5 L distilled water with continuous stirring to remove residual chemicals. After drying for 24 h at room temperature under vertical sterile laminar flow, samples were stored as described in section 7.3.4. The following disinfectants were tested in various concentrations: H_2O_2, NaOCl, potassium propionate and sodium sorbate solutions, Quatrol T® (QAC) and acetic acid. For the microbiological disinfection with LAB cell-suspension and cell-free supernatant the same procedure was applied, only the final washing step in distilled water was omitted.

Furthermore, CAPP treatments were investigated using a prototype plasma generator provided by Teagasc Ashtown, Dublin Ireland. Therefore, a single layer of grains was exposed to non-thermal plasma for 5 – 20 minutes with a distance of 1 cm between the plasma generator and the grain surface. After the treatment grains were packed and stored for 0 and 6 weeks as described in section 7.3.4.

7.3.7 Physical disinfection

The following treatments were investigated for physical decontamination: high hydrostatic pressure (HHP), ultrasound and microwaves, as well as storage under vacuum. Each procedure is detailed below.

7.3.7.1 Vacuum

For the vacuum treatment, grain portions of approximately 200 g were packed into polyethylene bags and vacuum sealed (Foodsaver Vacuum sealer FFS002, Lakeland, UK). The samples were stored for 0 and 6 weeks, as described in section 7.3.4, only the pipette tips with barrier filter were omitted during storage.

7.3.7.2 High pressure

Grain portions of 200 g were divided into 3 plastic bags and filled up with sterile distilled water to cover the grain surface with liquid. The bags were sealed and

positioned in another plastic bag, which was sealed under vacuum. This procedure was repeated 4 times.

The packed grains were placed in the high pressure machine and treated at 30°C for 10 – 600 seconds with 100 - 300 MPa (High pressure Food processor ISO LAB 900 5, IL- 100-250-9-W, Stansted Fluid Power Ltd., Stansted, UK). Afterwards, the grains were dried for 24 h under sterile vertical laminar flow before storage for 0 and 6 weeks.

7.3.7.3 Ultrasound

For the ultrasonic treatment, grain portions of approximately 200 g were used. Therefore, the grains were filled in plastic bags and 300 mL of distilled water or disinfectant was added to each bag. Afterwards, the bags were sealed and positioned in the ultrasonic bath for 5 - 20 minutes at 45 kHz and 120 W (Ultrasonic Cleaner USC- 600 TH, VWR, Radnor, Pennsylvania, United states). Subsequently, the grains were dried under sterile vertical laminar flow for 24 h and stored as described in section 7.3.4. In addition to distilled water, the following disinfectants were tested: H_2O_2 (10%), NaOCl (5%), acetic acid (10%) and Quatrol T® (20%).

7.3.7.4 Microwaves

For the microwave treatment, grain portions of approximately 200 g were divided in 3 beakers, covered and placed in the microwave. The grains were treated for 10 – 300 seconds at 800 W using a R209 microwave (sharp, Osaka, Japan).

In addition, chemical and microwave treatments were combined. The same procedure was used but each beaker was filled up with 300 mL of disinfectant before treated in the microwave. Subsequently, the grains were washed in 2.5 L distilled water with continuous stirring to remove residual disinfectant and dried at room temperature for 24 hours with vertical sterile laminar flow. The following disinfectants were tested in combination with the microwave treatment: H_2O_2 (10%), NaOCl (5%), acetic acid (10%) and Quatrol T® (20%).

7.3.8 Determination of Ergosterol

Ergosterol content (free and ester bound) was determined using the method of Jedličková et al. (2008). In brief, 10 g of milled grains were extracted with 50 mL of methanol. The cleaned extract (25 mL) was added to 3 g KOH and shaken until all KOH was dissolved. After addition of 10 mL n-hexane the mixture was incubated for 30 minutes at 65°C and cooled to room temperature. Upon addition of 5 mL water the upper layer was transferred and the procedure repeated 3 times. The combined supernatants were evaporated till dry and re-dissolved in 5 mL of methanol, before measurement by reverse phase high performance liquid chromatography (RPHPLC). The injection volume was 20 µL, the mobile phase consisted of 100% methanol with a flow rate of 1 mL/min and detection was carried out at 282 nm. The RPHPLC column used was a Nova-Pak C_{18} (300 x 3.9 mm, 4 µm). Peak-identity was verified using the UV- spectra recorded by the DAD. The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the signal/noise (s/n) ratio. The LOD was set for s/n of 3:1 and the LOQ was set for s/n of 10:1. For calibration, ergosterol standards between 1.0 and 200 mg/mL in methanol were prepared and analysed. The recovery was determined by spiking an ergosterol free sample with a standard solution and found to be 95±1%.

7.3.9 Mycotoxin analysis

The analysis of mycotoxins was carried out by UHPLC-MS/MS according to the method of Annunziata et al. (2017). In brief, 2 g milled, homogenised sample was extracted by manual shaking in the presence of 10 mL acetic acid (0.1%, v/v) for 1 min. Subsequently, 10 mL of acetonitrile was added and again shaken for 1 min. Magnesium sulphate (4 g) and sodium chloride (1 g) were subsequently added to the tubes and they were shaken for 1 min. Samples were centrifuged (3,500 rpm, 10 min) and 2.5 mL of the resulting supernatants were transferred into 15 mL polypropylene centrifuge tubes, which were evaporated to dryness under nitrogen at 40°C. The residues were resuspended in 0.2 mL of a water/methanol mixture (90/10, v/v), and filtered through a 0.2 mm filter, before 2.5 mL was injected into the UHPLC-MS/MS system. The separation was carried out with a Waters Binary Acquity™ UHPLC System, using an Acquity BEH® C_{18} (100 x 2.1 mm,1.7 mm) column at 40°C. The mobile phase consisted of A) 5 mM ammonium acetate in water/methanol (90:10, v/v) and B) 5 mM ammonium acetate þ 0.1% acetic acid in

methanol. The gradient used was 0 - 4 min, 100% A; 4 - 10 min 10% A, 90% B; 10 - 12 min 100% A. The flow rate was 0.4 mL/min and the run time 12 min. Detection was carried out using a Waters Quattro Premier XE™ with ESI interface. The LOD and LOQ were determined from the s/n ratios. The LOD was set for s/n of 3:1 and the LOQ was set for s/n of 10:1. Standard curves for each mycotoxin analysed were obtained by matrix calibration. The samples were measured as single determinations. Mycotoxins analysed include deoxynivalenol (DON), nivalenol (NIV), ochratoxin A (OTA), ochratoxin B (OTB), zearalenone (ZEA), patulin (PAT), enniatin B (ENB), enniatin B1 (ENB1), enniatin A1 (ENA1), T-2 toxin (T-2) and HT-2 toxin (HT-2) with the limits of detection being 10, 62.5, 1.5, 0.375, 6.25, 12.5, 5, 5, 25, 2.5 and 2.5 µg/kg (ppb), respectively.

7.3.10 Flour analysis

The disinfection treatments showing the best results in terms of fungal growth inhibition and mycotoxin reduction were repeated on uninfected grains to evaluate important flour quality parameters. Flour analysis was carried out on the whole grain flour obtained from natural grains treated with the different disinfection procedures (particle size < 0.5mm).

Changes in enzymatic activities due to the treatments were evaluated by determination of β-amylase activity (Megazyme Int., Ireland), total protease activity, using a haemoglobin standard (Brijs et al., 2002) and lipase activity by the copper soap assay (Rose and Pike, 2006). The relative amount of damaged starch was determined with the damaged starch assay (Megazyme Int., Ireland).

Proteins were sequentially extracted from freeze-dried flour, following the method described by Oliveira, Zannini and Arendt (2014). Each extraction was carried out in duplicate. Protein concentrations of the joined extracts were determined by the protein dye binding method of Bradford (Bradford, 1976), using bovine serum albumin as a standard. The extracts obtained from the Osborne fractionation were analysed further by SDS gel electrophoresis, using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA), according to the instructions given in the manual for the Protein80+ chip. The analysis was carried out in a molecular weight range of 4.5 - 95 kDa. The results are shown in a gel-like image.

Gluten quantity and quality were analysed using the Glutomatic (Perten Instruments GmbH, Hamburg, Germany) and the GlutoPeak (Brabender GmbH & Co. KG,

Duisburg, Germany) as described by Schmidt, Zannini and Arendt (2017). Briefly, the determination of wet and dry gluten contents, water-binding capacity and gluten index was carried out according to the AACC method 38-12.02 for wholemeal wheat flour. For further characterisation of the gluten quality, in terms of its development time and maximum strength, the GlutoPeak was used. Therefore, the sample was mixed with water under high shear force until the gluten network was formed and broken down again. The viscosity was recorded graphically as a function of time. Hence, strong flours, as desired for bread making, developed fast, showing high peaks with short peak times. The test was performed according to the manufacturer's recommendations reference. Briefly, 9 g of sample (14.0% moisture content) and 9 g of distilled water were weighed into the mixing chamber and equilibrated. Sample and water were mixed with 2500 rpm at 27°C. After the maximal mixing resistance was detected, or latest after 10 min, the measurement was stopped. Peak time and height were recorded to evaluate the gluten quality.

7.3.11 Baking trials and bread analysis

Baking experiments were carried out with the whole grain flour obtained from the natural grains treated with the different disinfection procedures (particle size < 0.5 mm). The percentage of water (based on flour weight) required to yield a dough consistency of 500 Brabender units (BU) (determined using a Brabender Farinograph according to the AACC method 54-21.02) was equal to 83% for the untreated sample. This amount of water was used for all the wholemeal wheat flour samples baked. Additionally, 2% yeast, 3% oil, 3% sugar and 2% salt (each based on flour weight) were used in the bread recipe. Prior to dough preparation, yeast was reactivated, using the whole amount of water (30°C) of the recipe, and placed in a proofer (KOMA sunriser, Roermond, Netherlands) at 30°C and 80% relative humidity for 10 min. Afterwards, the yeast suspension and the dry ingredients were combined in a mixer (Kenwood Chef Classic KM336). Mixing was carried out with a dough hook at speed I for 1 min, followed by scraping down the sides of the bowl and further mixing at speed II for 7 min. After mixing, the dough was divided into portions of 65 g, manually rounded, placed in non- stick baking tins (dimensions-top inside, 50 mm × 90 mm; bottom outside, 45 mm × 85 mm; inside depth, 30 mm; Sasa UK, Enfield Middlesex, UK) and proofed for 75 min (30 °C, 85% RH). Subsequently, the tins were transferred to the oven (Belling, Prescott, UK) and baked for 27 min at 175 °C (top and bottom). Afterwards, the bread loaves were

immediately removed from the tins and allowed to cool to room temperature for 120 min before further analysis.

After cooling to room temperature, the breads were weighed to determine the bake loss (weight reduction during baking). The specific volume was measured using a Volscan profiler (Stable Micro Systems, UK). After slicing the breads (25 mm width), the four slices from the centre of each loaf were used for further analysis. First, the crumb colour was analysed using a chroma meter CR-400 (Konica Minolta Inc., Tokyo, Japan) with CIE standard illuminant D65. The L* value, as calculated by the software, was used to evaluate the crumb lightness. The C-cell bread Imaging System (Calibre Control International Ltd., UK) was used to characterise the crumb structure. The following parameters were evaluated: number of cells per slice, total area of cells as a percentage of the total slice area and average diameter of the cells. The crumb texture was characterised by texture profile analysis (TPA), using a TA-XT2i texture analyser (Stable Micro Systems, Surrey, UK), equipped with a 25-kg load cell and a 20-mm aluminium cylindrical probe. A speed of 5 mm/s and a force of 0.98 N were applied to compress the middle of the crumb to 50% of its original height. 8 slices per batch were analysed on day 0, 3 and 5 after baking, respectively and the crumb hardness evaluated.

7.3.12 Statistical analysis

Baking trials and all analyses were carried out in three independent batch replicates, unless stated otherwise. Statistical analysis was performed using Minitab 17 software. Data points were checked for outliers (Grubb's test) and evaluation of significant differences was performed using one-way analysis of variances (ANOVA). All differences were considered significant at $P < 0.05$. Where F-values were significant, pairwise comparisons were carried out with the help of Tuckey Post Hoc test to describe the statistical significance between the different treatments.

7.4 Results and Discussion

7.4.1 Pre-screening of grain decontamination approaches

Table 18: Optimised processing parameters for each decontamination treatment.

Treatment	Optimised processing parameters
natural (uninfected control)	Not treated
5% infected control	Not treated
dry microwave	2 min at 800 watt in a covered beaker
microwave +NaOCl	2 min at 800 watt in a covered beaker with 5% NaOCl solution
high pressure treatment	10 min at 300 MPa and 30°C
sorbate	5% in water, washing for 10 min
propionate	5% in water, washing for 10 min
NaOCl	5% in water, washing for 10 min
H_2O_2	10% in water, washing for 10 min
Quatrol T®	20% in water, washing for 10 min
MRS+1.5% Phe + R29	Applied as cell suspension, washing for 10 min
ultrasound	20 min at 30°C in water
cold plasma	20 min at 25°C, plasma generator 1 cm above grains
acetic acid	10% in water, washing for 10 min

In this study various microbial, physical and chemical approaches have been screened for their suitability for post-harvest microbial decontamination of cereal grains to reduce food losses due to fungal spoilage. Treatments investigated include microbial decontamination, using antifungal active LAB, washing with disinfectant solutions, such as H_2O_2, NaOCl, Quatrol T®, acetic acid, sorbate and propionate, ultrasound, high hydrostatic pressure, microwaves, vacuum and non-thermal (cold) plasma. However, for each treatment, the optimal processing parameters had to be determined first to ensure optimal application. Therefore, a pre-screening was carried out testing various treatment times, temperatures and concentrations/intensities. *Fusarium culmorum* was chosen as indicator strain, as it is the primary reason for cereal spoilage, particularly in Central and Western Europe (Parry, Jenkinson and McLeod, 1995). Fungal growth inhibition was recorded visually over 3 weeks of storage (results not shown). For each treatment parameters

showing the best results are summarised in Table 18 and were used for further investigation in the following section.

7.4.2 Screening of grain decontamination approaches

The parameters showing the most promising results for each treatment in the pre-screening were investigated further by storage for 6 weeks. Ergosterol content (indicator for fungal bio-mass) and mycotoxins were determined for each treatment before and after the 6 weeks of storage. The results are summarised in Table 19.

Table 19: Ergosterol and mycotoxin contents for the treated wheat samples before and after storage[#].

	ergosterol content [mg/kg][1]		mycotoxins detected (with quantity [ppb])[2]	
Treatment	week 0	week 6	week 0	week 6
natural (negative control)	<LOD[a]	<LOD[a]	n.d.	n.d.
5% infected (positive control)	<LOD[a]	12.6±0.5[b]	n.d.	DON:523 OTA: 25.0 OTB: 4.0
dry microwave	<LOD[a]	<LOD[a]	n.d.	n.d.
microwave +NaOCl	<LOD[a]	<LOD[a]	n.d.	n.d.
vacuum packaging	<LOD[a]	<LOD[a]	n.d.	n.d.
high pressure treatment	<LOD[a]	<LOD[a]	n.d.	n.d.
sorbate	<LOD[a]	<LOD[a]	n.d.	n.d.
propionate	<LOD[a]	<LOD[a]	n.d.	n.d.
NaOCl (5%)	<LOD[a]	20.2±1.2[c]	n.d.	DON: 743 OTB: 61 OTA: 12
H_2O_2 (10%)	<LOD[a]	18.4±1.5[c]	n.d.	DON: 257 ENB: 19.1
Quatrol T® (20%)	<LOD[a]	24.8±3.2[d]	n.d.	ZEA: 38.4 ENB1: 32.5 ENA1: 18.3

MRS+1.5% Phe + R29 cells	<LOD[a]	15.4±1.1	n.d.	DON:1390
ultrasound	<LOD[a]	26.3±2.7[d]	n.d.	DON: 127 OTB: 43 ZEA: 55
cold plasma	<LOD[a]	28.1±2.4[d]	n.d.	DON: 92 OTA: 21.5 OTB: 5.3
acetic acid	<LOD[a]	12.5±1.3[b]	n.d.	DON: 482 ZEA: 39

[#] Ergosterol results shown are mean values ± standard deviation. Values in one column followed by the same lower case letter are not significantly different (p<0.05).

