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Isolation and characterisation of antifungal compounds from lactic acid bacteria and their application in wheat and gluten-free bread

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Under the supervision of
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To obtain the degree of
Doctor of Philosophy – PhD in Food Science and Technology

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Table of Contents

Table of Contents ..... **i**

List of Figures ..... **vi**

List of Tables ..... **ix**

Declaration ..... **xi**

Abbreviations ..... **xii**

Abstract **1**

Acknowledgements **2**

Chapter 1 Introduction **3**

1.1 Introduction 4

1.2 References 8

Chapter 2 Literature Review – Mould spoilage of bread and its biopreservation: A review of current strategies for bread shelf life extension **13**

2.1 Abstract 14

2.2 Introduction 15

2.3 Cereals and their contamination with fungi and mycotoxins 17

2.4 Conventional food preservatives 19

2.5 Alternative preservation techniques 21

2.5.1 Fermentation 21

2.5.1.1 Lactic acid bacteria 21

2.5.1.2 Yeast 29

2.5.2 Plant derive compounds 31

2.5.3 Protective packaging 33

2.5.4 Further applications 36

2.6 Conclusion 38
2.7 Acknowledgements 39
2.8 References 40
Chapter 3 Quantification of cyclic dipeptides from cultures of *Lactobacillus brevis* R2Δ by HRGC/MS using Stable Isotope Dilution Assay 54
3.1 Abstract 55
3.2 Introduction 56
3.3 Material and Methods 59
3.3.1 Chemicals 59
3.3.2 Synthesis of isotopically labelled cyclo-dipeptides 59
3.3.3 Standard wort production 61
3.3.4 MRS-broth and wort fermentations and sample extractions 61
3.3.5 Quantification of cyclic dipeptides by HRGC/MS 61
3.3.6 Statistical analysis 62
3.4 Results and Discussion 63
3.4.1 Deuteration of cyclic dipeptides 63
3.4.2 HRGC/MS cyclic dipeptide analysis method performance 67
3.4.3 Quantification of proline-based cyclic dipeptides in *Lb. brevis* R2Δ fermented MRS-broth and wort 70
3.5 Conclusion 77
3.6 Acknowledgements 77
3.7 References 78
Chapter 4 Application of *Lactobacillus amylovorus* DSM19280 in gluten-free sourdough bread to improve the microbial shelf life ... 85
4.1 Abstract 86

4.2 Introduction 87
4.3 Materials and Methods 90
4.3.1 Materials 90
4.3.2 Cultures, media and growth conditions 90
4.3.3 Starch hydrolysis test 91
4.3.4 Flour characterisation 91
4.3.5 Sourdough Type II preparation and analysis 91
4.3.6 Metabolite analysis in sourdough 92
4.3.7 Bread preparation 93
4.3.8 Bread evaluation 94
4.3.9 Shelf life 94
4.3.10 Statistical analysis 95
4.4 Results and Discussion 96
4.4.1 Flour composition and fermentation 96
4.4.2 Microbial bread shelf life 100
4.4.3 Bread properties 106
4.5 Conclusion 109
4.6 Acknowledgements 109
4.7 References 110
Chapter 5 Antifungal carboxylic acids produced by *Lactobacillus reuteri* R29 during sourdough fermentation: Substrate dependency and *in situ* antifungal activity in bread 115
5.1 Abstract 116
5.2 Introduction 117
5.3 Material and Methods 119
5.3.1 Materials 119

5.3.2 Flour characterisation 119
5.3.3 Sourdough Type II preparation 119
5.3.4 Sourdough analysis 120
5.3.5 *In situ* antifungal activity 121
5.3.6 Statistical analysis 121
5.4 Results and Discussion 122
5.4.1 Flour analysis 122
5.4.2 Characterization of the antifungal LAB sourdough 124
5.4.2.1 Carbohydrate metabolism 124
5.4.2.2 Impact of flour substrate on LAB viability and acidification rate 125
5.4.2.3 Influence of flour substrate on the quantity of antifungal compounds 127
5.4.3 *In situ* application of sourdough biopreservative 132
5.5 Conclusion 135
5.6 Acknowledgements 135
5.7 References 137
Chapter 6 Antifungal activity of three different *Lactobacillus* species and their production of antifungal carboxylic acid in wheat sourdough. 143
6.1 Abstract 144
6.2 Introduction 145
6.3 Materials and Methods 147
6.3.1 Materials 147
6.3.2 Cultures, media and growth conditions 147
6.3.3 Amylolytic and proteolytic activity test 148

6.3.4 Preparation of culture cell-free supernatant 148
6.3.5 Sourdough Type II preparation and analysis 148
6.3.6 Metabolite analysis in sourdough 149
6.3.7 Evaluation of *in vitro* antifungal activity 150
6.3.8 Evaluation of *in situ* antifungal activity 150
6.3.9 Statistical analysis 151
6.4 Results 153
6.4.1 Enzymatic activity 153
6.4.2 *In vitro* antifungal activity 153
6.4.3 Sourdough characterisation 155
6.4.4 *In situ* antifungal activity and antifungal compounds of LAB fermented sourdough 157
6.5 Discussion 162
6.6 Acknowledgements 166
6.7 References 167
Chapter 7 General Discussion 172
7.1 General Discussion 173
7.2 References 180
Appendix 184
Appendix A- Additional Table 185
Appendix B- Publications and presentations 186

List of Figures

Figure 2-1 Fumonisin B2 with marked free amino group..... 36

Figure 3-1 Mass spectra (MS-EI) of cyclo(Leu-Pro) (A), [²H]-cyclo(Leu-Pro) (B), cyclo(Pro-Pro) (C), [²H]-cyclo(Pro-Pro) (D), cyclo(Met-Pro) (E), [²H]-cyclo(Met-Pro) (F), cyclo(Phe-Pro) (G), [²H]-cyclo(Phe-Pro) (H). 65

Figure 3-2 Chromatogram obtained from the cyclic dipeptide standards: 1, cyclo(Leu-Pro) at 17.80 min (RI 2301.2); 2, cyclo(Pro-Pro) at 17.86 min (RI 2302.4); 3, cyclo(Met-Pro) at 20.97 min (RI 2706.8); 4, and cyclo(Phe-Pro) at 21.83 min (RI 2823.6)..... 68

Figure 3-3 Calibration curves obtained by mass chromatography of defined labelled and unlabelled cyclic dipeptides; peak areas of the selected isotopomers of cyclic dipeptides are always related to the peak area of the isotopic labelled internal standard; R^2 values for each curve were above 0.998 (cf. Table 3-3). 69

Figure 3-4 Amplified chromatogram of the *Lb. brevis* R2Δ MRS-broth CFS extract: Total ion chromatogram (A), chromatogram of m/z 154 with peak 1 for cyclo(Leu-Pro) (B), chromatogram of m/z 194 with peak 2 for cyclo(Pro-Pro) (C), chromatogram of m/z 228 with peak 3 for cyclo(Met-Pro) (D) and chromatogram of m/z 244 with peak 4 for cyclo(Phe-Pro) (E). 71

Figure 3-5 Mass spectra obtained from the extract of MRS-broth fermented with *Lb. brevis* R2Δ: mass spectrum at 17.84 min (RI 2302) resulted from peak 1 + 2 (A), mass spectrum at 20.97 min (RI 2706.8) resulted from peak 3 (B) and mass spectrum at 21.83 min (RI 2823.6) resulted from peak 4 (C). 72

Figure 4-1 Evaluation of fungal outgrowth on quinoa bread slices..... 95

Figure 4-2 In-vitro starch digestibility agar plate test, a) positive *Lb. amylovorus* 19280; b) negative *Lb. brevis* R2Δ..... 98