[1]LOD (limit of detection) = 0.5mg/kg

[2] n.d. – no residue detected above the reporting limits of the method (see section 7.3.9)

7.4.2.1 Microbial decontamination

Lactobacillus reuteri R29, a LAB strain previously reported to express high antifungal activity against a broad spectrum of food spoilage organisms was applied to reduce fungal growth. Furthermore, the authors previously demonstrated how this strains antifungal activity can be optimised upon the increased production and accumulation of phenyllactic acid (PLA) (Schmidt et al. 2018). From the preliminary trials it became evident that the application of the cell suspension with increased PLA content shows better results than the use of cell-free supernatant (Table 18). However, despite a substantially retarded fungal development during 3 weeks of storage, after 6 weeks no fungal inhibition compared to the untreated control was evident. The LAB treated sample contained with 15.4±1.1 mg/kg a higher Ergosterol content than the untreated control (12.6.±0.5 mg/kg). Also the analysis of mycotoxins showed unsatisfying results for the microbial decontamination. In week 0 the samples contained no detectable amounts of any of the mycotoxins tested. But after 6 weeks of storage deoxynivalenol (DON) was found at a concentration of 1390 µg/kg. Again this indicates even worse fungal spoilage than the untreated (5% infected) control which showed a DON concentration of 523 µg/kg.

L. reuteri R29 has been shown in various studies to be highly efficient against various spoilage fungi, including *Fusarium culmorum*, *in vitro* and *in situ* in a cereal matrix (Axel et al., 2016; Oliveira et al., 2015; Peyer et al., 2016; Varsha and Nampoothiri, 2016). This is in sharp contrast to the results obtained here where LAB treated samples showed even more fungal spoilage compared to the untreated ones. In the present study the infected grains in the sample were already overgrown with fungal hyphae before treatment, while previous studies primarily investigated the antifungal activity against fungal conidia. As a consequence, the exact amount of fungus present in the samples is unknown making it possible that the minimal inhibitory concentration of PLA was not reached. Furthermore, the mode of action and inhibitory activity is different for conidia and hyphae (Dalié, Deschamps and Richard-Forget, 2010). In addition, the residual medium on the grains also provided nutrients for the fungus promoting the initial growth to overcome the inhibitory effects of LAB and PLA. To the best of the authors knowledge antifungal activity of LAB has never been investigated over such a long period of time (6 weeks). This also explains the initial fungal inhibition in the 3 week pre-screening. However, in the second half of the storage the inhibitory effects subsided and fungal development occurred.

In terms of mycotoxin accumulation, the increased values for the LAB treated samples are due to the environmental stress created by the bacteria and PLA on the grains. Hence, mycotoxin production to overcome this stress factor was promoted. Furthermore, the residual medium on the samples provided nutrients and therefore energy for the fungus to produce the toxins. Therefore, the microbial decontamination could not be successfully applied for grain protection during storage and was not investigated further.

7.4.2.2 Physical decontamination approaches

The five approaches for physical grain decontamination with their specific treatment parameters are shown in Table 18. These are vacuum packaging, high hydrostatic pressure (HHP) (300 MPa, 10 min), ultrasonication (20min) and microwave treatments (on dry grains and in 5% NaOCl solution) for 2 min. As shown in Table 19, in week 0 all samples contained ergosterol amounts below the LOD. The high pressure, vacuum and both microwave treatments were found to fully inhibit the growth and development of *F. culmorum* over the six weeks of storage. This becomes evident, as the ergosterol levels determined for these four samples

remained even after week 6 below the LOD of 0.5 mg/kg. At the end of the same storage period, the untreated control was found to contain 12.6±0.5 mg/kg ergosterol. In contrast, the ultrasound treated samples presented after 6 weeks with an ergosterol content of 26.3±2.7 mg/kg an even higher ergosterol value than the untreated control.

Also in terms of mycotoxin accumulation, the HHP, and microwave treatments, as well as the vacuum packaging showed very good results. All these treatments resulted in mycotoxin concentrations below the LOD for each mycotoxin analysed after 0 and 6 weeks of storage. However, the ultrasound treatment, while having no substantial amounts of mycotoxins in week 0, was found to contain OTA, OTB, ZEA and DON in concentrations of 43, 2.6, 55 and 127 µg/kg, respectively.

The success of the vacuum packaging was predominantly based on the lack of oxygen. As spoilage moulds, such as *Fusarium culmorum* generally require oxygen to develop, the anaerobic storage conditions prevented fungal development (Gregori et al., 2013). Due to the reduced viability of the fungus, production of mycotoxins could not occur either. The dry microwave treatment was found to efficiently inhibit *F. culmorum* also, based on the denaturation of proteins due to internal heating (Aron Maftei et al., 2014; Jubeen et al., 2012). As a consequence of the fungal inhibition due to protein denaturation, also the production of mycotoxins was prevented. However, the internal heating is likely to also affect the wheat proteins having a negative impact on the technological performance of the grains, which is investigated further in section 7.4.3. Also, the microwave treatment combined with NaOCl resulted in a positive outcome. While heating has a contribution to the antifungal effect, the inhibition can be primarily attributed to the microwave induced creation of a hypochlorite radical (Forsberg, 2004; D'Ovidio et al., 2007). Due to the very high reactivity of the radical, it degrades the fungal cell membrane, resulting in the complete inhibition of *F. culmorum* development during storage. Furthermore, HHP was found to inhibit microbial spoilage very effectively. This is in good correlation with the results of Martínez-Rodríguez et al. (2014), who reported inhibition of fungal development and reduced spore viability due to HHP treatments of *P. roqueforti*. With the parameters chosen here *F. culmorum* was fully inhibited and as a consequence no mycotoxins were detected. Finally, the ultrasonic treatment was highly inefficient in terms of microbial protection. Existing literature reports controversial results for the antifungal efficiency of ultrasound treatments. While Chemat, Zill-E-Huma and Khan (2011) reported fungal inhibition, Butz and Tauscher (2002) found results similar to the present study. However, even

distribution of the grains and sufficient cavitation increased the difficulty of the treatment due to the solid nature of the sample. As a result no fungal inhibition or reduction in mycotoxins could be achieved and the treatment was not investigated further. It has to be mentioned however that in-field produced mycotoxins are unlikely to be affected by the treatments investigated here. Hence, reduced mycotoxin contents after 6 weeks of storage are attributed to the reduced fungal viability.

7.4.2.3 Chemical decontamination approaches

For the seven approaches of chemical decontamination, optimal parameters as shown in Table 18 were used for further investigation over 6 weeks of storage in this section. The results of ergosterol and mycotoxin analysis for each treatment are shown in Table 19.

In week 0 all samples were found with an ergosterol content below the LOD of 0.5 mg/kg. Washing with propionate and sorbate solutions showed very good results in terms of fungal growth inhibition, as the ergosterol concentrations after week 6 were still below the LOD. In contrast, the other 5 treatments resulted in substantial fungal growth during storage. As a result ergosterol concentrations determined for H_2O_2, NaOCl, acetic acid, Quatrol T® and cold plasma treatments were 18.4±1.5, 20.2±1.2, 12.5±1.3, 24.8±3.2 and 28.1±2.4 mg/kg, respectively. Results of the mycotoxin analysis are in good correlation with the ergosterol contents. Substantial amounts of toxins typical for *Fusarium* species were found for H_2O_2, NaOCl, acetic acid, Quatrol T® and cold plasma after 6 weeks of storage (Table 19). Washing with potassium sorbate or calcium propionate resulted in no detectable amounts of mycotoxins throughout the storage period, correlating well with the suppressed development of fungal bio-mass.

The antifungal activity of the propionate and sorbate treatments is in good correlation with results previously reported (Belz et al., 2012; Le Lay et al., 2016; Varsha and Nampoothiri, 2016). Both salts are known to contribute to the antifungal activity of various LAB and propioni bacteria. In addition, due to the good water solubility of the salts, they are easily removed after the treatment by rinsing with water. Hence, both treatments have potential for further application to inhibit fungal development and mycotoxin accumulation. On the other hand, acetic acid is also known for its antifungal properties but could not be applied successfully here. It is

likely that the fungal hyphae in the samples were more resistant to the acid than the commonly treated fungal conidia. Also the lack of other organic acids for synergistic effects reduced the antifungal performance of the acetic acid (Corsetti et al., 1998).

Hydrogen peroxide and sodium hypochlorite are commonly used disinfectants and therefore were expected to result in good inhibition of the fungal development during storage. However, it appears that under the experimental setup used here the concentration which could be applied was not sufficient to inhibit the fungal growth. As a result, higher amounts of mycotoxins than in the untreated control were found in these samples. This is due to the environmental stress of sub-lethal doses of the disinfectants promoting the production of mycotoxins. QACs, although previously reported for their antimicrobial activity, were not efficient. It is possible that Quatrol T®, which was used here is a formulation with lower antifungal activity. Furthermore, to date QACs were primarily investigated for antibacterial rather than antifungal performance. In addition, surrounding cereal matrix is likely to reduce the activity even further. Finally, the plasma treatment was not found to be successful. This is primarily due to the uneven surface of the grains and loose pieces of bran functioning as shield from the plasma (Butscher et al., 2016). As a consequence, the generated plasma cannot get in contact with the entire grain surface, leading to insufficient decontamination. Another problem is the high reactivity of the plasma, making it possible that it reacted with the air and bran before reaching the fungal hyphae. All in all, washing with propionate and sorbate solutions were the only chemical treatments found to be successful and with a perspective for industrial application.

7.4.3 Analysis of grain quality parameter

In order to obtain a deeper understanding of the effects the various treatments have on the grains, a characterisation of the flours obtained after milling was carried out. Therefore, only the treatments resulting in efficient fungal inhibition were investigated further.

7.4.3.1 Enzymatic activities and starch damage

Enzymatic activities determined include β-amylase, total protease and total lipase

activities. Furthermore, the degree of starch damage was analysed. The results are summarised in Table 20.

Table 20: Mean enzymatic activities and soluble protein concentrations for the treated wheat samples[#].

Treatment	protease	lipase	β-amylase	starch damage
natural (control)	1.07 ± 0.06^{ab}	0.54 ± 0.03^{a}	48.5 ± 0.6^{a}	4.9 ± 0.5^{a}
dry microwave	0.55 ± 0.02	0.17 ± 0.02^{b}	10.2 ± 0.4	6.5 ± 0.8
microwave +NaOCl	1.00 ± 0.03^{ab}	0.17 ± 0.02^{b}	33.3 ± 1.0^{b}	4.6 ± 0.2^{a}
vacuum packaging	1.00 ± 0.03^{ab}	0.49 ± 0.02^{a}	34.5 ± 0.3^{b}	4.8 ± 0.4^{a}
high pressure treatment	0.96 ± 0.05^{ab}	0.43 ± 0.02^{ac}	49.4 ± 0.2^{a}	5.2 ± 0.4^{a}
sorbate	0.93 ± 0.01	0.39 ± 0.04^{c}	47.8 ± 0.6^{a}	4.7 ± 0.3^{a}
propionate	1.15 ± 0.08^{a}	0.72 ± 0.05	50.9 ±1.6^{a}	4.6 ± 0.6^{a}

[#] **Results shown are mean values ± standard deviation. Values in one column followed by the same lower case letter are not significantly different ($p<0.05$).**

Despite the good results in terms of fungal inhibition, the dry microwave treatment was found to have a substantial impact on all enzymatic activities tested. Activities of β-amylase, lipases and proteases were substantially reduced compared to the untreated control, as well as the other treatments. Better results were found for the combined treatment of microwave with NaOCl. While the lipase activity was reduced similarly to the dry microwave treatment, no significant difference in terms of protease activity was found compared to the untreated control. The β-amylase activity was reduced by 30% compared to the untreated control, but much less affected then after the dry microwave treatment (activity reduced by 80%). High pressure and sorbate treatments were found to have just little impact on the enzymatic activities tested. Vacuum packaging had no influence on the lipase and protease activities but reduced the β-amylase activity by 28% (34.5±0.3 U/g). The degree of damaged starch was found to be 4.9±0.5% for the untreated sample and remained without significant changes for all treatments apart from the dry microwave, which resulted in a substantially increased starch damage content (6.5±0.8%).

These results show that microwave treatment, in particular applied to the dry grains, has a negative impact on seed quality and viability, as all important enzyme activities are reduced. The reason behind is in the internal heat generated by the microwaves which does not only lead to inhibition of the fungus but also to

denaturation of the wheat proteins, including inactivation of the enzymes (Aron Maftei et al., 2014). An alternative to avoid this problem would be the repeated application of sub-lethal doses of high-frequency microwaves. According to Aron Maftei et al. (2014) this technique can inhibit microbial development based on disruption of the cell membrane and induced DNA damage, rather than internal heating. However, more studies are required to investigate this technique further. Then microbial inactivation using microwaves without grain quality deterioration could be possible. The increased degree of starch damage due to the microwave treatment can be attributed to the heating effect as well. Microwave heating of starches is reported to cause damage due to its heat induced gelatinisation (Fan et al., 2017). With regards to technological quality, enzymatic activities and a low degree of damaged starch are essential for a good baking performance of the resulting flour. The combination of the microwave treatment with a NaOCl-solution showed slightly better results. Starch damage and proteolytic activity remained unaffected while amylase and lipase appeared to be more sensitive and got inactivated similar to the dry microwave treatment. The explanation behind is in the reduced internal heating in the grains due to the liquid surrounding. However, microbial inactivation was still achieved as the microwaves provided the energy necessary to activate the hypochlorite radical which then was responsible for the fungal inactivation (Oms-Oliu, Martín-Belloso and Soliva-Fortuny, 2010).

Propionate, sorbate, vacuum and HHP treatments had no substantial effect on the grains enzymatic activities since they only affected the grains externally to inhibit fungal growth. The enzymes in the inner grain layers remained unaffected. Hence, despite its good microbial inhibition microwave treatments appear to be less suitable for industrial grain protection compared to the other treatments discussed here.

7.4.3.2 Soluble protein content and seize distribution

In order to determine the treatments impact on the various wheat proteins, soluble proteins were extracted using Osborne fractionation and the protein content of each fraction was determined using the method previously described by Bradford (1976). The fractions obtained include metabolically active proteins, such as enzymes, (albumins), structure proteins (globulins) as well as hydrophilic and hydrophobic storage proteins (prolamins and glutelins, respectively). The protein content of each fraction is shown in Table 21.

In correlation with the enzymatic activities discussed above, the albumin content determined was significantly reduced due to the dry microwave treatment. Likewise, the amount of globulins got substantially reduced due to the dry microwave treatment. Also the HHP treatment resulted in a decreased content of albumins although the enzymatic activities were not affected. However, it is possible that enzymes other than the tested lipases, β-amylase and proteases are sensitive to the HHP treatment. Also an increased solubilisation due to the treatment without actual inactivation is possible. Interestingly, in agreement with the increased enzymatic activities, the propionate treatment resulted in increased albumin content also. However, also the microwave + NaOCl treatment led to an increased albumin content but decreased concentration of globulins. The storage protein fractions, prolamins and glutelins, remained unaffected by the dry microwave treatment as well as the other treatments investigated. As for the total soluble protein content, no effects from the treatments could be determined apart from the dry microwave treatments, which resulted in substantially reduced protein solubility. Again, this is due to the heat denaturation of the grain proteins during treatment, affecting the grain quality and likely to be an issue in terms of technological performance.

Table 21: Mean soluble protein concentrations for the treated wheat samples[#].

Treatment	Bradford			
	albumins	globulins	prolamins	glutelins
natural (control)	7.7 ± 0.8^{a}	3.5 ± 0.5^{ab}	15.9 ± 0.6^{a}	1.8 ± 0.2^{a}
dry microwave	3.8 ± 0.1	1.5 ± 0.1	13.0 ± 0.7	1.9 ± 0.1^{a}
microwave +NaOCl	9.3 ± 0.1^{b}	2.9 ± 0.5^{ab}	15.8 ± 1.0^{a}	1.7 ± 0.1^{a}
vacuum packaging	7.8 ± 0.4^{a}	3.9 ± 0.2^{a}	16.2 ± 0.6^{a}	1.6 ± 0.1^{ab}
high pressure treatment	6.9 ± 0.1^{a}	3.7 ± 0.3^{ab}	15.8 ± 0.7^{a}	1.4 ± 0.1^{b}
sorbate	7.9 ± 0.5^{a}	3.5 ± 0.4^{a}	16.1 ± 0.8^{a}	1.6 ± 0.1^{ab}
propionate	10.3 ± 0.2^{b}	2.5 ± 0.3^{b}	14.6 ± 0.7^{a}	1.5 ±0.3^{ab}

[#] Results shown are mean values ± standard deviation. Values in one column followed by the same lower case letter are not significantly different (p<0.05).

Furthermore, each fraction was analysed using the Agilent Bioanalyzer. However, no visible differences were determined for the prolamin and glutelin fractions of each treatment compared to the control (data not shown). This is in correlation with the results of the Bradford assay, which showed significant but practically irrelevant

differences in terms of protein content of these 2 fractions. Figure 12 shows the obtained gel-images for the albumin (A) and globulin (B) fractions.

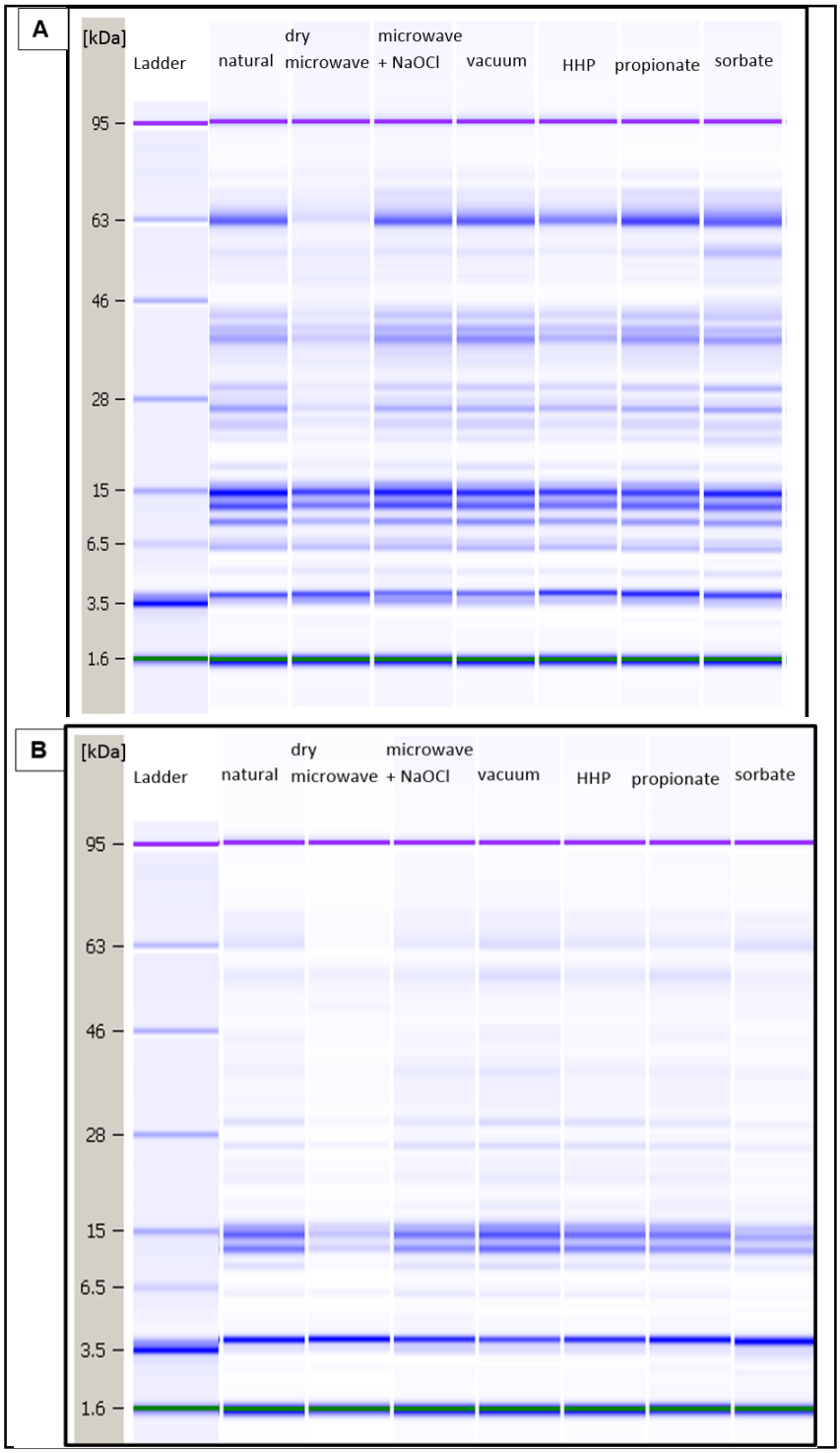

Figure 12: Gel-like images obtained from Bioanalyzer using an Agilent 80+ protein chip for A) albumin fraction and B) globulin fraction of the treated wheat samples.

In correlation with the Bradford assay it is visible that microwave and HHP treatments reduced the amount of albumins extracted. Furthermore, the propionate treatment resulted in increased intensity of a band at approximately 60 kDa, explaining the increased albumin content after the treatment. On the globulin gel however, the band at 60 kDa is visibly less intense compared to the other samples. Hence, it is likely that the salt exposure during the treatment has altered the solubility of the protein, shifting it from the globulin to the albumin fraction. Like on the albumin gel, both microwave treatments, in particular on dry grains, resulted in reduced intensity of all bands compared to all other samples. In conclusion, the results obtained from the Bioanalyzer are correlating well with the findings from the Bradford assay and the enzymatic activities. HHP and microwave treatments lead to protein denaturation and enzyme inactivation and therefore result in reduced visibility of the protein bands on the gel-image. Considering the importance of the enzymes and storage proteins for the technological performance of a flour, the results show a substantial quality deterioration. This applies mainly to the microwave treatment but to a lesser extent also to the HHP treatment. How much this affects the final product quality will be investigated further in section 7.4.4.

7.4.3.3 Gluten characterization

Due to the primary industrial use of cereals, and wheat in particular, being in the baking industry, the amount and quality of gluten is of major importance for the technological performance. Therefore, this section is to evaluate the impact of the various treatments on the gluten content and quality of the grains, using the Glutomatic and GlutoPeak. The results are summarised in Table 22.

Firstly, wet and dry gluten contents were determined to 33.1±2.0 g/100g and 11.3±0.2 g/100g, respectively. The only treatment resulting in significantly different results was the dry microwave decontamination. This treatment induced such severe damage to the gluten proteins that no network could form anymore and hence, no dry and wet gluten content could be determined. As a consequence, the water binding capacity and gluten index (GI), a measure of the network strength, could not be determined either. For all the other treatments however, no significant change in terms of water binding capacity or GI was found compared to the control. Furthermore, the gluten network strength and development time under high sheer force were determined using the GlutoPeak. Similarly to the Glutomatic results no gluten network could be formed after the microwave treatment, resulting in no

maximum network strength and development time determined. Furthermore, a slight decrease in gluten development time was found for the sorbate and propionate treatments compared to the control and the other treatments. This is likely due to residual propionic and sorbic acid from the treatment, decreasing the network development time (Farahnaky and Hill, 2007). No noteworthy changes in terms of maximum network strength were found for the treatments. All in all, with the exception of the dry microwave treatment, no substantial changes to the amount and quality of the gluten network were found due to the decontamination treatments. Hence it can be expected that these samples show good results not only in terms of microbial decontamination, but in technological performance in form of bread making quality.

Table 22: Mean results of gluten characterisation by Glutomatic and GlutoPeak for the treated wheat samples[#].

Treatment	Glutomatic				GlutoPeak	
	dry gluten content [g/100g]	wet gluten content [g/100%]	water binding capacity [g/100g]	Gluten index	peak time [s]	peak height [BU]
natural (control)	11.3 ± 0.2^{a}	33.1 ± 2.0^{a}	32.0 ± 2.0^{a}	94 ± 3^{a}	59 ± 2^{a}	70 ± 2^{a}
dry microwave	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
microwave +NaOCl	12.6 ± 0.7^{ab}	35.9 ± 1.7^{ab}	34.7 ± 1.6^{ab}	90 ± 7^{a}	60 ± 6^{ab}	68 ± 2^{a}
vacuum pakaging	11.5 ± 0.5^{ab}	34.4 ± 1.8^{ab}	33.6 ± 1.8^{ab}	97 ± 1^{a}	62 ± 1^{a}	70 ± 2^{a}
high pressure treatment	11.7 ± 0.5^{ab}	35.3 ±1.6^{ab}	34.1 ± 1.6^{ab}	97 ± 1^{a}	55 ± 2^{ab}	68 ± 2^{a}
sorbate	11.7 ± 0.3^{ab}	34.9 ± 0.4^{ab}	33.7 ± 0.3^{a}	96 ± 1^{a}	55 ± 1^{b}	69 ± 4^{a}
propionate	12.2 ± 0.3^{b}	36.6 ±0.4^{b}	35.4 ± 0.3^{b}	96 ± 1^{a}	53 ± 2^{b}	70 ± 3^{a}

[#] Results shown are mean values ± standard deviation. Values in one column followed by the same lower case letter are not significantly different (p<0.05).

7.4.4 Baking trials

The six treatments found to be efficient in fungal growth inhibition during the storage period were repeated on uninfected grains, in order to investigate their impact on the grains technological performance in terms of bread making. Following the treatment, the grains were milled to a fine whole grain flour and used for bread baking trials. The resulting breads are shown in Figure 13.

Figure 13: Pictures of the breads obtained from A) natural (untreated control), B) microwave (dry), C) microwave + NaOCl, D) high hydrostatic pressure, E) vacuum packed, F) propionate and G) Sorbate.

Subsequently, the following quality parameters were analysed: bake loss, specific loaf volume, crumb lightness (Table 23), as well as crumb texture and physical crumb structure (Table 24).

Table 23: Mean results of loaf characteristics for the treated wheat samples[#].

Treatment	bake loss [%]	specific loaf volume [mL/g]	crumb lightness [%]
natural (control)	5.3 ± 0.3^{a}	3.1 ± 0.2^{a}	63 ± 2^{a}
dry microwave	4.0 ± 0.2^{b}	1.4 ± 0.1	66 ± 2^{ab}
microwave +NaOCl	4.5 ± 0.3^{bc}	2.4 ± 0.3^{b}	65 ± 3^{ab}
vacuum packaging	5.1 ± 0.4^{ac}	2.7 ± 0.2^{ab}	63 ± 3^{a}
high pressure treatment	4.8 ± 0.3^{ac}	2.3 ± 0.2^{b}	63 ± 3^{a}
sorbate	4.0 ± 0.3^{a}	2.9 ± 0.3^{a}	69 ± 2^{b}
propionate	4.5 ± 0.2^{c}	2.4 ± 0.1^{b}	70 ± 2^{b}

[#] Results shown are mean values ± standard deviation. Values in one column followed by the same lower case letter are not significantly different ($p<0.05$).

Table 24: Mean results of crumb characteristics for the treated wheat samples[#].

Treatment	no of cells / slice area	area of cells [%]	cell diameter [mm]	crumb hardness		
				day 0	day 3	day 5
natural (control)	1180 ± 95[a]	53 ± 1[a]	2.8 ± 0.5[a]	6.8 ± 1.5[a]	16.0 ± 3.5[a]	17.9 ± 3.6[ab]
dry microwave	785 ± 58	51 ± 1[a]	3.1 ± 0.5[a]	48.3 ± 20.4	92.8 ± 28.6	115.6 ± 47.9
microwave +NaOCl	1199 ± 121[a]	52 ± 2[a]	2.4 ± 0.6[a]	9.9 ± 2.5[ab]	22.8 ± 6.6[a]	28.6 ± 9.1[ab]
vacuum packaging	1067 ± 69[a]	53 ± 1[a]	2.8 ± 0.4[a]	7.8 ± 2.2[ab]	17.6 ± 3.9[a]	23.2 ± 8.0[ab]
high pressure treatment	1027 ± 118[a]	52 ± 1[a]	2.5 ± 0.3[a]	10.7 ± 4.0[b]	22.6 ± 6.0[a]	27.7 ± 10.3[ab]
sorbate	1310 ± 119[a]	50 ± 1	1.9 ± 0.4	9.6 ± 2.4[ab]	21.5 ± 3.6[a]	26.3 ± 5.2[ab]
propionate	1231 ± 146[a]	52 ± 2[a]	2.3 ± 0.4[a]	5.8 ± 2.1[a]	14.6 ± 4.1[a]	16.9 ± 3.1[a]

[#] Results shown are mean values ± standard deviation. Values in one column followed by the same lower case letter are not significantly different (p<0.05).

Firstly, the bake loss (weight reduction during baking) and specific loaf volume were determined for the breads resulting from each treatment. The flour from untreated grains resulted in a specific volume of 3.1±0.2 mL/g. A significantly reduced specific volume was found due to both of the microwave treatments, HHP and the propionate treatment. However, as Figure 13B shows, by far the biggest impact was a result of the dry microwave treatment, resulting in a specific loaf volume of 1.4±0.1 mL/g. In general a high specific volume is desired by industry and consumers, meaning a compromised quality and marketability due to the decontamination treatment. However, changes in terms of bake loss due to the various treatments were found to be insignificant.

Furthermore, the colour of the bread crumb was evaluated in terms of its brightness. Compared to the untreated control, only the sorbate and propionate treatments showed a significant difference. These two treatments resulted in darker colour of the bread, most likely due to residual acids on the grains from the washing treatment, increasing the rate of Maillard reaction. Analysis of the bread crumb structure using C-cell analysis shows that no noteworthy differences in terms of percentile cell area per slice result from any of the treatments compared to the control. However, the dry microwave treatment caused a more open structure with fewer but bigger cells compared to the control. In contrast, the sorbate treatment resulted in a rather closed crumb structure with significantly smaller cells compared

to the control, as can be seen in Figure 13 as well. The other treatments had no significant impact on the bread crumb structure. Finally, the physical crumb texture was analysed using texture profile analysis (TPA) on day 0, 3 and 5 after baking. Most noticeably is the substantially increased crumb hardness after dry microwave treatment, compared to all other samples. The other treatments showed no significant differences to the control over the 5 days of analysis. This shows that the treatments did not result in an accelerated rate of staling and therefore did not compromise the product quality.

As it was expected from the flour analysis, the dry microwave treatment resulted in significantly reduced final bread quality. This is primarily attributed to the heat induced denaturation of gluten and metabolically active enzymes as well as the increased starch damage (Goesaert et al., 2005). The other treatments were found to have no substantial impact on the final bread quality for all parameters tested. So the impact on the proteins due to the HHP and microwave + NaOCl treatments had no practical relevance and did not compromise the final bread quality. This proofs that vacuum packaging, HHP, sorbate, propionate and microwave + NaOCl treatments were able to successfully inhibit fungal development during storage without compromising the bread making ability of the grains.

7.5 Conclusions

In conclusion, out of the 13 treatments tested for their suitability as post-harvest decontamination treatment for wheat grains prior to storage 5 were found to provide promising results. Unfortunately, microbial decontamination, using antifungal LAB was not found to be suitable to inhibit fungal development during storage. Of the 7 chemical treatments, only propionate and sorbate salts inhibited the fungal growth and mycotoxin production. However, both washing procedures require chemicals which have to be removes afterwards. Despite the fact that both are salts derived from organic acids, this would result in low consumer acceptance. Furthermore, an up-scaling would be expensive and relatively impractical. The other washing procedures were not effective and CAPP was found to be unsuitable for solid samples with uneven surfaces, as found with the grains here.

While most physical treatments showed good results in terms of fungal inhibition during storage, the microwave treatments, in particular on dry grains, had a negative impact on the grain quality. Damage due to the microwave induced heating was so severe that enzymes and gluten proteins got severely damaged, resulting in poor flour and bread quality. The ultrasound treatment showed no satisfying results either, as it could not inhibit the fungal development during storage. This can be attributed to the impracticality of the procedure for solid samples like cereal grains. HHP treatments, despite the fungal inhibition with no substantial impact on the final bread quality would be difficult to apply for industrial use. The inability of application in an on-line process and the requirement of subsequent drying could proof impractical and expensive during industrial application.