Figure 4-3 Shelf life of quinoa bread against environmental moulds during a 14-day storage period. Bread spoilage is indicated as percentage of the total surface area of each of the 12 slices where fungal outgrowth occurred: Mould free slices (white area), <10 % mouldy (grey diagonally striped area), 10–24 % mouldy (grey area), 25-49 % mouldy (black horizontally

striped area) and >50 % mouldy (black area). Mean values are shown (n = 2), error bars indicate standard deviations. 101
Figure 4-4 Chromatographic profile of a 15 µg/mL standard mix (dashed line); freeze dried quinoa sourdough samples, fermented with *Lb. amylovorus* DSM19280 (black line) and chemical acidified control (grey line) extracted according to Brosnan et al. (2014). Compounds listed in Table 4-4. 103
Figure 4-5 Crumb hardness for quinoa and wheat sourdough breads and their controls; mean values ± confidence interval (α = 0.05). 106
Figure 5-1 Cell growth after sourdough fermentation (48 h) with *Lb. reuteri* R29 using different substrates; mean values ± confidence interval (α = 0.05), different lower case letters indicate statistical difference (P < 0.01). 126
Figure 5-2 Chromatograms with identified compounds in samples of quinoa, rice and wheat sourdough doughs fermented with *Lb. reuteri* R29; A) 4-hydroxyphenyllactic acid, B) hydrocaffeic acid, C) 4-hydroxybenzoic acid, D 2-hydroxyisocaproic acid, E) vanillic acid, F) caffeic acid, G) 3-phenyllactic acid, H) phloretic acid, I) hydroferulic acid, J) benzoic acid, K) p-coumaric acid, L) ferulic acid, M) salicylic acid, N) azelaic acid; chromatograms from quinoa sample mainly with B and C from rice and H from wheat sample. 131
Figure 5-3 Shelf life of quinoa, rice and wheat control breads and sourdough breads fermented with *Lb. reuteri* R29 after challenging against environmental moulds during a 14-day storage period. Bread spoilage is indicated as percentage of the total surface area of each of the 12 slices where fungal outgrowth occurred: Mould free slices (white area), < 10 % mouldy (grey diagonally striped area), 10–24 % mouldy (grey area), 25-49 % mouldy (black horizontally striped area) and > 50 % mouldy (black area). Mean values are shown (n = 2), error bars indicate standard deviations. 134
Figure 6-1 Proteolytic activity on milk agar (left) and amylolytic activity on starch agar (right) of *Lb. amylovorus* DSM19280 (a), *Lb. brevis* R2Δ (b) and *Lb. reuteri* R29 (c)..... 153

Figure 6-2 Inhibitory zones formed around *Lb. amylovorus* DSM19280, *Lb. brevis* R2Δ and *Lb. reuteri* R29 against *F. culmorum* TMW4.2043 after 2 days of incubation in comparison with substrate control (only mMRS5-agar). 154

Figure 6-3 Growth of *F. culmorum* TMW4.2043 (10^4 cfu/mL) in malt extract broth (pH 4) containing: 15 % (--●--), 7.5 % (—x—), 3.75 % (—○—) or 0 % (•••▲•••) of cell-free supernatant of (A) *Lb. amylovorus* DSM19280; (B) *Lb. brevis* R2Δ and (C) *Lb. reuteri* R29. Data represents the average of a duplicate with standard deviations. 155

Figure 6-4 Chromatograms of 3-phenyllactic acid (I) and 2-hydroxyisocaproic acid (II) derived from extracts of wheat sourdough fermented with *Lb. reuteri* 29 (A), *Lb. brevis* R2Δ (B) and *Lb. amylovorus* DSM19280 (C). Mass spectra of 3-phenyllactic acid (ID) and 2-hydroxyisocaproic acid (IID)..... 159

Figure 6-5 Shelf life of wheat bread against environmental moulds during a 14-day storage period. Bread spoilage is indicated as percentage of the total surface area of each of the 12 slices where fungal outgrowth occurred: Mould-free slices (white area), <10 % mouldy (grey diagonally striped area), 10–24 % mouldy (grey area), 25-49 % mouldy (black horizontally striped area) and >50 % mouldy (black area). Mean values are shown (n = 2); error bars indicate standard deviations. 161

List of Tables

Table 2-1 Antifungal lactic acid bacteria used as starter for biopreservatives, their tested activity and related antifungal compounds, over the last 15 years. 23

Table 2-2 Antifungal yeasts applied in bread baking, their tested activity and related antifungal compounds. 30

Table 3-1 Isotopomeric distribution of unlabelled and deuterium labelled cyclic dipeptides (molecular m/z-ion of the unlabelled cyclic dipeptide in bold). 66

Table 3-2 Determination of the concentration of the labelled compounds using methyl octanoate as internal standard (1 μg/mL)..... 67

Table 3-3 Selected m/z of unlabelled compounds and labelled standards and calibration functions used for the quantification of cyclic dipeptides. ... 69

Table 3-4 Concentration of cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Met-Pro) and cyclo(Phe-Pro) in cultures of *Lb. brevis* R2Δ, acid treated broth and in unfermented controls. 73

Table 4-1 Quinoa and wheat flour composition (values based on fresh weight of samples)[a]. 97

Table 4-2 Carbohydrate levels, lactate formation, TTA and pH in sourdoughs made from quinoa and wheat flour (DY 200) after 48 h fermentationa. 99

Table 4-3 pH and TTA values of quinoa sourdoughs breads[a]. 102

Table 4-4 Concentration of antifungal compounds (ppm)[a]. 105

Table 4-5 Bread and crumb characteristics of sourdough breads and control breads made from quinoa and wheat flour[a]. 108

Table 5-1 Quinoa, rice and wheat flour composition (values based on fresh weight of samples)[a]. 123

Table 5-2 Initial and residual sugars [mmol/kg flour] from flours and sourdoughs (SD) fermented with *Lb. reuteri* R29[a]. 125

Table 5-3 Values for pH, total titratable acid (TTA) and organic acids (lactate and acetate) of flours and sourdough (SD) after fermentation using *Lb. reuteri* R29[a]. 127

Table 5-4 Antifungal compounds [ppm] analysed in freeze dried quinoa, rice and wheat sourdoughs fermented with *Lb. reuteri* R29[a]. 129
Table 6-1 Sourdough characterisation of the fermentation of wheat flour (DY 200) after 48 h fermentationa different species of LAB[a]. 156
Table 6-2 Antifungal compounds [ppm] analysed in freeze dried sourdoughs[a] and their single MIC. 157

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.



Signature

Abbreviations

AP,	Active packaging
a_W,	Water activity
CD,	Coeliac disease
cfu,	Colony forming unit
DON,	Deoxynivalenol
DY,	Dough yield
EFSA,	European Food Safety Authority
GRAS,	Generally regarded as safe
HRGC/MS,	High resolution gas chromatography-low resolution mass spectrometry
LAB,	Lactic acid bacteria
MAP,	Modified atmosphere packaging
MIC,	Minimal inhibition concentration
OTA,	Ochratoxin A
PTV,	Programmable Temperature Vaporizer
QPS,	Qualified Presumption of Safety
RID,	Refractive index detector
SD,	Sourdough
SIDA,	Stable isotope dilutions assay
TTA,	Total titratable acidity
UV/DAD	Ultra violet-diode array detector

Abstract

As part of the "free-from" trend, biopreservation for bread products has increasingly become important to prevent spoilage since artificial preservatives are more and more rejected by consumers. A literature review conducted as part of this thesis revealed that the evaluation of more suitable antifungal strains of lactic acid bacteria (LAB) is important. Moreover, increasing the knowledge about the origin of the antifungal effect is fundamental for further enhancement of biopreservation. This thesis addresses the investigation of *Lactobacillus amylovorus* DSM19280, *Lb. brevis* R2Δ and *Lb. reuteri* R29 for biopreservation using *in vitro* trials and *in situ* sourdough fermentations of quinoa, rice and wheat flours as biopreservatives in breads. Their contribution to quality and shelf life extension on bread was compared and related to their metabolic activity and substrate features. Moreover, the quantity of antifungal carboxylic acids produced during sourdough fermentation was analysed. Overall a specific profile of antifungal compounds was found in the sourdough samples which were strain and substrate dependently different. The best preservative effect in quinoa sourdough and wheat sourdough bread was achieved when *Lb. amylovorus* DSM19280 fermented sourdough was used. However, the concentration of the antifungal compounds found in these biopreservatives were much lower when compared with *Lb. reuteri* R29 as the highest producer. Nevertheless, the artificial application of the highest concentration of these antifungal compounds in chemically acidified wheat sourdough bread succeeded in a longer shelf life than achieved only by acidifying the dough. This evidences their partial contribution to the antifungal activity and their synergy. Additionally, a HRGC/MS method for the identification and quantification of the antifungal active compounds cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Met-Pro) and cyclo(Phe-Pro) was successfully developed by using stable isotope dilutions assays with the deuterated counterparts. It was observed that the concentrations of cyclo(Leu-Pro), cyclo(Pro-Pro), and cyclo(Phe-Pro) increased only moderately in MRS-broth and wort fermentation by the activity of the selected microorganism, whereas the concentration of cyclo(Met-Pro) stayed unchanged.

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Chapter 1 Introduction

1.1 Introduction

Bread belongs to one of the major staple foods and is consumed daily in all parts of the world (Hager et al., 2012). Additionally, the interest in the application of alternative gluten-free crops for the production of gluten-free bread is growing constantly, mainly due to the rising diagnosis of coeliac disease (CD) as well as other allergic reactions and non-coeliac gluten sensitivity have risen over the last years (Catassi et al., 2013; Fasano and Catassi, 2012). CD is an immune-mediated enteropathy caused by the ingestion of the prolamin fraction of gluten (present in wheat, rye and barley) in genetically sensitive individuals (Gallagher, 2009). The adherence to a gluten-free diet is still the only accepted treatment for CD (Arendt et al., 2011). Consequently, a growing market exists in gluten-free cereal products (Zannini et al., 2012).

Microbial spoilage of bread and the consequent waste problem still causes large economic losses for both the bakery industry and the consumer. Sourdough is the foremost fermentation method used for baking purposes and it has been proven to be ideal for improving the texture, aroma, flavour, and nutritional value; furthermore, it retards staling and extends the microbial shelf life of gluten-containing and gluten-free breads (Arendt et al., 2007; Gänzle et al., 2007; Moore et al., 2008; Moroni et al., 2009). Traditional sourdough, classified as Type I, is a mixture of flour and water, which is fermented spontaneously by lactic acid bacteria (LAB) and yeasts, refreshed and propagated in various fermentation steps (Corsetti, 2013). Whereas, Type II is obtained through a unique fermentation step using a portion of the mature sourdough from Type I or defined starter cultures (Corsetti, 2013). Sourdough fermented with specific antifungal strains of LAB serves as a high-potential biological ingredient to produce breads with an extended shelf life. Chapter 2 gives an overview of the current status of bread spoilage and reviews possible methods for bread biopreservation.

LAB represent a group of Gram-positive bacteria which are associated with a range of metabolic and physiological characteristics. Their use as starter cultures in the manufacture of fermented foods relies on a long tradition. However, in the last recent decades the research of LAB has gained great prominence. One part of this research is to study the antifungal activity of LAB. Driven by the increasing demand of

consumers for more natural and minimal-processed foods, the application of specific antifungal LAB strains is promising for biopreservation. As such, LAB fermented food products fit perfectly in the healthy life style trend and offer a natural way to extend shelf life ensuring food safety.

Many species of the genus *Lactobacillus* but also some *Lactococcus*, *Pediococcus* and *Leuconostoc* species were assessed for safety from the European Food Safety Authority (EFSA) without raising safety concerns. As a result, they have been included in the QPS (Qualified Presumption of Safety) list for authorised use in the food and feed chain within the European Union (EFSA, 2012). Conclusively, this promotes these starter cultures to be categorised as safe biological agents guaranteeing a clean label by the EU regulation on food additives (EEC, 2008). The same applies to the U.S., where they enjoy the generally regarded as safe (GRAS) status regulated by the U.S. Food and Drug Administration.

In the last decades, considerable effort has been directed to screen the antifungal activity of LAB in order to find suitable starter cultures for biopreservation. This content was recently reviewed by a number of studies (Crowley et al., 2013; Dalié et al., 2010; Oliveira et al., 2014; Pawlowska et al., 2012). However, *in situ* applications, where antifungal LAB starter cultures were used, are still scarce but include milk, meat, fruit, vegetable and cereal fermentations.

Lactic and acetic acid are the main fermentation products by LAB. In addition to these, further low-molecular weight compounds have been isolated from LAB fermentates which have been associated with antifungal activity. They include other organic acids (e.g., formic, propionic, and butyric acids), carbon dioxide, diacetyl, hydrogen peroxide, 3-hydroxy fatty acids, cyclic dipeptides, phenyl and substituted phenyl derivates (e.g., 3-phenyllactic, 4-hydroxyphenyllactic, benzoic acid), reuterin, fungicins and other proteinaceous compounds (Batish et al., 1997; Magnusson et al., 2003; Ryan et al., 2011; Schnürer and Magnusson, 2005; Sjögren et al., 2003; Ström et al., 2002; Yang and Chang, 2010). However, some studies showed that the concentrations of several of these metabolites in the culture broths are much lower than their minimal inhibition concentration (MIC), which is required for non-synergistic antifungal activity. Therefore, a complex synergistic mechanism between

the metabolites has been proposed to play the key role in antifungal action (Cortés-Zavaleta et al., 2014; Miescher Schwenninger et al., 2008; Ndagano et al., 2011; Niku-Paavola et al., 1999).

The obligate homofermentative strain *Lactobacillus amylovorus* DSM19280 has previously been reported to be effective against a range of different food spoilage (Belz et al., 2012; Lynch et al., 2014; Oliveira et al., 2015; Ryan et al., 2011; Waters et al., 2012). *Lactobacillus reuteri* R29, as an obligate heterofermentative strain, revealed antifungal activity in malting trials (Oliveira et al., 2015). The aim of this thesis was to further investigate these strains for *in vitro* and *in situ* cereal fermentations along with another strain, *Lactobacillus brevis* R2Δ, selected for its antifungal traits.

Cyclic dipeptides provide a multitude of bioactive properties (e.g., anticancer, antiviral, antihyperglycemic, and antimicrobial) which make them to remarkable tools for industrial and clinical applications (Borthwick et al., 2012). Several proline-based and two leucine-based cyclic dipeptides have been isolated from culture broths of different LAB (Dal Bello et al., 2007; Li et al., 2011; Magnusson et al., 2003; Niku-Paavola et al., 1999; Ryan et al., 2011; Ström et al., 2002; Yang and Chang, 2010). The presence of these cyclic dipeptides in LAB fermentates was associated with antifungal activity although the quantity of these compounds were not assessed by these studies. To investigate the concentration level of cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Met-Pro) and cyclo(Phe-Pro), deuterium-labelled (^{2}H-labelled) counterparts of these four cyclic dipeptides were synthesized in order to develop stable isotope dilutions assays (SIDA) in combination with high resolution gas chromatography-low resolution mass spectrometry (Chapter 3). The developed method was then applied to analyse MRS-broth and wort fermentates using the selected strains.