The most efficient method found in this study is the vacuum packaging. It resulted in total fungal inhibition combined with no noteworthy grain and bread quality deterioration. Furthermore, it is a cost effective, easy to apply method, allowing a “clean label” for the resulting product. So the approach is suitable even for underdeveloped countries. On the other hand, the absence of chemical preservatives ensures great consumer acceptance worldwide. Hence, the comparison of traditional methods with the novel approaches revealed that the traditional methods are still superior to the emerging ones. In particular the storage under vacuum is more efficient, easy to apply and cost effective than the new approaches investigated here. These results make it evident that emerging technologies, such as plasma and ultrasound require further research and optimisation before industrial application becomes feasible.

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Chapter 8: Characterisation of the antifungal activity of the cowpea defensin Cp-thionin II after partial purification

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8.1 Abstract

As a result of the rapidly growing human population, reducing post-harvest crop losses of cereals due to microbial pests has major importance. Plant defensins have the potential to fulfil these demands, beeing highly specific and efficient antimicrobial agents. Hence, this study aimed to extract and characterise a peptide from cowpea seeds and investigate its antifungal performance. After extraction and partial purification, N-terminal sequencing was used to identify the primary peptide in the extract as cowpea-thionin II. Antifungal activity was found against *Fusarium culmorum* (MIC = 50 µg/mL), as well as *Aspergillus niger* and *Penicillium expansum* (MIC > 500 µg/mL against both). The extract was resistant against heat treatment (100°C, 15 min) but lost its antifungal activity in presence of cations (Na^+, K^+, Ca^{2+} and Mg^{2+}, respectively). Membrane permeabilization of fungal hyphae was evident at 25 µg/mL, while induction of oxidative stress only had minor contribution to the antifungal performance. The extract did not induce haemolysis at all concentrations tested (up to 200 µg/mL). Finally, it was successfully used to protect stored wheat grains from fungal spoilage (determined via ergosterol content) when applied at 100 µg/mL. In conclusion, the defensin Cp-thionin II showed the potential for future application as food bio-preservative.

8.2 Introduction

Satisfying the nutritional demands of the rapidly growing global population has, during recent decades, turned into an ever increasing challenge. Due to the limited resources and agricultural area available, research has focused on improved efficiency in terms of food production and preservation. It was estimated that with the current amounts of food waste, the food production has to increase by 60 - 110% until 2050, in order to feed the global population (Ray et al., 2013; Tilman et al., 2011). The reason behind this rapid increase is not only the growing population, but also the predicted increase in food consumption per capita (Tilman and Clark, 2014). In addition, recent studies have demonstrated that the approaches to increase the yield of agricultural crops, such as cereals, are not sufficient and sustainable to satisfy the global demands of future generations (Ray et al., 2013). Considering the importance of cereals for human nutrition over the last centuries (approximately 70 kg per person per year), this poses a major problem (Albertson et al., 2016). Hence, research and industry are trying to reduce crop losses and increase the sustainability.

One of the main reasons of food waste is the microbial spoilage of the crops in-field, as well as post-harvest. According to Freita-Silva, de Oliveira and Freire Júnior (2014), approximately 15% of the global cereal production is lost due to microbial spoilage. While the reduction of in-field spoilage is intensely investigated (Mannaa and Kim, 2017), the equally important microbial protection after harvest and during storage is often overlooked. However, up to 20% of the harvested cereals worldwide turn into food waste, mainly as a result of microbial spoilage during storage and downstream processing (Ridolfi, Hoffman and Siddhartha, 2018). Microbial contaminants include bacteria, yeasts and filamentous fungi. For cereals, fungi belonging to the genera of *Fusarium*, *Aspergillus* and *Penicillium* are the most commonly found spoilage organisms (Russo et al., 2017). Growth and development of these fungi during storage result in grain quality deterioration and, if not disposed, potential consumer health hazards (Schmidt et al., 2016; Schmidt et al., 2018; Tournas and Niazi, 2018). In parallel, consumer acceptance for conventional food preservation is decreasing continuously. Consumers demand “clean-label” products combined with high standards in terms of food safety and quality (Figiel and Kufel, 2016). Therefore, research has to focus on new, natural approaches to ensure microbial safety, in order to meet consumer`s desire and nutritional demands.

One approach of bio-preservation that recently received a lot of research interest are plant-derived antimicrobial peptides (AMPs). The overexpression of plant defensins as a response to pathogen exposure and the localisation in tissues with preferential cell-wall location in epidermal cells suggest that they play a major role in the defense mechanisms of the plants (Garcia-Olmedo et al., 1998). As a consequence, AMPs are highly specific against spoilage organisms while usually harmless for humans (Javadpour et al., 1996; Thevissen et al., 2004), making them highly promising candidates for efficient bio-preservatives.

Commonly, plant AMPs are 5 – 8 kDa peptides, comprised of 45 to 54 amino acid residues and have an isoelectric point around 9 (Thery and Arendt, 2018). The global fold of plant AMPs consists of one α-helix and one β-sheet, which are stabilised by disufide bonds (Lay, Brugleria and Anderson, 2003). The mode of action of plant defensins is manifold and not fully understood yet (Thevissen et al., 2004). It usually involves binding of the peptide to the microbial cell membrane. The peptide then can induce inhibiting mechanisms either on the cell surface or after internalisation into the cell. Inhibitory mechanisms include modification of ion fluxes or the activation of enzyme pathways.

One such peptide, previously reported as natural antibacterial agent is the cowpea-thionin II (Cp-thionin II). It can be found in various tissues of the plant, with highest concentrations in the seeds. During germination, the peptide concentration was found to decrease (Franco et al., 2006). This suggests that the peptide is part of the natural plant defense mechanism, making antifungal activity likely. To the best of the authors` knowledge, the antifungal activity of natural Cp-thionin II against *F. culmorum*, *A. niger* and *P. expansum* has not been studied previously. However, a recent study with a synthetic linear analogue of the peptide showed promising results (Thery and Arendt, 2018).

Previous studies have shown the thermal stability of numerous plant defensins (Broekaert et al., 1995; Terras et al., 1992), which increases their potential as food preservatives. On the other hand, plant defensins often are sensible to the presence of cations (Vriens, Cammue and Thevissen, 2014), which could be a major drawback for food applications. Regarding the mode of action of the peptide against fungal hyphae, the induction of membrane permeabilization and oxidative stress towards the fungal cells are discussed (Thery and Arendt, 2018). Another important consideration for the application as preservative is the consumer safety. Although plant defensins are usually nontoxic towards mammalian cells (Thevissen et al.,

2004), depending on the amount of disulfide bonds and their hydrophobicity and amphipathicity some toxic effects have been reported (Hollmann et al., 2016; Jenssen, Hamill and Hancock, 2006). Therefore, the haemolytic activity against mammalian red blood cells is of further interest to characterise the peptide. Finally, the application of natural AMPs as food preservatives was reported by several researchers (Lucera et al., 2012; Rai et al., 2016; Rydlo, Miltz and Mor, 2006). However, the environmental conditions and sample matrix play an essential role for the efficiency of the peptide. Hence, it is uncertain if the here investigated application as preservative during cereal storage can be successful.

The results of this study provide important information regarding the potential of AMPs in general and Cp-thionin II in particular as bio-preservative. Hence, it increases the knowledge regarding this highly promising approach to naturally reduce food losses and increase sustainability to satisfy the global nutritional requirements.

8.3 Materials and Methods

8.3.1 Extraction and partial purification of the peptide

Extraction of the peptide from commercial organic cowpea (*Vigna unguiculata*) seeds was based on the method described by Franco et al. (2006), with some modifications. In brief, organic cowpea seeds were milled to a fine flour, using a coffee grinder, and extracted with 0.1 M HCl / 0.15 M NaCl buffer (meal : buffer ratio 1:5) under continuous stirring for 4 h at 5°C. Subsequently, the supernatant was neutralised (using NaOH), filtered (pore size 0.45 µm) and saturated with 60% ammonium sulphate. The precipitate, formed overnight, was extensively dialysed against distilled water (2.0 kDa upper cutoff, Sigma-Aldrich), lyophilised and resuspended in equilibration buffer (0.15 M Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$). The obtained crude extract was applied to anion exchange chromatography, using a HiTrap™ DEAE FF (1 mL) column (GE Healthcare). Chromatography was carried out using an AKTA protein purification system (GE Healthcare Life Sciences). The flow rate used was 1.0 mL/min and the eluted fractions (1.0 mL) were collected. Equilibration buffer (buffer A) was used to elute the non-retained fraction, while retained proteins were displaced from the column using buffer B (0.15 M Tris/HCl buffer + 1 M NaCl, pH 7, containing 5 mM $CaCl_2$). The non-retained fraction was applied to cation exchange chromatography, using a HiTrap™ SP HP (1 mL) column (GE Healthcare) with the same conditions. The retained protein fractions were eluted with a gradient of 0 - 100% buffer B, applied over 40 min, and collected for further analysis.

After further dialysis at room temperature against distilled water and lyophilisation, the residue was redissolved in distilled water and used as stock solution for further analysis. The Pierce™ BCA Protein Assay Kit (Thermo-Fischer Scientific) was used according to the supplier`s instructions to determine the protein concentration of the solution. The purity of the extract was assessed by SDS gel electrophoresis, using a Tris/tricine precast gel (Bio-Rad), stained with Coomassie blue G-250. Sample preparation and electrophoresis of the native and denatured (heated for 7 min in presence of 9.5 mg dithiotreitol/mL) sample were carried out according to the supplier`s instructions (Bio-Rad). The band migrating at approximately 6 kDa was used for N-terminal sequencing by Edman degradation (5 residues), carried out by LakePharma (Belmont, USA). A BLAST analysis on UniProt protein database was

used to identify the peptide based on the first five amino acids determined by Edman sequencing.

8.3.2 Circular Dichoism (CD)

The analysis of the secondary structure of the extracted peptide was carried out using circular dichroism (CD) spectroscopy according to the method described by Liu et al. (2008). In brief, the extract was diluted in deionized water or 20 mM sodium dodecyl sulphate (SDS) to a final protein concentration of 1 mg/mL. CD measurements were performed using a Chirascan CD Spectrometer (Applied Photophysics), at 27°C within a wavelength range of 180-260 nm. Each solution was measured in triplicate and the solvent CD was subtracted from the sample CD.

8.3.3 Fungal strains

Three different species of filamentous fungi commonly found on cereal products, namely *Fusarium culmorum*, *Aspergillus niger* and *Penicillium expansum,* were investigated. The fungal strains *F. culmorum* FST 4.05, *A. niger* FST4.21 and *P. expansum* FST 4.22 originated from the culture collection of School of Food and Nutritional Sciences, University College Cork (Cork, Ireland).

8.3.4 Antifungal activity assay

The antifungal activity of the cowpea extract was determined by following germination and growth of fungal conidia in a microtiter plate assay, as described by Van Der Weerden, Lay and Anderson (2008). Briefly, fungal conidia were collected from colonies grown for 72 h on potato dextrose agar (PDA) (Sigma Aldrich) at 25°C and diluted to a final concentration of 10^4 spores/mL in half strength potato dextrose broth (½ PDB), using a haemocytometer. Filter sterilised extracts (20 µL) and fungal spore suspension (180 µL) were combined in the wells of a 96-well microtiter plate. Final peptide concentrations in the mixture were ranging from 500 µg/mL to 6 µg/mL. Fungal growth was followed over 96 h at 25°C by measurement of the optical density (OD) at 620 nm (Multiscan™, Thermo Scientific). Addition of 20 µL of 0.1% acetic acid or sterile distilled water to the fungal spore suspensions were used as negative and positive control, respectively.

Additionally, the inhibition of fungal growth was controlled on PDA plates. Fungal spore suspension was added to warm ½ PDA to a final concentration of 10^4 spores/mL and poured into a sterile petri dish (20 mL). After solidification, 4 wells were cut into the agar and filled with 50 µL of extract, containing 0 – 200 µg protein/mL. A well containing 50 µL of 0.1% acetic acid was prepared similarly as negative control. The extract was allowed to diffuse into the agar and the plates subsequently incubated for 3 d at 25°C. Fungal growth inhibition was evaluated by measuring the halo around the wells.

8.3.5 Determination of the minimal inhibitory concentration (MIC) and half maximal inhibitory concentration (IC_{50})

After 96 h of incubation at 25°C, the MIC was determined as the lowest peptide concentration that completely inhibited fungal growth. The concentration required to inhibit the fungal development by 50% (IC_{50}) was determined by non-linear regression, using the software graph PRISM (GraphPad Software, Inc., La Jolla, CA) with the microplate reader data.

8.3.6 Thermal stability

In order to study the thermal stability of the extract, the peptide solution was heated at 100°C for 15 min. After cooling to room temperature (30 min), the antifungal activity of the extract at MIC was determined against *F. culmorum* in a 96-well microtiter plate assay, as described in section 8.3.4.

8.3.7 Effect of cations on the antifungal activity

The influence of various cations on the antifungal performance of the cowpea extract was investigated in an antifungal assay, performed in different salt solutions, as described by Terras et al. (1992). *F. culmorum* spores (10^4 spores/mL) were inoculated in ½ PDB, containing 100 mM NaCl, 50 mM KCl, 5 mM $CaCl_2$ or 5 mM $MgCl_2$, respectively. After addition of the peptide solution (50 µg/mL), fungal growth was followed in a microtiter plate assay, as described in section 4.3.4.

8.3.8 Membrane permeabilization assay

A membrane permeabilization assay was carried out on *F. culmorum* hyphae based on the method described by Van Der Weerden, Lay and Anderson (2008), with some modifications. Fungal hyphae were grown overnight at 25°C in ½ PDB from a suspension of 10^4 conidia/mL. Following centrifugation (10 min, 5,000 g), the hyphae were washed twice with and resuspended in synthetic fungal medium (SFM; prepared as described by Rodríguez et al. (2003)). The extract was added to a final peptide concentration of 100, 50 (MIC), 25 and 12.5 µg/mL, respectively. Solutions of hyphae without peptide or with 1% Triton X-100 (Sigma-Aldrich) were used as negative and positive controls, respectively. After incubation for 2 h at 25°C, the fluorophor propidium iodide was added to a final concentration of 0.5% and the mixture was incubated for 10 min at room temperature in the dark. Subsequently, fluorescence of fungal hyphae was measured using a fluospectrophotometer (Varioscan® LUX reader) with excitation and emission wavelengths of 488 nm and 538 nm, respectively or examined using confocal laser scanning microscopy (CLSM) (Olympus) (excitation wavelength 460 -490 nm).

8.3.9 Induction of reactive oxygen species (ROS)

The measurement of ROS was carried out based on the method of Van Der Weerden, Lay and Anderson (2008) with some modifications. *F. culmorum* hyphae (grown as described above) were treated with water or cowpea extract (containing various concentrations of peptide) for 12 h before incubation with dihydrorhodamine 123 (Sigma-Aldrich) (10 µg/mL) for 2 h. After extensive washing with 0.6 M KCl fluorescence of the hyphae was visualised using a fluorescence microscope (Olympus) (excitation wavelength 460 – 490 nm) and measured using a fluospectrophotometer with excitation and emission wavelengths of 488 nm and 538 nm, respectively. Hyphae treated with water and H_2O_2 (1% w/v) were analysed as negative and positive controls, respectively.

8.3.10 Haemolysis assay

The peptide solution was studied for its ability to induce haemoglobin release from fresh defibrinated sheep erythrocytes, as described previously by (Laverty et al. 2010). Fresh sheep red blood cells (Thermo Fischer Scientific) were washed three

times with equal volumes of phosphate buffered saline, pH 7.4 (PBS). After centrifugation for 15 min at 900 g, the erythrocytes were resuspended in PBS to a final concentration of 4% (v/v). In a 96-well microtiter plate, 20 µL of peptide solution (different concentrations in PBS) and 80 µL of the erythrocyte suspension were combined and incubated for 1 h at 37°C. Subsequently, the suspension was centrifuged (10 min, 1,000 g) and the supernatant was transferred to a new microtiter plate. The release of haemoglobin was measured spectrophotometrically at 405 nm. Erythrocytes treated with 0.1% Triton X-100 in PBS and PBS alone were treated similarly as positive and negative controls, respectively. The percentage of haemolysis was calculated as published by (Laverty et al. 2010).

$$\%\, Haemolysis = \frac{(Abs405\ peptide\ treatment) - (Abs405\ PBS)}{(Abs405\ 0.1\%\ triton\ X - 100) - (Abs405\ PBS)}$$

The release of haemoglobin was determined for six replicates.