Chapter 4, Chapter 5 and Chapter 6 evaluate the potential and suitability of the selected antifungal strains to improve the microbial shelf life of gluten-containing and gluten-free bread and consequently improve safety and quality. In order to fully exploit the antifungal efficacy of the starter cultures, Type II sourdough fermentations were applied. These are generally not suitable for achieving dough leavening but are used for dough acidification and as dough improvers (Corsetti, 2013). A further approach with these studies was to evaluate the metabolic activity, acidification

pattern and quantity of antifungal compounds associated with the LAB sourdough fermentation and their contribution to shelf life extension on bread.

1.2 References

Arendt, E.K., Moroni, A., Zannini, E., 2011. Medical nutrition therapy: use of sourdough lactic acid bacteria as a cell factory for delivering functional biomolecules and food ingredients in gluten free bread. Microbial Cell Factories 10 Suppl 1, S15. doi:10.1186/1475-2859-10-S1-S15

Arendt, E.K., Ryan, L.A.M., Dal Bello, F., 2007. Impact of sourdough on the texture of bread. Food Microbiology 24, 165–174. doi:10.1016/j.fm.2006.07.011

Batish, V.K., Roy, U., Lal, R., Grover, S., 1997. Antifungal attributes of lactic acid bacteria--a review. Critical Reviews in Biotechnology 17, 209–25. doi:10.3109/07388559709146614

Belz, M.C.E., Mairinger, R., Zannini, E., Ryan, L.A.M., Cashman, K.D., Arendt, E.K., 2012. The effect of sourdough and calcium propionate on the microbial shelf-life of salt reduced bread. Applied Microbiology and Biotechnology 96, 493–501. doi:10.1007/s00253-012-4052-x

Borthwick, A.D., Grove, T., Nw, L., 2012. 2,5-Diketopiperazines: Synthesis, Reactions, Medicinal Chemistry, and Bioactive Natural Products. Chemical Reviews. doi:10.1021/cr200398y

Catassi, C., Bai, J.C., Bonaz, B., Bouma, G., Calabrò, A., Carroccio, A., Castillejo, G., Ciacci, C., Cristofori, F., Dolinsek, J., Francavilla, R., Elli, L., Green, P., Holtmeier, W., Koehler, P., Koletzko, S., Meinhold, C., Sanders, D., Schumann, M., Schuppan, D., Ullrich, R., Vécsei, A., Volta, U., Zevallos, V., Sapone, A., Fasano, A., 2013. Non-celiac gluten sensitivity: The new frontier of gluten related disorders. Nutrients 5, 3839–3853. doi:10.3390/nu5103839

Corsetti, A., 2013. Chapter 4 Technology of Sourdough Fermentation and Sourdough Applications, in: Gobetti, M., Gänzle, M.G. (Eds.), Handbook on Sourdough Biotechnology. Springer, pp. 85–103. doi:10.1007/978-1-4614-5425-0

Cortés-Zavaleta, O., López-Malo, a, Hernández-Mendoza, a, García, H.S., 2014. Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. International Journal of Food Microbiology 173, 30–5. doi:10.1016/j.ijfoodmicro.2013.12.016

Crowley, S., Mahony, J., van Sinderen, D., 2013. Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. Trends in Food Science & Technology 33, 93–109. doi:10.1016/j.tifs.2013.07.004

Dal Bello, F., Clarke, C.I., Ryan, L.A.M., Ulmer, H., Schober, T.J., Ström, K., Sjögren, J., van Sinderen, D., Schnürer, J., Arendt, E.K., Strom, K., Sjogren, J., Schnurer, J., 2007. Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. Journal of Cereal Science 45, 309–318. doi:10.1016/j.jcs.2006.09.004

Dalié, D.K.D., Deschamps, A.M., Richard-Forget, F., 2010. Lactic acid bacteria – Potential for control of mould growth and mycotoxins: A review. Food Control 21, 370–380. doi:10.1016/j.foodcont.2009.07.011

EEC, 2008. Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. Official Journal of the European Union L 354, 16.

EFSA, 2012. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update). EFSA Journal 10, 3020. doi:10.2903/j.efsa.2012.3020.

Fasano, A., Catassi, C., 2012. Clinical practice. Celiac disease. The New England Journal of Medicine 367, 2419–26. doi:10.1056/NEJMcp1113994

Gallagher, E., 2009. Front Matter, in Gluten-Free Food Science and Technology. Wiley-Blackwell, Oxford, UK. doi: 10.1002/9781444316209.fmatter.

Gänzle, M.G., Vermeulen, N., Vogel, R.F., 2007. Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiology 24, 128–138. doi:10.1016/j.fm.2006.07.006

Hager, A.S., Wolter, A., Czerny, M., Bez, J., Zannini, E., Arendt, E.K., 2012. Investigation of product quality, sensory profile and ultrastructure of breads made from a range of commercial gluten-free flours compared to their wheat counterparts. European Food Research and Technology 235, 333–344. doi:10.1007/s00217-012-1763-2

Li, J., Wang, W., Xu, S.X., Magarvey, N.A., McCormick, J.K., 2011. *Lactobacillus reuteri* -produced cyclic dipeptides quench agr -mediated expression of toxic shock

syndrome toxin-1 in staphylococci. Proceedings of the National Academy of Sciences 108, 3360–3365. doi:10.1073/pnas.1017431108/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1017431108

Lynch, K.M., Pawlowska, A.M., Brosnan, B., Coffey, A., Zannini, E., Furey, A., McSweeney, P.L.H., Waters, D.M., Arendt, E.K., 2014. Application of *Lactobacillus amylovorus* as an antifungal adjunct to extend the shelf-life of Cheddar cheese. International Dairy Journal 34, 167–173. doi:10.1016/j.idairyj.2013.07.017

Magnusson, J., Ström, K., Roos, S., Sjögren, J., Schnürer, J., 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiology Letters 219, 129–135. doi:10.1016/S0378-1097(02)01207-7

Miescher Schwenninger, S., Lacroix, C., Truttmann, S., Jans, C., Spörndli, C., Bigler, L., Meile, L., 2008. Characterization of low-molecular-weight antiyeast metabolites produced by a food-protective *Lactobacillus-Propionibacterium* coculture. Journal of Food Protection 71, 2481–2487.

Moore, M.M., Dal Bello, F., Arendt, E.K., 2008. Sourdough fermented by *Lactobacillus plantarum* FST 1.7 improves the quality and shelf life of gluten-free bread. European Food Research and Technology 226, 1309–1316. doi:10.1007/s00217-007-0659-z

Moroni, A. V, Dal Bello, F., Arendt, E.K., 2009. Sourdough in gluten-free bread-making: an ancient technology to solve a novel issue? Food Microbiology 26, 676–84. doi:10.1016/j.fm.2009.07.001

Ndagano, D., Lamoureux, T., Dortu, C., Vandermoten, S., Thonart, P., 2011. Antifungal activity of 2 lactic acid bacteria of the *Weissella* genus isolated from food. Journal of Food Science 76, M305–311. doi:10.1111/j.1750-3841.2011.02257.x

Niku-Paavola, M.L., Laitila, A., Mattila-Sandholm, T., Haikara, A., 1999. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. Journal of Applied Microbiology 86, 29–35.

Oliveira, P.M., Brosnan, B., Furey, A., Coffey, A., Zannini, E., Arendt, E.K., 2015. Lactic acid bacteria bioprotection applied to the malting process. Part I: Strain

characterization and identification of antifungal compounds. Food Control 51, 433–443. doi:10.1016/j.foodcont.2014.07.004

Oliveira, P.M., Zannini, E., Arendt, E.K., 2014. Cereal fungal infection, mycotoxins, and lactic acid bacteria mediated bioprotection: from crop farming to cereal products. Food Microbiology 37, 78–95. doi:10.1016/j.fm.2013.06.003

Pawlowska, A.M., Zannini, E., Coffey, A., Arendt, E.K., 2012. "Green preservatives": combating fungi in the food and feed industry by applying antifungal lactic acid bacteria. Advances in Food and Nutrition Research 66, 217–238. doi:10.1016/b978-0-12-394597-6.00005-7

Ryan, L.A.M., Zannini, E., Dal Bello, F., Pawlowska, A., Koehler, P., Arendt, E.K., 2011. *Lactobacillus amylovorus* DSM 19280 as a novel food-grade antifungal agent for bakery products. International Journal of Food Microbiology 146, 276–283. doi:10.1016/j.ijfoodmicro.2011.02.036

Schnürer, J., Magnusson, J., 2005. Antifungal lactic acid bacteria as biopreservatives. Trends in Food Science & Technology 16, 70–78. doi:10.1016/j.tifs.2004.02.014

Sjögren, J., Magnusson, J., Broberg, A., Schnürer, J., Kenne, L., 2003. Antifungal 3-Hydroxy Fatty Acids from *Lactobacillus plantarum* MiLAB 14. Applied and Environmental Microbiology 69, 7554–7557. doi:10.1128/AEM.69.12.7554

Ström, K., Sjögren, J., Broberg, A., Schnürer, J., 2002. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(l-Phe-l-Pro) and cyclo(l-Phe-trans -4-OH-l-Pro) and 3-phenyllactic Acid. Applied and Environmental Microbiology 68, 4322–4327. doi:10.1128/AEM.68.9.4322

Waters, D.M., Parlet, C., Moroni, A., Arendt, E.K., 2012. Identification of the fungal microflora of coffee beans from different origins and evaluation of different decontamination concepts, in: 24th International Conference on Coffee Science, ASIC Costa Rica 24th. pp. 145–152.

Yang, E.J., Chang, H.C., 2010. Purification of a new antifungal compound produced by *Lactobacillus plantarum* AF1 isolated from kimchi. International Journal of Food Microbiology 139, 56–63. doi:10.1016/j.ijfoodmicro.2010.02.012

Zannini, E., Pontonio, E., Waters, D.M., Arendt, E.K., 2012. Applications of microbial fermentations for production of gluten-free products and perspectives.

Applied Microbiology and Biotechnology 93, 473–485. doi:10.1007/s00253-011-3707-3

Chapter 2 Literature Review – Mould spoilage of bread and its biopreservation: A review of current strategies for bread shelf life extension

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2.1 Abstract

Microbial spoilage of bread and the consequent waste problem causes large economic losses for both the bakery industry and the consumer. Furthermore the presence of mycotoxins due to fungal contamination in cereals and cereal products remains a significant issue. The use of conventional chemical preservatives has several drawbacks, necessitating the development of clean-label alternatives.

In this review, we aim to describe current research aiming to extend the shelf life of bread through the use of more consumer friendly and ecologically sustainable preservation techniques as opposed to chemical additives. Studies on the *in situ*-production/-expression of antifungal compounds are presented, with special attention given to recent developments over the past decade. Sourdough fermented with antifungal strains of lactic acid bacteria is an area of increasing focus and serves as a high-potential biological ingredient to produce gluten-containing and gluten-free breads with improved nutritional value, quality and safety due to shelf-life extension, and is in-line with consumers demands for more products containing less additives. In addition, alternative biopreservation techniques are briefly addressed. This review outlines recent progress that has been made in the area of bread biopreservation and future perspectives in this important area.

2.2 Introduction

Baked goods are considered to be one of the most important products of the food industry. Bread is a staple food in many countries and consumed daily all over the world. Within the European Union (EU) the production of bread is relatively stable showing low growth in most western countries. The Germans and Austrians consume the most bread averaging at 80 kg bread per person per year, while the UK and Ireland have the lowest average annual consumption, less than 50 kg (The Federation of Bakers, 2012). In contrast to Europe, the consumption of bread products shows an increasing trend in most developing countries (Elsanhoty et al., 2013).

Over the last decade, consumer behaviour in the baked goods category has changed. In particular, consumers' concerns about safety and additive contents in food have received much attention. Additionally, the number of health-conscious consumers has increased. Consequently, a high demand for "natural" and "wholesome" foods without chemical preservatives and additives exists on the market. Accordingly, bread manufacturers have increasingly moved to produce so called "clean label products" which fit this healthier lifestyle of customers. Food labels of such products have claims such as "no preservatives" or "natural". In fact, marketing the absence of additives/preservatives (21 % of new products launched in Europe 2013/2014) and the inclusion of wholegrain continues to drive health-focused new product developments (Mintel, 2014). In turn, such minimally processed food without chemical preservatives or other artificial additives should still be of high-quality and have an extended shelf life.

Spoilage of bread can be caused by bacteria, yeast and moulds. However, contamination originates predominantly post baking by fungal spores being deposited from the bakery environment (Knight and Menlove, 1961). The most critical factors controlling the growth of undesirable fungi on foodstuffs are oxygen, temperature, pH, and water activity (a_w). Generally, breads have a relatively high moisture content and aw between 0.94–0.97 at a pH of about 6 with sliced, prepacked and wrapped breads belong to the most susceptible bakery products for mould spoilage (Magan et al., 2003a). This is because wrapping prevents moisture loss from the bread slices allowing suitable growth conditions for fungi in a humid atmosphere. When wrapped,

freshly baked breads without any added preservatives have a shelf-life of only a few days at room temperature.

Microbial deterioration of bread is of serious concern and the consequent waste problem still causes large economic losses for both the bakery industry and the consumer (Melikoglu and Webb, 2013). In 2011, Novozymes surveyed over 4000 bread consumers throughout Europe and found evidence that the main reason that bread was thrown away was because it had become mouldy (van Sint Fiet, 2015). Apart from the unpleasant sight of visible mould growth, fungi are also responsible for the production of mycotoxins and off-flavours, which might be produced even before fungal outgrowth is visible (Magan et al., 2003b). Thus, spoiled breads represent a hazard which can be of a high risk to consumer's health.

Physical methods like ultraviolet light, infrared radiation, microwave heating or ultra-high pressure treatments can destroy post-baking contaminants in breads. However, few studies employing these methods have been done in the recent past. In light of the continuing consumer trend towards a healthier lifestyle, studies have increasingly set targets to replace traditionally used chemical preservatives with environmental friendly, "clean-label" alternatives. Thus the use of "biopreservation", defined as the extension of "shelf life by the use of natural or controlled microbiota and/or their antimicrobial compounds" (Stiles, 1996) has become an increasingly important field of research. Furthermore, this term can also be applied to active plant ingredients or plant extracts. Additionally, recent investigations have dealt with improving protective packaging. This paper reviews the potential for biopreservation and smart packaging to insure bread safety with an emphasis on the *in situ*-production/-expression of antifungal compounds by lactic acid bacteria (LAB).