8.3.11 Wheat grain spoilage protection

Wheat grains, supplied by Doves Farm Foods Ltd (Hungerford, UK), were disinfected according to the method described by Oliveira et al. (2012). Briefly, 300 g of grains were disinfected in 2 L 10% (w/v) hydrogen peroxide (H_2O_2) solution for 10 min with continuous stirring. Subsequently, the grains were washed for 5 min in 4 L distilled water. This procedure was repeated once, but with only 5 min of disinfection. Immediately, the grains were moved to sterile plastic boxes and dried at room temperature for 24 h under vertical sterile laminar flow. Finally, the grains were exposed to ultraviolet light (10 min) and collected aseptically for further use.

For preparation of contaminated wheat, disinfected grains were mixed with 2% (v/w) spore suspension of *F. culmorum* (10^4 spores/mL). After 10 days of incubation at 25°C, complete fungal proliferation of the grains was visible and the grains were defined as 100% infected.

Infected and disinfected grains were mixed to samples of 100 g (dry matter), containing 5% infected kernels. Subsequently, the samples were sprayed with 2% (v/w) of sterile-filtered extract (protein concentration of 0, 25, 50 and 100 µg/mL, respectively). Glacial acetic acid was applied similarly as a control. Each sample was then divided into 6 portions and filled into sterile plastic bags. The bags were sealed, perforated with two pipette tips, containing a barrier filter to allow gas

exchange, and stored at room temperature. After 0 and 6 weeks of storage, 3 portions of each sample were collected, milled to a whole grain flour (particle size < 2mm), homogenised and stored at -20°C until further analysis.

8.3.12 Determination of Ergosterol

The total ergosterol content before and after storage was determined based on the method of Jedlickova et al. (2008). In brief, 10 g of milled grains were extracted with 50 mL of methanol under constant shaking at room temperature for 30 min. After centrifugation, 25 mL of the supernatant were transferred into a tube containing 3 g KOH and shaken until the KOH had fully dissolved. Subsequently, 10 mL of n-hexane were added and the mixture was incubated for 30 min at 65°C. After cooling to room temperature, 5 mL distilled water were added and the upper layer collected. The extraction with n-hexane was repeated 3 times, the combined extracts evaporated till dry and the residue re-dissolved in 5 mL of methanol before analysis by HPLC.

The RP-HPLC column used was a Nova-Pak C_{18} (300 x 3.9 mm, 4 µm) (Agilent Technologies). Peak-identity was verified using the UV-spectra recorded by the DAD. The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the signal/noise (s/n) ratio. The LOD was set for s/n of 3:1 and the LOQ was set for s/n of 10:1. For calibration, ergosterol standards between 1.0 and 200 µg/mL in methanol were prepared and analysed.

8.3.13 Statistical analysis

Statistical analysis was carried out using Microsoft XLSTAT Version 2015.5.01. (Adinosoft Inc, New York, USA). Standard deviations were calculated for absorbance values at each peptide concentration of the extract based on triplicates, unless otherwise stated. The effect of the various salts and the heat treatment on the antifungal performance of the peptide was analysed with one-way ANOVA followed by a Tukey-Kramer HSD test to identify differences relative to the control. All cases with $p<0.05$ were considered as significant. The same statistical analysis was carried out to determine individual differences in haemolysis activity for each concentration of the peptide in comparison to the negative control.

8.4 Results and discussion

8.4.1 Extraction, partial purification and identification of the cowpea peptide

The purification of the crude extract containing Cp-thionin II, obtained from the cowpea seeds, was carried out by ion-exchange chromatography using Red-Sepharose columns. The anion exchange resulted in retention of one fraction which showed no antifungal activity (data not shown). The unbound fraction was further purified by cation exchange. After disposal of the unbound fraction, 6 peaks were eluted, using 1M NaCl solution, and collected. The first 5 peaks showed no antifungal activity (data not shown) and were not investigated further. The major peak (Fig. 14, black arrow) was used for further analysis and dialysed against distilled water, lyophilised and re-dissolved to a final concentration of 10 mg/mL. The purity of the so obtained fraction was analysed by SDS-gel electrophoresis (Fig. 14). It is visible in Figure 14 (bottom) that the denatured extract shows an intense band at approximately 6 kDa, as well as 2 bands with lower intensity at approximately 17 and 26 kDa. In contrast, the native sample has no band migrating at 6 kDa, but therefore a very broad band at 14.5 – 16 kDa, followed by 2 bands at approximately 17 and 26 kDa with very low intensity. This shows that the main peptide of the extract, when suspended in sample buffer, occurs primarily in di- and trimers. After denaturation, the dimers were broken into the monomers visible on the gel. The 2 bands at higher molecular weight show that the purification of the cowpea peptide was only partial. However, it is also visible that the band migrating at 6 kDa has the highest intensity and hence, contains the peptide primarily responsible behaviour of the extract during subsequent analysis. In addition, the UniProt database holds no information regarding a peptide from cowpea seeds with 17 or 26 kDa that exhiits antimicrobial activity. Therefore, the results of the following sections are primarily attributed to the band migrating at 6 kDa.

The N-terminal protein sequencing of the 6 kDa peptide by Edman degradation was used to identify the extracted peptide based on the first 5 amino acid residues. Table 25 shows a comparison of antimicrobial peptides previously extracted from cowpea seeds with the first 5 amino acid residues of the peptide characterised in this study. Based on the comparison of the sequences the extracted peptide was identified as cowpea-thionin II (Cp-thionin II).

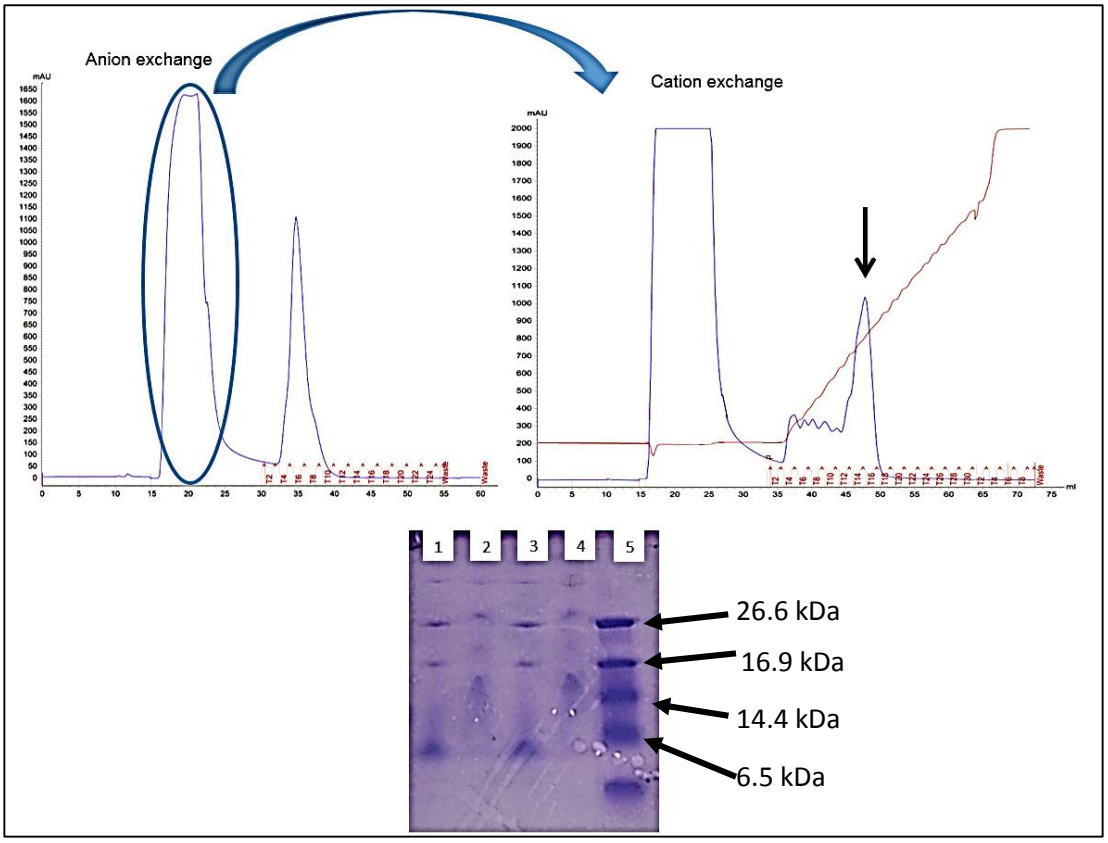


Figure 14: Top left: Chromatogram obtained from anion exchange column (HiTrap™ DEAE FF) with un-retained fraction (eluted with equilibration buffer containing 0.1 M HCl and 0.15 M NaCl) transferred to cation exchange column (HiTrap™ SP HP) (top right). The retained proteins were eluted with a mixture of equilibration buffer without (A) and with (B) 1 M NaCl added, using a gradient from 0 – 100% B over 40 minutes. The fraction used for further investigation is marked with a black arrow. Bottom: SDS gel electrophoresis of the fraction obtained from cation exchange chromatography. 1;3 – denatured sample, 2;4 – native sample, 5 – ladder.

In order to obtain further information regarding the structure of the extracted protein, circular dichroism spectroscopy was performed and the results compared to the proposed 3D model of Cp-thionin II (Figure 15). The 3D model shows the peptide as one single subunit containing 3 β-sheet and one α-helical structure. Overall, the conformation of the peptide is stabilized by 4 disulfide bonds between cysteine residues, which are displayed in ball-and-stick form (yellow). This motif (CSαβ) of Cp-thionin II is typical for native defensins (Almeida et al., 2002). In good correlation with the 3D model are the CD spectra of the extract. In both solvents (water and 20 mM SDS), a slightly positive peak was found at ~190 nm, followed by a crossover at ~200 nm and a minimum at ~210 nm. Furthermore, it is noteworthy that the positive peak is slightly bigger in water, while in SDS a much bigger negative peak was found. The shape of both graphs indicates good structured conformers, which is due

to the 4 disulfide bonds stabilising the peptide. Interestingly, Thery and Arendt (2018) found similar results in SDS but a much less structured conformation in water. The explanation behind is the lack of disulphide bonds in the synthetic linear analogue studied there. Furthermore, the peak minima indicate a propensity for helical conformations, as can be found in the 3D model. In addition, CD spectroscopy revealed a slightly higher percentage of helical conformation in SDS, combined with a slight reduction of random coils (data not shown). Overall, the differences between the conformations in the 2 solvents are marginal, as the disulphide bonds ensure a structured conformation in both solvents.

Table 25: Characteristics of the peptide extracted from cowpea seeds compared with related antimicrobial peptides (amino acids containing a disulfide bond are underlined).

Name	Source	Sequence	Activity	Function	Reference
peptide extracted here	*Vigna unguiculata*	KTCMT-	*F. cumorum* *A. niger* *P. expansum*		this study
Cp-thionin II	*Vigna unguiculata*	KTCMTKKEGWGRCLIDT TCAHSCRKYGYMGGKC QGITRRCYCLLNC	Gram-positive *S. aureus* Gram-negative *E. coli*, *P. syringae*	γ-tionin	(Franco et al., 2006)
Cp-thionin	*Vigna unguiculata*	RVCESQSGFKGACTGD HNCALVCRNEGFSGGN CRGFRRRCFCTLKC	unknown	Trypsin inhibitor	(Melo et al., 2002)
Linear peptide KT43C	synthetic peptide	KTCMTKKEGWGRCLIDT TCAHSCRKYGYMGGKC QGITRRCYCLLNC	*F. cumorum* *A. niger* *P. expansum*		(Thery and Arendt, 2018)

As shown by Franco et al. (2006), Cp-thionin II is a peptide consisting of 46 residues, including 8 cysteines arranged in a typical disulphide bond pattern (C1-C8 / C2-C5 / C3-C6 / C4-C7) (Lay, Brugliera and Anderson, 2003). It was reported to belong to the super family of γ-thionins, also known as defensins. The molecular weight determined is with approximately 6 kDa very close to the previously reported

5.2 kDa for both natural (Franco et al., 2006) and synthetic peptide (Kraszewska et al., 2016; Thery and Arendt, 2018). Due to the impurities in the extract and the limited accuracy of the SDS-PAGE it was not possible to determine the molecular weight more accurately. Additionally, the extraction method used by Franco et al. (2006) was found to result in monomers of the defensin only. In contrast, the extraction method applied here also resulted in di- and trimers. If this has an impact on the antimicrobial performance is unclear, as the inhibiting effect of residues is not fully understood yet. Franco et al., (2006) further demonstrated the antibacterial properties of the peptide. However, to the best of the authors` knowledge and according to the PhytAMP database, no studies regarding the antifungal performance of the peptide exist. In particular, regarding the potential application as food bio-preservative, the activity against common food spoilage fungi is of interest. Also the stability against environmental stress factors and the potential health risk due to consumption were investigated in this study.

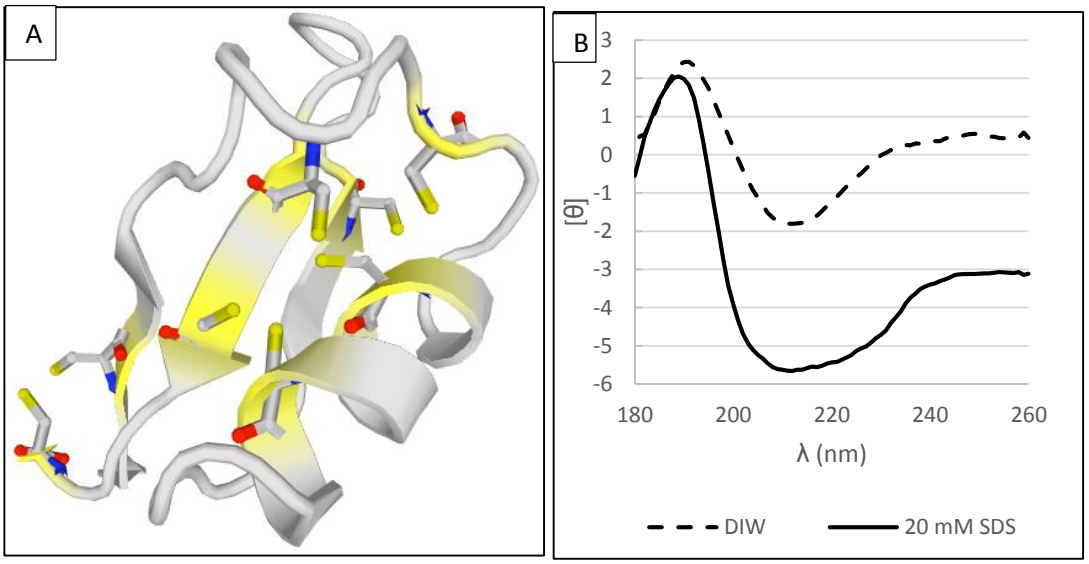


Figure 15: A) Swiss model P84920 for the predicted 3D structure of the peptide cowpea-thionin II. The disulfide bonds between the cysteine residues are shown in ball-and-stick form (yellow). B) Circular dichroism (CD) spectrum obtained for the cowpea extract in water (broken line) and 20 mM sodium dodecyl sulfate (solid line).

8.4.2 Antifungal activity of the cowpea extract

The extract containing Cp-thionin II showed antifungal activity against the spores of 3 major food spoilage fungi, namely *F. culmorum*, *A. niger* and *P. expansum* (Fig. 16). The strongest inhibition of fungal spore germination was found against *F. culmorum.* Substantial fungal growth inhibition after 96 h was achieved for concentrations of 25 µg/mL or higher. Based on the results of the microtiter plate assay, the IC_{50} and MIC were determined as 40 µg/mL and 50 µg/mL, respectively (Fig. 16A). Peptide concentrations required to inhibit growth of *P. expansum* and *A. niger* were found to be much higher compared to *F. culmorum.* MIC-values against both fungi were above the highest concentration investigated here (500 µg/mL, Fig. 16B and 16C). However, spore germination and fungal growth *of P. expansum* and *A. niger* after 96 h were substantially reduced for the highest peptide concentration tested (500 µg/mL). This demonstrates the antifungal activity of Cp-thionin II against all 3 fungi tested, despite the relatively low activity against *A. niger* and *P. expansum*. The inhibition of fungal spore germination was further assessed on PDA plates, which confirmed the results obtained from the microtiter plate assay (data not shown).