2.3 Cereals and their contamination with fungi and mycotoxins

Many species and varieties of cereals and pseudocereals are cultivated worldwide. Their products are an important source of nutrients for human consumption in both developed and developing countries. The major cereal crops produced worldwide are wheat, rice and maize (FAO, 2013). Most relevant for bread production in the western world is wheat followed by rye. Other relevant cereals include barley, oat, and the gluten-free crops, millet, sorghum, quinoa, amaranth and buckwheat. The latter three belong to the pseudocereals, which feature higher quality protein and the presence of abundant quantities of fibre, vitamins and minerals such as calcium and iron when compared to their cereal counterparts (Alvarez-Jubete et al., 2010).

Cereals and pseudocereal crops are susceptible to a wide spectrum of plant pathogens. During cultivation, cereal contamination by fungi can cause severe plant diseases which can cause enormous yield losses correlated with the reduction of grain quality. Common phytogenic pathogens include filamentous fungi such as *Alternaria*, *Aureobasidium*, *Cladosporium*, *Claviceps*, *Epicoccum*, *Fusarium* and *Helminthosporium*, with *Fusarium* infections seem to representing a major threat (Oliveira et al., 2014). Cereal grains and their products are exposed to contamination post-harvest, during storage and pre- and post-processing. The most common fungi related to losses in bakery products belong to the genera *Aspergillus*, *Cladosporium*, *Endomyces*, *Fusarium*, *Monilia*, *Mucor*, *Penicillium*, and *Rhizopus* (Dal Bello et al., 2007). Other bread ingredients, equipment or packaging material, when contaminated, could also represent a potential vehicle allowing spoilage-related and undesirable microorganisms into the baking environment (Reale et al., 2013). To guarantee food safety and quality along the food production chain, it is essential to follow guidelines like Codex Alimentarius including implementing such systems as good agricultural practices (GAP), good manufacturing practices (GMPs), good hygienic practices (GHPs) and Hazard Analysis and Critical Control Point (HACCP) systems.

In normal bakery practice moulds do not survive the bread-baking process (Knight and Menlove, 1961). In contrast, mycotoxins produced by crop pathogens and food-spoilage fungi are relatively heat stable (Vidal et al., 2015; Wu and Wang, 2015) and therefore often represent a more serious problem (Osborne and Stein, 2007). The

effect of processing on the mycotoxin content in grains was recently summarized by Kaushik et al. (2015). The author concluded that the reduction in mycotoxins during baking is relatively low and varies with the toxin. An EU regulation (No. 1881/2006) defines maximum levels for the mycotoxins, aflatoxin (2 μg/kg) and ochratoxin A (OTA) (3 μg/kg) in all cereal products; for the *Fusarium* mycotoxins, deoxynivalenol (DON) (500 μg/kg) and zearalenone (50 μg/kg) specific maximum levels have been set for bread and other bakery products such as biscuits or pastries (EEC, 2006). However, for some *Fusarium* mycotoxins like T-2 and HT-2 toxins, fumonisins, enniatins and nivalenol no maximum level has yet been set. A great number of articles are available in the literature for further reading about mycotoxins. Pereira et al. (2014) give an up-to-date review about the occurrence of mycotoxins specifically in cereals and cereal-related foodstuff along with their recent methods of analysis. A review by Oliveira et al. (2014) addresses cereal fungal infections in relation with bioprotection. The authors provide an extensive overview of mycotoxins found in cereal crops, their fungal source and their possible health hazards for humans and animals. Mycotoxins may cause severe health problems with carcinogenic, nephrotoxic, neurotoxic and/or immunosuppressive effects. Wu et al. (2014) provide a more detailed description about the adverse human health impacts associated with the major groups of mycotoxins, aflatoxin, fumonisins, DON and OTA. Co-occurrence of different mycotoxins is of great concern due to possible synergic toxic effects. Of further concern are conjugated mycotoxins, which are masked by transformation, conjugation or compartmentalization. Food processing can result in conjugation. To date, no regulatory levels exist for these conjugated mycotoxins in cereals and cereal-based foods.

The presence of mycotoxins due to fungal contamination in cereals and cereal products remains a significant problem worldwide, and although monitoring occurs in most countries, they are detected frequently in processed cereal products (Aldana et al., 2014; Błajet-Kosicka et al., 2014; Demirel and Sariozlu, 2014; Škrbić et al., 2012).

2.4 Conventional food preservatives

Weak organic acids such as propionic and sorbic acid are commonly added as chemical preservatives to suppress the growth of undesired microorganisms and to lengthen the shelf life of bakery products. Generally at lower pH values, these acids are in their undissociated form and can easily penetrate the plasma membrane. Once intracellular, the acid dissociates and due to the release of charged protons, the cell cytoplasm gets acidified. Consequently, the drop in pH decreases phosphofructokinase activity, a key enzyme of glycolysis, and hence reduces the ATP yield (Krebs et al., 1983). Recent investigations have suggested that sorbic acid may act additionally as a membrane-active antimicrobial compound through the inhibition of the plasma membrane H+-ATPase proton pump (Stratford et al., 2013a, 2009).

Within the EU, the use of chemical preservatives in bakery wares like bread and rolls is limited (EEC, 2008). Because of their higher water solubility and easier handling than their respective corrosive acids, potassium, sodium or calcium salts of propionic and sorbic acid are the forms generally used (Magan et al., 2003a). Sorbate is allowed up to 0.2 % (w/w) and propionate can be added to a maximum 0.3 % (EEC, 2008). To define it more precisely, these levels pertain only to prepacked sliced bread and rye bread. A maximum of only 0.1 % propionate is permitted for prepacked unsliced bread. This means that for unpacked bread and in particular wheat bread made only from wheat flour, water, yeast or sourdough and salt, neither the addition of sorbate nor propionate is allowed. The addition of benzoic acid to bakery products is, although sometimes examined in studies (Guynot et al., 2005; Suhr and Nielsen, 2004), not authorized.

Suhr et al. (2004) conducted an *in vitro* screening experiment over a wide pH and aw range using different concentrations of weak acid preservatives in rye agar and wheat agar, against 9 fungal isolates. On wheat agar, a high propionate concentration (0.3 %) generally had a strong inhibitory effect on all fungi tested at pH 4.5 and a_w 0.95, but not at pH 6, which is generally the pH of standard wheat bread. However, on rye agar (high a_w and low pH), the growth of *P. roqueforti* even occurred up to the addition of 0.3 % propionate. Thus, the authors concluded that the addition of propionate to rye sourdough bread is not recommended due to the resistance of *P. roqueforti*.

Furthermore, in our opinion, those results also indicate that propionate has only slight effect in mould inhibition when included in breads at pH 6.

In another study, sorbate completely inhibited fungal growth at a_w 0.90 and pH 4.5 when compared to calcium propionate assayed at the highest concentration in bread analogues at 0.3 % level (Guynot et al., 2005). The current legislated maximum level of 0.2 % was not tested. However, although sorbate seems to be more efficient at inhibiting spoilage, it is rarely used in bread due to its negative impact on bread volume (Lavermicocca et al., 2000). The use of 0.3 % calcium propionate *in vitro* only partially prevented germination of conidia, in 12 tested fungal species (Lavermicocca et al., 2000). Nevertheless, some *in situ* experiments on the use of propionate to control bread spoilage showed shelf life prolongation and some further examples are given throughout this review.

Overall, high concentrations of sorbate or propionate are desired for antifungal activity, but this can also alter the sensory properties of the product. Prolonged usage of the same preservatives against spoilage fungi may lead to the development of fungal resistance to those chemicals (Levinskaite, 2012; Stratford et al., 2013b; Suhr and Nielsen, 2004). Moreover, concentrations below the maximum level of preservative must be chosen carefully. Sub-optimal concentrations, lower than 0.03 % can result in an enhancement of fungal growth (Marin et al., 2002) and higher mycotoxin production (Arroyo et al., 2005).

2.5 Alternative preservation techniques

The disadvantages outlined regarding the use of chemical preservatives has encouraged researchers to finding alternative agents to control spoilage fungi in food products. Furthermore, strategies need to be developed to reduce mycotoxins levels ensuring food safety and consumer health.

2.5.1 Fermentation

2.5.1.1 Lactic acid bacteria

Microbial fermentation is one of the oldest and most economically and ecologically friendly methods of preserving foods (Zannini et al., 2012). Among bakery products the microorganisms most widely used as starter cultures, applied for example in sourdough production, are LAB. Since active compounds responsible for biopreservation are produced or released by LAB *in situ*, use of sourdough technology can replace chemical preservatives, guaranteeing a clean label while imparting additional positive effects, such as improved flavour, texture and nutritional properties, coupled with higher consumer acceptance (Pawlowska et al., 2012). A further benefit of LAB is that many species from the genera *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* have been referred to the European Food Safety Authority (EFSA) for safety assessment without raising safety concerns. As a result, they have been included in the QPS (Qualified Presumption of Safety) list for authorised use in the food and feed chain within the European Union (EFSA, 2012). The same applies to the US, where they enjoy the Generally Regarded as Safe (GRAS) status regulated by the U.S. Food and Drug Administration.

Recently there have been several comprehensive reviews examining LAB with respect to their antifungal activity spectrum, effective metabolites and their interactions with mycotoxins (Crowley et al., 2013; Dalié et al., 2010; Oliveira et al., 2014).

This review however, describes how specific antifungal LAB strains have been applied to extend the shelf life of bread. In general, a low preservative effect can be achieved through the use of LAB-fermented sourdough due to the pH drop and acidification associated with the production of organic acids, mainly lactic and acetic acid. However, it has been reported that chemical acidification either has no influence on

mould inhibition or can only prolong shelf life to a limited extent (Axel et al., 2015; Ryan et al., 2011). Accordingly, this suggests that LAB, in addition to producing lactate and acetate, produce or release other active compounds during fermentation, which contribute to the antifungal activity when sourdough is included in bakery products. These metabolites are usually low molecular mass compounds such as phenyl and substituted phenyl derivates (3-phenyllactic, 4-hydroxyphenyllactic, and benzoic acid), cyclic dipeptides, hydroxy fatty acids or antifungal peptides. Coupled to their relatively high minimal inhibition concentration (MIC), they are produced at low levels in the fermentation substrate. Thus, the antifungal inhibitory mechanism is believed to originate from a complex synergy effect among these low molecular mass compounds. Moreover, the production of the antifungal compounds during fermentation is substrate specific (Axel et al., 2015; Vermeulen et al., 2006). Therefore, it is necessary to measure the levels of these compounds produced both in nutrient growth media and the sourdough environment.

In the last decades, considerable effort has been directed to screen the antifungal activity of LAB in order to find suitable starter cultures for sourdough fermentation. In terms of biopreservation, these studies first rely on *in vitro* assays which either include dual culture plate assays and or micro titre plate assays (Magnusson and Schnürer, 2001). If a strain shows broad activity against a range of bread spoilage organisms, it is further evaluated in sourdough fermentation and bread baking to assess both its potential to extend shelf life and its effects on bread quality. Such an approach was undertaken by several research groups and findings are presented in Table 2-1. This table summarizes which LAB have been applied as antifungal starter culture in sourdough and bread fermentation. It also presents the mould activity spectrum and the antifungal compounds analysed which were related to the biopreservative extension of shelf life Furthermore, it includes a few studies that assess whether the level of mycotoxins can be decreased by means of fermentation.

Table 2-1 Antifungal lactic acid bacteria used as starter for biopreservatives, their tested activity and related antifungal compounds, over the last 15 years.

Starter culture used	Activity spectrum	Antifungal compounds in biopreservative	Substrate/ Application	Reference
Lb. amylovorus DSM19280	*A. niger, F. culmorum*, *P. expansum*, *P. roqueforti,* environmental moulds[b]	PLA, OH-PLA, hydroferulic acid, phloretic acid	Wheat sourdough, Quinoa sourdough	(Axel et al., 2015; Belz et al., 2012; Ryan et al., 2011)
Lb. plantarum *Lb. brevis (DI-PROX MTTX,* commercial starter mix*)*	Deoxynivalenol	n.d.[a]	Wheat sourdough	(Banu et al., 2014)
Lb. plantarum CRL 778	*A. niger* (a_w= 0.971, 60% lower Ochratoxin A production)	Organic acids, PLA, OH-PLA	Wheat slurry, Quinoa slurry and mixtures	(Gerez et al., 2014)
Not specified	Deoxynivalenol, ochratoxin A	n.d.[a]	Traditional wheat sourdough type I	(Vidal et al., 2014)
Lb. plantarum FST 1.7	*A. niger*, *F. graminearum*, *F. culmorum*, *F. oxysporum,* (no activity against *P. roqueforti*), environmental moulds[b]	Cyclic dipeptides, PLA	Wheat sourdough, Gluten-free sourdough (mixture of brown rice, corn starch, buckwheat and soya flour), Gluten-free sourdough (quinoa, teff, buckwheat, oat, sorghum)	(Dal Bello et al., 2007; Moore et al., 2008; Ryan et al., 2009a, 2009b, 2008; Wolter et al., 2014)
Lactococcus BSN	*A. niger, F. oxysporum, F. moniliforme*	n.d.[a]	Wheat sourdough	(Varsha et al., 2014)
Lb. hammesii DSM16381	*A. niger*, *P. roqueforti,* environmental moulds[b]	Monohydroxy octadecenoic acid	Wheat sourdough supplemented with linoleic acid	(Black et al., 2013)
Lb. rhamnosus TISTR 541	*A. flavus*/ aflatoxin (B1, B2, G1, G2)	Aflatoxin binding cell wall	Baladi bread (whole wheat flour)	(Elsanhoty et al., 2013)

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Starter culture used	Activity spectrum	Antifungal compounds in biopreservative	Substrate/ Application	Reference
Pc. acidilactici KTU05-7 *Pc. pentosaceus* KTU05-8 *Pc. pentosaceus* KTU05-10	*A. fumigatus,* *A. niger,* *A. versicolor,* *F. culmorum,* *P. chrysogenum,* *P. expansum,* environmental moulds[b]	Bacteriocins like inhibitory substances, organic acids	Single LAB cell suspension $5x10^4$ cfu/cm^2) sprayed on the surface of the baked wheat bread	(Cizeikiene et al., 2013)
Commercial sourdough starter	Beauvericin, enniatin,	n.d.[a]	Wheat and rye sourdough	(Hu et al., 2013)
Leuconostoc citreum C5 *W. confusa* HO24	*Cladosporium sp.,* *Neurospora sp.,* *P. crustosum*	Organic acids	Rice sourdough for steamed rice cake	(Baek et al., 2012)
Leu. citreum HO12 *W. koreensis* HO20	*A. niger,* *P. roqueforti*	Ethanol, organic acids	Whole wheat sourdough	(Choi et al., 2012)
Lb. paralimentarius PB127 *Lb. rossiae* LD108	*A. japonicus*	Antifungal peptides, organic acids, PLA	Wheat sourdough, Panettone	(Garofalo et al., 2012)
Lb. plantarum ATCC 20179 *Lb. acidophilus* ATCC 20079	Environmental moulds[b]	Organic acids	Sourdough in traditional Iran flat bread (Sangak)	(Najafi et al., 2012)
Lb. fermentum Te007 *Lb. paracasi* D5 *Lb. pentosus* G004 *Pc. pentosaceus* Te010 (Co-cultures)	*A. niger,* *A. oryzae*	protein-like compounds	LAB cells or supernatants added to bread dough	(Muhialdin et al., 2011)
Lb. acidophilus *Lb. sakei* (Co-culture)	Environmental moulds[b]	Organic acids	Wheat sourdough	(Plessas et al., 2011)
Lb. plantarum LB1 + *Lb. rossiae* LB5 (Co-culture)	*P. roqueforti,* environmental moulds[b]	Antifungal peptides, organic acids; acetic acid, formic acid, lactic acid, PLA	Sourdough fermented wheat germ (freeze dried) in wheat bread	(Rizzello et al., 2011)