In agreement with previous studies, the partially purified cowpea extract showed substantial antifungal activity against all three fungi investigated. The peptide was previously reported by Franco et al. (2006) and Kraszewska et al. (2016) for its antibacterial activity against both Gram-negative and Gram-positive strains. Furthermore, Thery and Arendt (2018) demonstrated the antifungal activity of a synthetic, linear analogue of Cp-thionin II against fungi belonging to the genera of *Fusarium*, *Aspergillus* and *Penicillium*. An explanation for the antifungal performance of the defensin is suggested to be in the structural similarity with the human beta-defensin 3 (Kraszewska et al., 2016). However, it was further reported by Kraszewska et al. (2016) that, apart from structural similarity, the overall net charge of the peptide has a major impact on the antimicrobial performance. Additionally, other researchers reported the importance of disulfide bounds, hydrophobicity and amphipaticity for the overall antimicrobial performance (Hollmann et al., 2016, Jenssen, Hamill and Hancock, 2006).

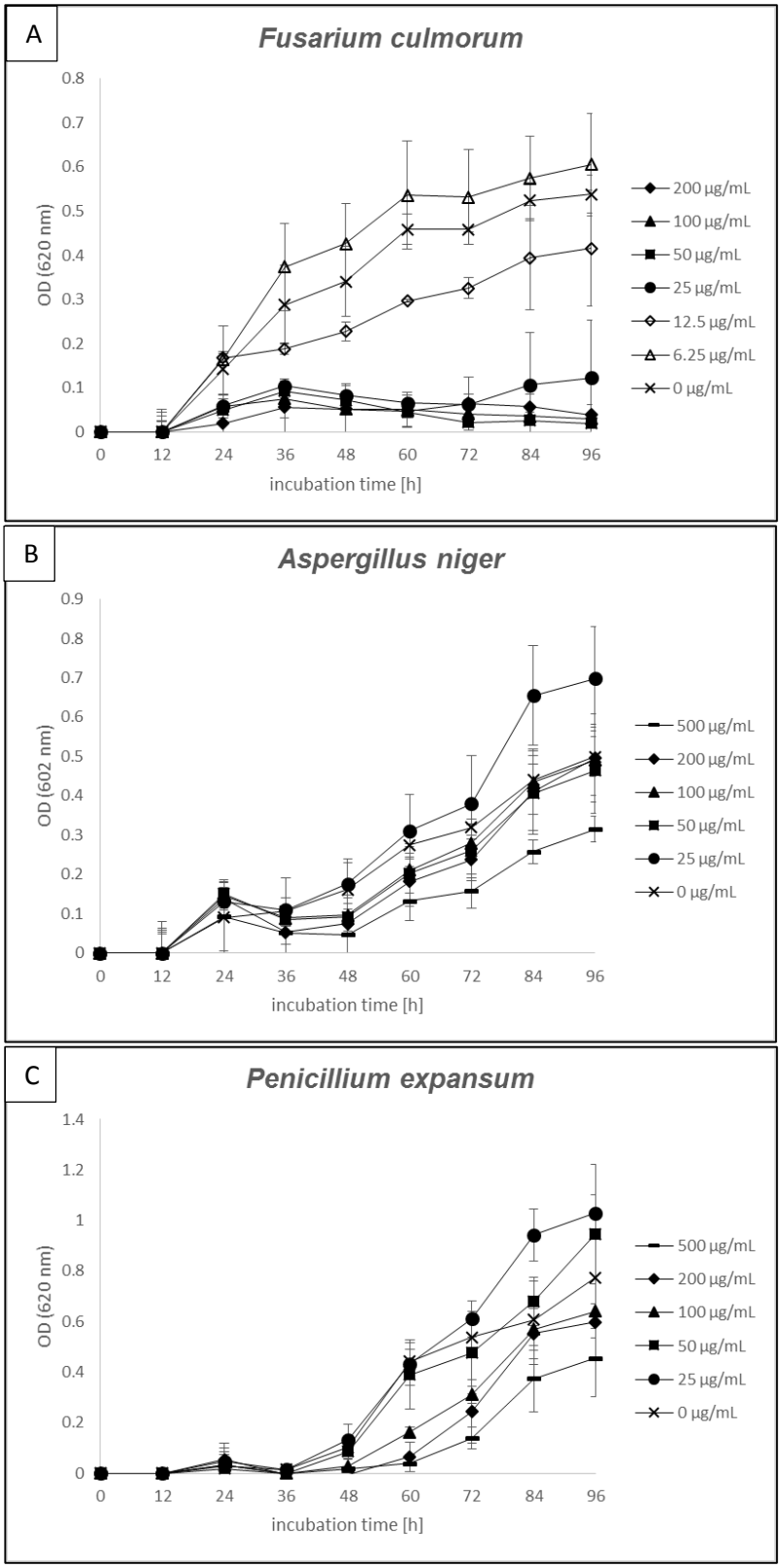


Figure 16: Fungal growth curves in presence of various concentrations of Cp-thionin II (in µg/mL) against A) *Fusarium culmorum* FST 4.05, B) *Aspergillus niger* FST 4.22, C) *Penicillium expansum* FST 4.21. All values for optical density (OD) are the mean values of three independent replicates ± standard deviation.

From an evolutionary point of view, the localisation of the peptide in the seeds suggests that the peptide is originally produced to protect the germinating seed from environmental pathogens, such as the fungi studied here (Franco et al., 2006). Hence, high antimicrobial activity against the tested pathogens is plausibe, making the peptide potentially a promising candidate for bio-protection.

Compared to KT43C, the linear analogue of the peptide studied by Thery and Arendt (2018), the extract containing the natural peptide showed similar antifungal activity against *P. expansum* and *A. niger*. However, the MIC against *F. culmorum* was with 50 µg/mL significantly higher than reported for the linear analogue (20 µg/mL). This discrepancy can be explained by the structural differences between synthetic and natural peptide. Firstly, as indicated by SDS electrophoresis, the natural peptide occurs not only as monomer but also as di- and trimer. As a consequence double the peptide concentration (in µg/mL) results in a similar amount of active molecules available. In addition, the peptide was only partially purified in this study and impurities in form of other peptides are visible on the SDS-gel. As a result, the true concentration of Cp-thionin II would be lower than the protein concentration determined for the extract, explaining the higher concentrations required for fungal inhibition.

8.4.3 Effect of heat and cations on the antifungal activity of the cowpea extract

In order to gain further information regarding the properties of the cowpea extract and to estimate its potential for food applications the resistance against environmental stress factors, such as heat and ionic strength, was investigated. Therefore, the impact of these stress factors on the antifungal activity against *F. culmorum* was studied. Figure 17 shows the fungal growth curves over 96 h, incubated with the cowpea extract, containing 50 µg/mL of protein (MIC), after heat treatment (100°C, 15 min) and in presence of various cations (Na^+, K^+, Ca^{2+}, Mg^{2+}). No differences in spore germination inhibition were evident between the heated and unheated peptide solutions. Both treatments resulted in total inhibition of germination (10^4 conidia/mL) over 96 h in ½ PDB, while the controls (no peptide, heated and unheated) resulted in normal fungal development.

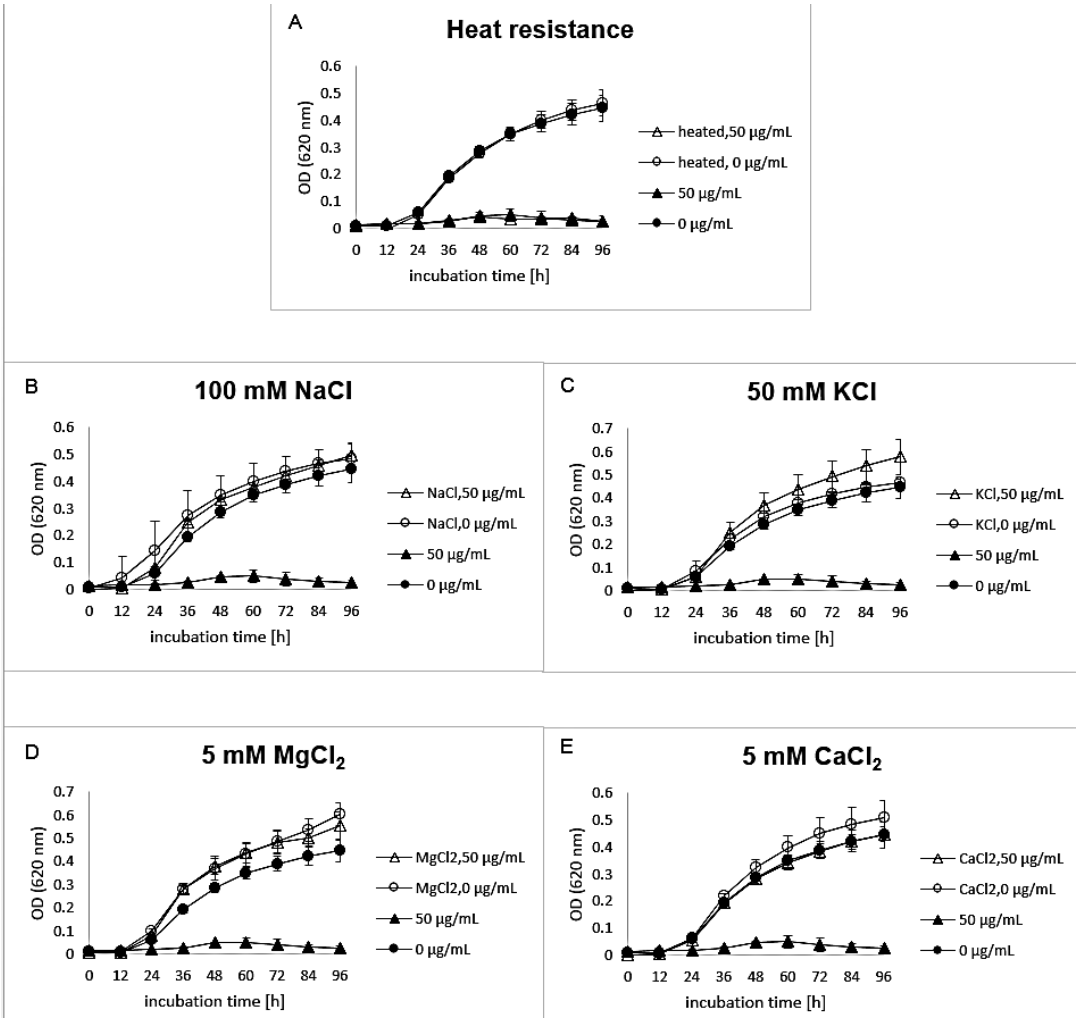


Figure 17: Heat and cation sensitivity of the antifungal activity of the cowpea extract (50 µg/mL). Fungal growth curves of *Fusarium culmorum* after heat treatment (15 minutes at 100°C) (A) and in presence of various salts (B – E) are mean values of three independent replicates ± standard deviation.

In the presence of cations, the extract lost most of its antifungal activity against *F. culmorum* when applied at its MIC (50 µg/mL). The monovalent cations Na^+ (100 mM) and K^+ (50 mM) caused a complete loss of antifungal activity. Likewise, the divalent cations Ca^{2+} and Mg^{2+} (both at 5 mM) were also found to reduce the antifungal activity of Cp-thionin II substantially.

The antifungal activity remained unaffected by heat treatment, demonstrating the thermal resistance of Cp-thionin II. The heat resistance of AMPs, including synthetic Cp-thionin II and its linear analogue was previously demonstrated by Kraszewska et al. (2016) and Thery and Arendt (2018), respectively. Different factors influencing the thermal stability of a peptide are discussed. Cp-thionin II is a small peptide with flexible order when in aqueous solution (Franco et al., 2006). This attribute

contributes to a higher thermal stability, as the molecules have more freedom of movement when heated.

In agreement with the studies of Kraszewska et al. (2016) and Vriens, Cammue and Thevissen (2014), the presence of cations reduced the antifungal activity of Cp-thionin II. The increased net charge of the peptide further disturbs the hydrophilic/hydrophobic balance of the peptide, compromising its antifungal activity. As a consequence of the coverage of the peptide surface, the protein interactions are reduced, inducing a lowered antimicrobial activity. Furthermore, as a result of the ions binding to the fungal membrane, it becomes more difficult for the peptide to find receptors on the fungal membrane to bind and exert its antifungal activity (Wu et al., 2003). Finally, the presence of cations in the medium can change the overall configuration of the peptide, causing substantial deviation from its original structure and so explaining the loss in antifungal performance (Oard and Karki, 2006).

For possible future applications, the salt sensitivity of the extract can be a major drawback, in particular for preservation of food products. However, it has to be mentioned that the concentration of NaCl (100 mM = 5.8 g/100g), the most commonly found salt in food products, was higher than found in most foods. An investigation regarding the level of salt tolerated by the peptide could reveal further details regarding possible food application. Furthermore, the good thermal stability found in this study would make the extract accessible for a large field of application, including processed foods.

8.4.4 Mode of action

In order to identify some characteristics regarding the mode of action of Cp-thionin II in the extract, a membrane permeabilization assay was performed on *F. culmorum* hyphae. The permeabilization of the fungal cell membrane was achieved with peptide concentrations of 12.5 µg/mL ($^1/_4$ MIC) and higher (Fig. 18). The membrane permeabilization of the peptide was found in a dose-dependent manner, hence the increased fluorescence for higher concentrations. However, substantial fungal growth inhibition was only achieved for peptide concentrations of 25 µg/mL or higher. Permeabilised hyphae had significant cytoplasmic granulation at higher concentrations. However, Cp-thionin II induced permeabilization appeared to be required for inhibition but was not sufficient to cause cell death, as membrane permeabilization appeared already at concentrations which were insufficient for

fungal growth inhibition. It also has to be considered, that MIC-values for the inhibition of spore germination and the killing of hyphae can vary.

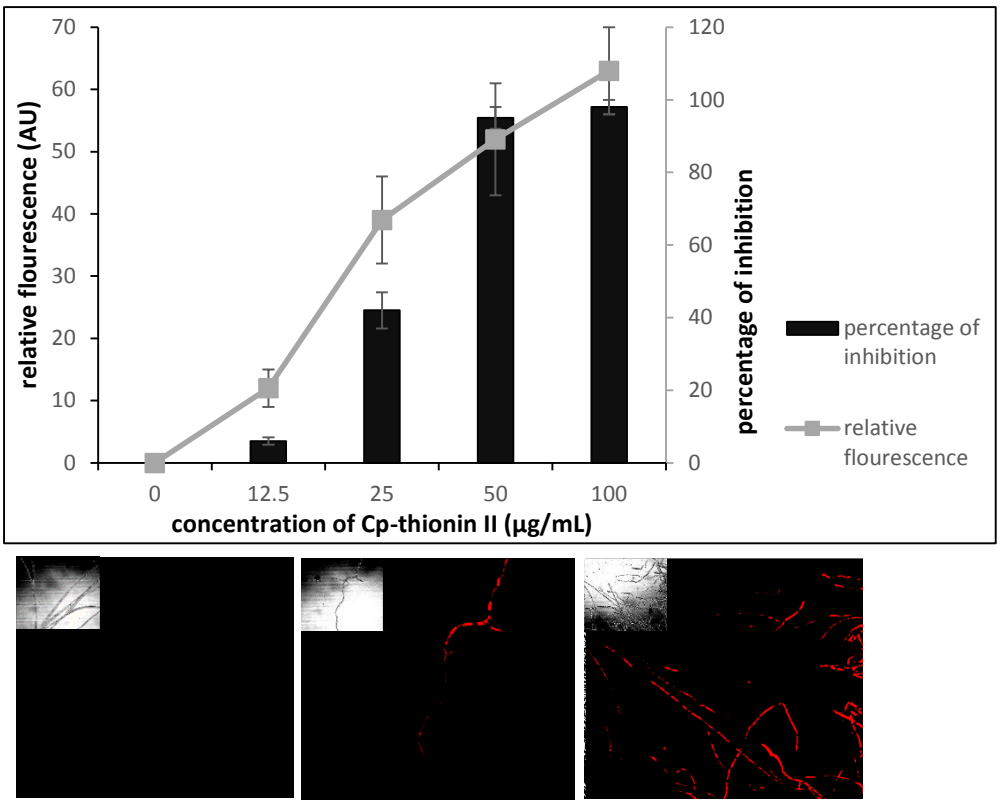
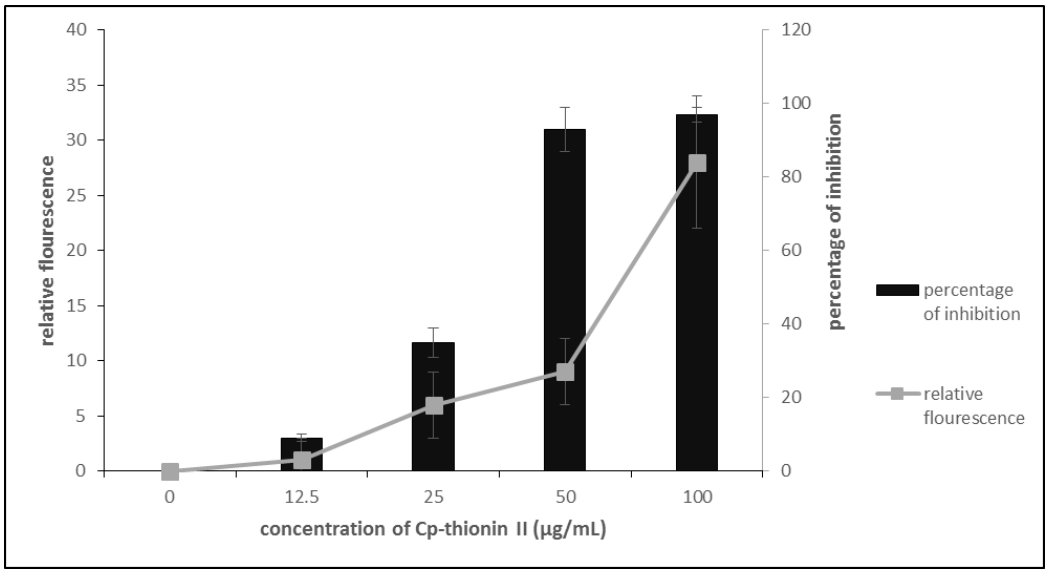


Figure 18: Membrane permeabilization assay. Top: After 18 h of growth, *F. culmorum* hyphae were treated for 2 hours with cowpea extract (0; 12.5; 25; 50 and 100 µg peptide/mL). Permeabilization of the fungal membrane was determined by fluorescence with propidium iodide (0.5%) (excitation 488 nm, emission 538 nm) and correlated to the percentage of inhibition. Values are the mean of three independent replicates. Bottom: *F. culmorum* hyphae were observed with a confocal laser scanning microscope, with an excitation wavelength 460 – 490 nm. Left: no extract; middle: 25 µg/mL Cp-thionin II; right: 100 µg/mL.

Another inhibitory mechanism is the increased generation of free radicals, usually from mitochondrial source, which can lead to an excessive level of reactive oxygen species (ROS), commonly known as oxidative stress. Plant defensins have been reported to induce an overproduction of ROS as part of their antifungal performance (Vriens, Cammue and Thevissen, 2014). Substantial production of ROS appeared only at the highest peptide concentration (100 µg/mL) (Figure 19), which is well above the concentration required for fungal growth inhibition. This suggests that the overproduction of ROS is not a primary mechanism in the antifungal performance of Cp-thionin II against *F. culmorum*. However, at high concentrations it may be a

supportive mechanism, further enhancing the activity of the peptide (Hayes et al., 2013).



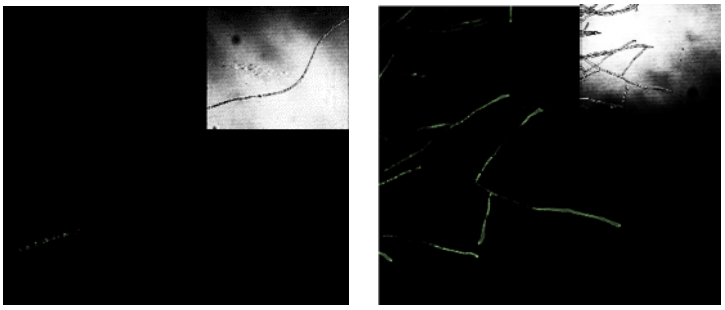


Figure 19: Detection of reactive oxygen species (ROS) production. Top: After 24 hours of growth, *F. culmorum* hyphae were treated for 12 hours with cowpea extract (0; 12.5; 25; 50 and 100 µg peptide/mL). Production of ROS was determined by fluorescence of DHR 123 (excitation 488 nm, emission 538 nm) and correlated to the percentage of fungal growth inhibition. Each value is the mean of three independent replicates. Bottom: *F. culmorum* hyphae were observed using a confocal laser scanning microscope, with excitation wavelength 460 - 490 nm. Left: no peptide; right: 100 µg/mL.

It is evident that the inhibitory activity of Cp-thionin II against fungal hyphae involves the permeabilization of the hyphae, leading to leakage and granulation of the plasma. Interaction of the peptide with intracellular targets, inducing oxidative stress appears only as a supportive mechanism at high concentrations. However, variations regarding the mode of action against fungi of a different class, such as *A. niger* or *P. expansum,* are likely (El-Mounadi et al., 2016). Differences in morphology and cell/wall composition could change the involvement of ROS in the antifungal performance against different fungi. The results obtained are generally in

good agreement with the findings of Thery and Arendt (2018) and the values obtained for the inhibition of fungal spores.

8.4.5 Haemolytic activity of the cowpea extract

For possible applications as food preservative, it is essential to combine high antimicrobial activity with consumer safety. Therefore, the cytolytic activity of the extract against red blood cells of sheep was determined in order to estimate its haemolytic activity against mammalian cells. The haemolytic activity was evaluated as the release of haemoglobin after treatment with the extract in a microtiter plate assay. All concentrations investigated (up to 200 µg/mL) did not lyse the red blood cells (data not shown).

It has been shown that the presence of cholesterol is the primary reason behind the reduced sensitivity of mammalian cells towards lysis by AMPs. In contrast, fungal cell membranes contain primarily ergosterol which is more easily targeted by AMPs (Mason, Marquette and Bechinger, 2007). Furthermore, it has been reported by Thevissen et al. (2004) that the cytotoxicity of plant defensins is very low. However, it needs to be mentioned, that natural AMPs are often more cytotoxic compared to their linear analogues, where the disulfide bridges are removed (Liu et al., 2008). Despite the use of a natural peptide, containing disulphide bridges, a lack of haemolytic activity was found for Cp-thionin II, which is in good agreement with the results obtained by Thery and Arendt (2018). On the other hand, it has to be considered that the assay had to be carried out in PBS buffer, which contains different salts. As shown above, the extract lost its antifungal activity in presence of cations. Hence, a possible haemolytic activity could be lost as well. Consequently, further studies regarding the safety of the extract are required before food application can be considered.

8.4.6 Wheat grain spoilage protection

The application of the extract as food preservative was illustrated by its use on artificially infected (*F. culmorum*) wheat grains prior to storage. The ergosterol content was measured as a marker of fungal bio-mass, to evaluate the efficiency of the peptide treatment. The results of the determination of ergosterol content in the samples are summarised in Figure 20. The untreated controls showed a substantial

increase in ergosterol content during the storage period. The 5% infected, untreated control, in particular, had an ergosterol content below detection (<LOD) in week 0 and 42.3 ± 1.3 ppm at week 6. In contrast, the acetic acid treatment resulted in total fungal inhibition with contents < LOD before and after storage. The use of the cowpea extract reduced the fungal development at all concentrations. The use of solutions with peptide concentrations of 25 µg/mL and 50 µg/mL resulted 38.7 ± 1.4 ppm and 25.0 ± 0.7 ppm ergosterol, respectively. This indicates a significantly reduced fungal bio-mass compared to the untreated control. The highest concentration tested (100 µg/mL) resulted in the total inhibition of fungal development during storage (<LOD after week 6).

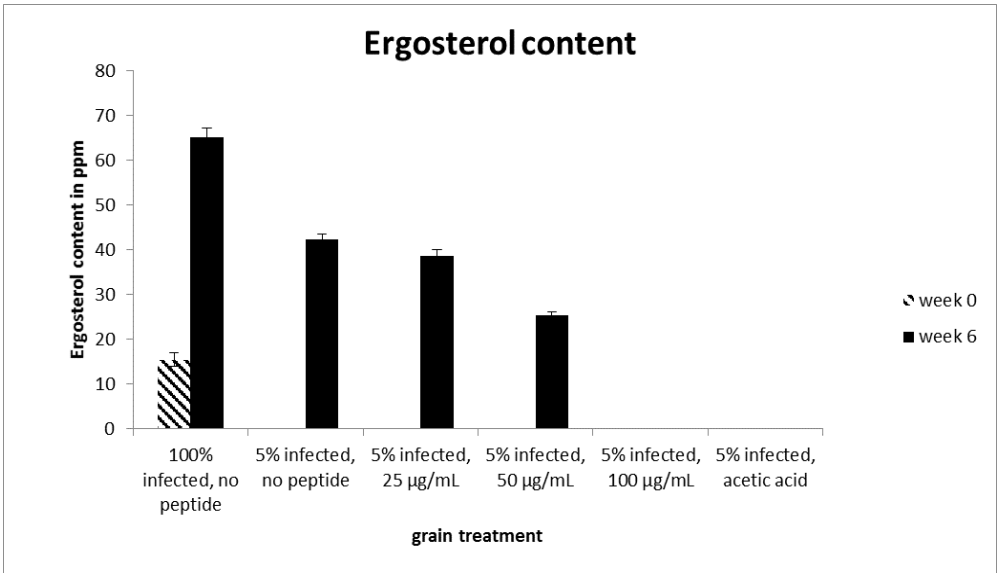


Figure 20: Ergosterol contents of the grain samples before (week 0, black stripes) and after storage (week 6, black) with standard deviation. The limit of detection (LOD) was determined as 0.75 ppm and ergosterol contents determined to <LOD are shown as "0 ppm" in the chart.

The extract successfully protected the wheat grains from fungal spoilage during incubation. The use of other natural AMPs to prevent food spoilage has been previously reported to be efficient against spoilage fungi (Lucera et al., 2012; Rai et al., 2016). However the concentrations required for total inhibition of fungal development were higher than the MIC against *F. culmorum* obtained *in vitro*. This can be explained by the differences in matrices between the grains and PDB. In particular, compounds present in the outer layers of the wheat kernels could compromise the antifungal performance. Furthermore, the fungus was allowed to

proliferate over the kernels before the defensin solution was applied. This can result in an increase of the concentration required for inhibition. It also has to be considered that the MIC for inhibition of spore germination varies from the MIC for the inhibition of hyphae. However, the extract containing Cp-thionin II demonstrated great potential as a bio-preservative during cereal storage, as fungal development was completely inhibited when applied at 100 µg peptide/mL.

8.5 Conclusions

The cowpea extract containing Cp-thionin II, showed antifungal activity against three spoilage fungi commonly found on cereals and cereal products. The peptide showed typical characteristics of plant-derived defensins, including heat stability and sensitivity towards cations. Furthermore, it was found to be harmless towards sheep erythrocytes. These results are in good agreement with previous studies of the natural cowpea-thionin II (Franco et al., 2006) and its synthetic linear analogue (Kraszewska et al., 2016; Thery and Arendt, 2018). The application to protect fungal contaminated wheat grains during storage was successful, as fungal development could be completely inhibited using a solution with a peptide concentration of 100 µg/mL. However, despite the highly promising results of this study extensive further research is required. Improvement of the extraction efficiency and purity of the extract are of high importance. Also a deeper understanding of the mode of action to increase the antifungal performance is required. The efficiency against other food pathogens would be of high interest to fully understand the potential and limits of Cp-thionin II. On the other hand, the interactions with other food constituents (e.g. proteolytic enzymes) need to be considered before the application as bio-preservative. Furthermore, the safety of the defensin towards the consumers has to be investigated for both, acute and long-term toxicities.

In conclusion, despite the need for further extensive investigation, the extract containing Cp-thionin II showed great potential for the possible application as bio-preservative. As such it could be a very important tool to reduce food waste and increase sustainability. Hence, it can become a valuable contribution to satisfy the global nutritional requirements of future generations.

8.6 Acknowledgements

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Chapter 9: Overall discussion and conclusions

9.1 Overall discussion and conclusions

In the quest of feeding the global population today and in future years to come, the reduction of food waste and increased sustainability has become of major interest for both, food industries and researchers. Due to the essential contribution of cereal based products to human nutrition, as well as other uses including animal feed and bio-fuel, crop protection to reduce losses is of particular importance. Extensive research has been undertaken to identify efficient methods to prevent microbial spoilage of cereal crops pre- and post-harvest (Mannaa and Kim, 2017), while satisfying the consumer demand for high quality and safety in "clean-label" products (Figiel and Kufel, 2016). Therefore, two literature reviews have been conducted as part of this thesis to outline recent advances in post-harvest microbial protection of cereals, using physical (**chapter 2**), chemical or microbiological (**chapter 3**) approaches.

A wide variety of approaches for physical decontamination methods receive great research interest and show promising results. Conventional treatments, such as dry heat can only achieve sufficient decontamination with several negative effects on the grain quality (Clear et al., 2002; Gilbert et al., 2005). As a consequence, the interest in emerging, more efficient technologies is high. Hence, wet instead of dry heat, using steam or super-heated steam, was found to inhibit microbial spoilage more efficient without grain quality deterioration (Ban and Kang, 2016; Hu et al., 2016). In addition, super-heated steam was also found to be more efficient against already accumulated mycotoxins (Cenkowski et al., 2007). Also radiation approaches, using ionizing (gamma or electron beam radiation) or non-ionizing (microwaves, UV-light or ultrasound) are intensely investigated. However, despite partially promising results (in particular for ionizing radiation) doses reported for compete fungal inhibition cause severe damage to the treated crop or exceed the limit of generally permitted radiation (Aziz et al., 2007; Kottapalli,Wolf-Hall and Schwarz, 2006). Likewise, microwaves and UV-light can effectively decontaminate cereal crops but cause severe quality deterioration and reduced seed viability (Aron Maftei et al., 2014; Schmidt-Heydt, Cramer and Graf, 2012). The use of high hydrostatic pressure (HHP) has sown good microbial inhibition and degradation of mycotoxins without product quality deterioration (Black et al., 2007). On the down side however, the need to suspend the grains in liquid and the inability to use on-line processing make it unfeasible for industrial use.

As reviewed in **chapter 3**, new approaches for chemical decontamination, such as cold plasma and electrolysed water, are intensely researched as well. While both methods have shown promising results by inhibiting spoilage organisms without negative impact on the sample quality further research is required (Audenaert et al., 2012; Selcuk, Oksuz and Basaran, 2008). The success of each treatment is strongly dependent on numerous factors. Hence, further optimisation is required before industrial use becomes feasible. The use of bio-preservatives, in form of protective cultures or isolated antimicrobial compounds, is a popular approach to replace conventional preservatives. In particular, lactic acid bacteria (LAB) are frequently reported to extend the microbial self-life of cereal products (Axel et al., 2016; Le Lay et al., 2016; Melin et al., 2007). However, to-date the exact mechanisms behind the antimicrobial performance of LAB are not fully understood yet. Hence, further investigation is required to optimise the use of LAB for industrial use. The isolation and characterisation of bacterial antifungal compounds includes carboxylic and phenolic acids, reuterin, fatty acids and proteinaceous compounds among others. However, it is understood that complex synergistic mechanisms between the individual compounds are responsible for the antifungal performance (Axel et al., 2016; Schnürer and Magnusson, 2005). As a consequence, the use of a protective culture or bacterial cell-free supernatant (cfs) is the most promising approach. Also the use of bacteria to bind and remove mycotoxins is under investigation but only partially understood yet (Ahlberg, Joutsjoki and Korhonen, 2015). Further research will reveal the true potential of this approach.

For both purposes, microbial decontamination and mycotoxin detoxification, each treatment reviewed in **chapters 2** and **3** encompasses associated difficulties for the successful application to cereals. Not only does the efficiency of each treatment depend on numerous factors, also the homogenous treatment of the entire sample provides a technological challenge. Thus, in order to make industrial application possible the treatment conditions and settings must be optimised for each crop. However, even with ideal process conditions no one single existing treatment can provide sufficient microbial decontamination and detoxification without substantial grain quality deterioration. In particular the removal of in-field produced mycotoxins displays an enormous challenge. In many cases, the treatment time and intensity must be much higher for mycotoxin degradation compared to the inhibition of living organisms. Thus, successful degradation of these toxins without compromising the grains quality and seed viability is difficult. In addition, of major concern related to the decay of mycotoxins are the resulting degradation products. The vast majority of

research has examined the reduction in the level of the original parent mycotoxin, paying little attention to the degradation products or mechanism of degradation. Consequently, the combination of several treatments to find synergies appears to represent a very promising approach for optimal results. Research also has to focus on understanding and optimisation of the mechanisms responsible for microbial inhibition, in order to discover new ways of microbial shelf-life extension. Then it will be possible to produce products that meet the highest standards in terms of food quality and safety.

In **chapter 4** the characterisation of wheat grains, carrying various degrees of contamination with *Fusarium culmorum*, before and after storage demonstrated how fast spoilage can occur if crops are not treated appropriately. For all levels of contamination, fungal bio-mass increased rapidly during the storage period. Furthermore, mycotoxins such as deoxynivalenol and zearalenone were accumulated, causing potential consumer health hazard if the crop is not disposed completely. Interestingly, the mycotoxin concentrations of all samples decreased during storage. This can be explained with the fact that mycotoxins are released as response to environmental stress, i.e. to overcome the grains defense mechanisms (Hestbjerg, Felding and Elmholt, 2002; Oliveira et al., 2012). Hence, mycotoxins are produced during the initial infection, while afterwards the energy is used to increase the bio-mass. A similar reaction would be expected as response to insufficient decontamination (Buffet-Bataillon et al., 2012). This demonstrates the importance of complete microbial inhibition before storage and processing. Furthermore, the samples with the lowest degree of initial infection contained the most fungal bio-mass after storage. The reason behind is the over-production of mycotoxins during the initial infection and less oxygen available (relative to the fungal bio-mass) in the higher infected samples, leading to self-inhibition of the fungus (Atalla et al., 2003). These results clearly highlight the importance of complete microbial decontamination before grain storage, but also suggest that use of sub-lethal doses of antimicrobial treatment might promote the production and accumulation of mycotoxins, due to the stress imposed on the organisms. Furthermore, the inverse relationship between fungal bio-mass and mycotoxin content shows that also grains without visible spoilage can cause consumer health hazards. From a technological point of view, severe grain quality deterioration occurred as a result of the fungal spoilage. The degradation of polysaccharides and storage proteins was determined and visualised with scanning electron microscopy. Determination of selected enzymatic activities revealed that the fungus after the initial infection releases

numerous hydrolytic enzymes to penetrate into deeper grain layers where more nutrients are available. The expression of such enzymes was identified as the tool to penetrate into the grains and release nutrients from proteins and polysaccharides present in the endosperm. Hence, as a result of the fungal spoilage, after 6 weeks of storage the grains did not fulfil the requirements for whole grain flour anymore.

Subsequently, in **chapter 5** the infected and stored samples were characterised further with regards to their gluten quality and quantity, as well as the fungal impact on resulting dough and bread making qualities. Due to the fungal proliferation gluten quantity and network strength were significantly reduced. The hydrolytic enzyme activities determined in **chapter 4** were found responsible for the degradation and therefore reduced quantity and network strength of the gluten (Wang et al., 2005). As a result of the gluten degradation, also the resulting dough and bread quality were substantially reduced, due to the lower water binding ability of the dough. Furthermore, the increased amylase activity resulted in the degradation of water absorbing polysaccharides and a delay in starch gelatinisation (Tester, Qi and Karkalas, 2006). All these factors finally led to high dough stickiness, bake loss and poor crumb structure and texture compared to the control. Interestingly, these quality deteriorations were found to be independent from the level of initial contamination. As a consequence, microbial decontamination has to eliminate spoilage organisms completely in order to provide sufficient protection.

The research conducted in **chapters 4** and **5** provides important knowledge regarding the mechanisms and kinetics of fungal spoilage when crops are not appropriately decontaminated. Consumer health hazards due to mycotoxins occur already before fungal bio-mass is visible. Hydrolytic enzymes were identified as the primary tool of the fungus to proliferate into the grains. Hence, an inhibition of the fungal enzymes could possibly be used to inhibit spoilage. Furthermore, mycotoxins are known to be very heat resistant and mostly water soluble (Bretz et al., 2005) and can be expected to transfer into the final bread, causing substantial consumer health hazard. This work demonstrates the importance of appropriate microbial decontamination of cereal crops prior to storage and downstream processing. Hence, underlining the importance of further research regarding the approaches reviewed in **chapters 2** and **3**.

Lactic acid bacteria have been subject to intense research as potential food bio-preservatives with beneficial effects for the technological performance. Phenyllactic acid (PLA) was recognised as one of the key components responsible for the

antifungal performance of *Lactobacillus reuteri* R29 (Oliveira et al., 2015). Thus, the objective of **chapter 6** was to increase and optimise the antifungal performance of the strain as a result of increased accumulation of PLA. Supplementation of the bacterial growth medium with phenylalanine promoted the Ehrlich pathway for amino acid metabolism, resulting in increased production and accumulation of PLA. With the increase in PLA levels after fermentation, the antifungal activity of the cfs against *F. culmorum*, *A. niger* and *P. expansum* was increased substantially. Furthermore, the antifungal performance was not compromised by heat treatment. The use of the bacterial cfs as natural preservative during bread making resulted in 4 days (100%) of microbial shelf-life extension. In addition, numerous researchers have demonstrated the high antimicrobial activity of 3-hydroxypropionaldehyde, also known as reuterin (Lüthi-Peng, Dileme and Puhan, 2002). However, this study was the first to investigate the strain *L. reuteri* R29 for its ability to produce reuterin. After careful adjustment of the conditions during fermentation, *L. reuteri* R29 produced substantial amounts of reuterin, which was highly efficient against the three fungi investigated in this study when applied *in vitro*. In contrast, the use of reuterin containing cfs during the bread making process did not extend the microbial shelf-life. The reason behind is the high reactivity of reuterin with the thiol groups present in the dough, in particular at high temperatures (Reinbold et al., 2008). In addition, no antifungal metabolites, other than reuterin are present in this cell-free supernatant, further decreasing its antifungal activity *in situ*. As a consequence, reuterin was found to be not suitable as bio-preservative.

This chapter contributes essential knowledge regarding the optimisation of the bacterial fermentation to increase the yield of PLA and reuterin, respectively. Furthermore, the potential of PLA as bio-preservative was further highlighted. Future research should aim at an increased PLA production *in situ* or from a natural medium, as was attempted by Le Lay et al. (2016) already. Also the use of PLA producing strains with proteolytic activity could lead to high PLA production *in situ* and strong antifungal activity. Furthermore, promotion of the Ehrlich-pathway of other strains with a broader spectrum of antifungal compounds could result in even better antimicrobial activity. Also the production of PLA in a batch (Valerio et al., 2016) or fed-batch process (Rodriguez et al., 2012) from phenylpyruvic acid has delivered promising results. In addition to the here tested activity against fungal spores, the activity against fungal hyphae would be of further interest. On the other hand however, phenolic acids like PLA only show antimicrobial activity at low pH, which is a major drawback for its use.

As shown in **chapters 2** and **3**, numerous emerging technologies are discussed to replace conventional preservatives. However, to-date it is still unclear how applicable these novel methods are and if further optimisation is required. To investigate this topic fungal contaminated wheat grains were treated with various physical, chemical and microbial decontamination methods and analysed for fungal spoilage and technological quality before and after storage (**chapter 7**). Interestingly, microbial decontamination using LAB with the optimised conditions for PLA production (determined in **chapter 6**) was found to be inefficient for this approach. The bacterial fermented medium did not inhibit fungal proliferation and accumulation of mycotoxins in the sample. The primary explanation is that the grains were partially overgrown with fungal hyphae before the treatment. As pointed out earlier, the mode of action for inhibition is different for conidia and hyphae (Dalie, Deschamps and Richard-Forget, 2010), hence the difference in antifungal performance compared to **chapter 6**. Regarding the physical treatments, vacuum packaging, microwaves and HHP were found to successfully inhibit fungal growth and production of mycotoxins. In contrast, ultrasound was found to be inefficient against both, fungal growth and production of mycotoxins. However, despite its efficiency in microbial decontamination, microwave treatment was found to compromise the grain quality substantially. This is primarily due to the heating of the grains, inducing damage to the proteins, i.e. enzymes present in the kernel (Aron Maftei et al., 2014). In addition, the internal heating induced gelatinisation of the starch granules, which further reduced the bread making ability of the grains (Fan et al., 2017). As a consequence, the breads baked from these grains were of poor quality. Interestingly, the combination of microwaves with sodium hypochlorite solution resulted in fungal inhibition without grain quality deterioration. Inactivation was achieved by activation of the hypochlorite radical by the microwaves (Oms-Oliu, Martin-Belloso and Soliva-Fortuny, 2010), while the liquid prevented the grains from excessive heating. Regarding the chemical treatments, only washing with sorbate and propionate solutions was successful to inhibit fungal growth and mycotoxin accumulation. In addition, the grain quality was not found to be compromised, resulting in high quality breads. Overall, it can be concluded that the established methods are still superior to the new emerging technologies investigated here, which require further research.

The research contributes important knowledge regarding weaknesses of novel approaches compared to the still superior established ones of industrial application as grain decontamination. While several reports regarding the potential of all

methods tested here exist (Bilek and Turantas, 2013; Heinz and Buckow, 2009; Aron Maftei et al., 2014; Mir, Shah and Mir, 2016), the successful application prior to cereal storage requires further optimisation and modification before any of the methods becomes industrially feasible. In addition, future research also should investigate for the additional ability to remove or destroy mycotoxins already produced in-field. As reported in **chapter 4**, consumer health hazards due to mycotoxins are present before quality deterioration from the fungal bio-mass occurs. Furthermore, a stronger focus has to be placed on the efficient combination of methods, as it appears unlikely that any single method can successfully protect cereal crops without quality deterioration. This would allow to optimise the decontamination with little to no impact on the grains. The combination of microwaves with NaOCl already showed significantly better results than each of the two treatments individually. Furthermore, it is remarkable how different treatments trigger the production of different mycotoxins by the fungus. Only little research was conducted on this topic so far, but comparable results were reported by Vaquera, Patriarca and Pinto (2016). However, this topic needs further investigation and careful consideration, to ensure grain processing does not induce the release of unexpected mycotoxins.

Finally, an approach for bio-preservation that currently receives a lot of research interest is the use of antimicrobial peptides (AMPs). In **chapter 8** of this thesis the peptide Cp-thionin II was extracted from cowpea seeds, partially purified and characterised. It was identified as γ-thionin, which are classified as plant derived defensins. This suggests that the peptide is part of the defense mechanism of the plant (Franco et al., 2006). Thus, it is very efficient and specific against natural pathogens (Garcia-Olmedo et al, 1998). The cowpea extract was found to show strong antifungal activity against *F. culmorum*, *P. expansum* and *A. niger*, which was not compromised by heat treatment at 100°C. In agreement with Kraszewska et al. (2016), the peptide solution was found to be sensitive towards the presence of cations, losing its antifungal activity. At the same time, it had no haemolytic activity against sheep erythrocytes. The mode of action against fungal hyphae was partially characterised to be based on membrane permeabilisation, while the induction of oxidative stress only has a minor contribution. These results are in agreement with the characterisation of the synthetic linear analogue of the peptide done by Thery and Arendt (2018). Finally, the extract was tested to decontaminate fungal infected wheat grains during storage. A concentration of 100 µg/mL was successful to fully inhibit fungal development during cereal storage. This finding is in good agreement

with the results previously reported by other researchers (Lucera et al., 2012; Rai et al., 2016).

The knowledge obtained in this chapter is a helpful contribution to the research regarding AMPs. This was the first study demonstrating inhibitory activity of the peptide Cp-thionin II against conidia and hyphae of filamentous fungi. Furthermore, the characterisation of the peptide confirmed preceding studies on the natural (Franco et al., 2006) and synthetic (Kraszewska et al., 2016; Thery and Arendt, 2018) peptide and added further knowledge. In particular, regarding the mode of action and haemolytic activity against mammalian erythrocytes new information were gathered. Finally, the defensin was successfully applied to protect fungal contaminated wheat grains during storage. Future research should aim to fully understand the mode of action of the peptide against various spoilage organisms. This would allow more efficient and specific application of the peptide. However, extensive investigations regarding the consumer health safety of the peptide have to be conducted before industrial use becomes a possibility. If further investigated, Cp-thionin II could potentially become a new industrially used preservative. The results of this study contribute valuable knowledge regarding the potential of Cp-thionin II as bio-preservative.

Overall, this thesis highlighted the importance of appropriate microbial decontamination of cereal crops before storage to prevent consumer health hazards and ensure high product quality. To achieve this goal, several approaches were investigated, including chemical, physical and microbiological decontamination. Despite the promising results of emerging bio-preservatives, to date the established methods were still found superior. However, the research undertaken here can be a valuable contribution to establish new bio-preservatives.

9.2 References

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Appendix

List of Publications and Presentations

First author publications

Schmidt, M., Zannini, E., Lynch, K.M. and Arendt, E.K. (2018). Novel approaches for Chemical and Microbiological shelf life extension of cereal crops. *Critical Reviews in Food Science and Nutrition.* DOI: 10.1080/10408398.2018.1491526 (*in press*)

Schmidt, M., Zannini, E. and Arendt, E.K. (2018). Recent Advances in Physical Post-Harvest Treatments for Shelf-Life Extension of Cereal Crops. *Foods* 7 (4), pp. 45.

Schmidt, M., Lynch, K.M., Zannini, E. and Arendt, E.K. (2018). Fundamental study on the improvement of the antifungal activity of *Lactobacillus reuteri* R29 through increased production of phenyllactic acid and reuterin. *Food Control* 88, pp.139-148.

Schmidt, M., Zannini, E. and Arendt, E.K. (2017). Impact of post-harvest degradation of wheat gluten proteins by Fusarium culmorum on the resulting bread quality. *European Food Research and Technology* 243(9), pp.1609-1618.

Schmidt, M., Horstmann, S., De Colli, L., Danaher, M., Speer, K., Zannini, E. and Arendt, E.K. (2016). Impact of fungal contamination of wheat on grain quality criteria. *Journal of Cereal Science* 69, pp.95-103.

Oral presentations

Schmidt, M., Zannini, E. and Arendt, E.K. (2015). Impact of fungal contamination on grain and bread quality. 4. Fruehjahrstagung des Weihenstephaner Instituts fuer Getreideforschung (WIG), WIG, Freising, Germany, 21-22nd April 2015

Schmidt, M., Zannini, E. and Arendt, E.K. (2016). Optimization of antifungal activity of *Lactobacillus reuteri* R29 and potential application in food production. IUFoST, 18th World Congress of Food Science and Technology, Dublin, Ireland, 21 - 25th August 2016

Poster presentations

Schmidt, M. and Arendt, E.K. (2018). Optimization of antifungal activity of *Lactobacillus reuteri* R29 and potential application in food production. VII International Symposium on Sourdough; Sourdough for Health, Cork, Ireland, 06 - 08th June 2018

Schmidt, M. and Arendt, E.K. (2017). Optimization of antifungal activity of *Lactobacillus reuteri* R29 and potential application in food production. LAB12, 12th International Symposium on Lactic Acid Bacteria, Egmond Aan Zee, The Netherlands, 27 – 31st August 2017