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Starter culture used	Activity spectrum	Antifungal compounds in biopreservative	Substrate/ Application	Reference
Lb. buchneri FUA 3252 *Lb. diolivorans* DSM14421 (Co-cultures)	*A. clavatus, Cladosporium sp., Mortierella sp., P. roqueforti,* environmental moulds[b]	Acetic acid, ethanol, lactic acid, propionic acid	Wheat and whole wheat sourdough	(Zhang et al., 2010)
Lb. reuteri CRL1100 *Lb. plantarum* CRL778 *Lb. brevis* CRL772 *Lb. brevis* CRL*796* (single + co-culture)	*A. niger, Penicillium sp.*	Organic acids, PLA	Wheat sourdough	(Gerez et al., 2010, 2009)
Lb. brevis AM7	*P. roqueforti,* environmental moulds[b]	Antifungal peptide and protein	Wheat sourdough + water- soluble extract (27 %, vol/wt, 5 mg of protein/mL) from *Phaseolus vulgaris cv. Pinto*	(Coda et al., 2008)
Lb. casei *Lb. fermentum* *Lb. plantarum*	Environmental moulds[b]	n.d.[a]	Wheat sourdough (Iranian Lavash bread)	(Fazeli et al., 2004)
Lb. plantarum 21B	*A. niger*	n.d.[a]	Wheat sourdough	(Lavermicocca et al., 2000)

[a]not determined; [b]Shelf life test without fungal spore inoculum/ or exposure to bakery air.

In an *in vitro* screening with 95 strains of different lactobacilli and pediococci against *A. niger* CH101, *Penicillium sp.* CH102 and *F. graminearum* CH103, *Lb. plantarum* CRL778 performed best (Gerez et al., 2009) and also *in situ* (Gerez et al., 2010). In a follow-up study by the same group, this same strain was applied *in situ* as a biopreservative culture inoculated into a slurry composed of water, wheat and quinoa flour (Gerez et al., 2014). The growth of *A. niger* 13D and levels of OTA on the resulting bread slices were decreased by 60 % independent to a_w. The antifungal effect was related to the production of lactic, acetic, 3-phenyllactic acid and 4-hydroxy-phenyllactic acids. In contrast, Vidal et al. (2014) found that OTA levels were confirmed to be quite stable during the bread making process, regardless of whether sourdough was added or not. Traditional wheat sourdough type I was used for the latter experiment, where the specific microbiota were not determined. Sourdough type I, is a mixture of flour and water, which is fermented spontaneously by LAB and

yeasts and continuously back-slopped in various fermentation steps (Corsetti, 2013). A Type II is obtained through a unique one-step fermentation using a portion of the mature sourdough from Type I or defined starter cultures (Corsetti, 2013). Elsanhoty et al. (2013) inoculated wheat grains with *A. flavus* to allow aflatoxin B and G production. Fermentation of the contaminated flour with *Lb. rhamnosus* TISTR541 and yeast resulted in the lowest level of aflatoxin in the bread. A sourdough fermentation using a commercial starter mix (*Lb. plantarum* and *Lb. brevis*) also gave a significant decrease (58.6-66.5 %) in DON content produced with *F. graminearum* MI113 artificially contaminated wheat flour (Banu et al., 2014). The detoxifying effect of LAB against mycotoxins is believed to be the result of binding to bacterial cell wall structures, with peptidoglycan being particularly important (Lahtinen et al., 2004). Other strategies for biological detoxification of mycotoxins using microorganism have been recently reviewed by Hathout and Aly (2014).

Lb. plantarum FST1.7 represents another antifungal LAB strain which has been applied to extend the shelf life of wheat and gluten-free (composite recipe using a mix of brown rice flour, buckwheat flour, corn starch and soya flour) sourdough breads, after it was shown to be active against spoilage moulds and bacteria *in vitro* (Dal Bello et al., 2007; Moore et al., 2008; Ryan et al., 2008). The antifungal activity of *Lb. plantarum* FST1.7 was related to lactic acid, 3-phenyllactic acid and the two cyclic dipeptides cyclo (L-Leu-L-Pro) and cyclo (L-Phe-L-Pro) production which were detected in the cell free supernatant. Subsequent analyses revealed the presence of 3-phenyl lactic acid and cyclic dipeptides also in the wheat sourdough (Ryan et al., 2009a, 2009b). Unfermented LAB growth media already contains cyclic dipeptides but their concentrations can increase upon fermentation with LAB (Axel et al., 2014). The level of cyclic dipeptides found in sourdough and broth are 1,000-fold lower than the MICs indicating a minor significance in the antifungal action, but synergistic effects with other compounds are possible (Axel et al., 2014; Niku-Paavola et al., 1999; Ryan et al., 2009a). In the gluten-free system, it must be stated however, that *Fusarium* outgrowth on the infected gluten-free bread slices started for all breads after 2 days. In comparison with the other gluten-free breads, the *Lb. plantarum* FST1.7 sourdough bread had the lowest increase in mould spoilage per day (Moore et al., 2008). The

same strain failed to extend the shelf life when single types of gluten-free flour was fermented and incorporated in a gluten-free bread system (Wolter et al., 2014).

The biological preservation of wheat sourdough bread fermented with *Lb. amylovorus* DSM19280 inhibited the growth of *A. niger*, *F. culmorum*, *P. expansum* and *P. roqueforti* more effectively than calcium propionate (Ryan et al., 2011). The successful inhibitory activity in salt-reduced wheat sourdough bread and gluten-free quinoa sourdough bread reinforces the potential of this strain as an antifungal starter culture (Axel et al., 2015; Belz et al., 2012). HPLC-UV/DAD analysis of the *Lb. amylovorus* DSM19280 fermented quinoa sourdough extracts revealed the presence of the antifungal compounds hydroferulic acid, 4-hydroxyphenyllactic acid, phloretic acid and 3-phenyllactic acid ranging from 13 to 86 ppm (Axel et al., 2015).

In fact, few studies have reported the use of sourdough in gluten-free products for mould inhibition (Axel et al., 2015; Baek et al., 2012; Moore et al., 2008). In contrast to wheat bread, gluten-free breads usually have higher water contents and a_w (Hager et al., 2012; Moore et al., 2004). This higher water availability makes gluten-free products more susceptible for fungal spoilage. In the absence of the gluten network, the movement of water from the gluten-free bread crumb to the crust is enhanced (Sciarini et al., 2010). Thus, combating fungal contaminations in gluten-free systems is more difficult. The same applies for salt-reduced bread. Salt acts as a preservative agent due to its ability to reduce a_w.

An interesting and so far unique approach was undertaken by Rizzello et al. (2011), exploiting the potential of sourdough fermented wheat germ to delay fungal outgrowth on wheat bread. Wheat germ is the embryo containing part of the grain and one of the main by-products of the milling process. It features high quality proteins, lipids and is rich in vitamins. Use of sourdough biotechnology stabilised and improved some nutritional characteristics of wheat germ making it suitable for food processing (Rizzello et al., 2010). The use of the freeze-dried wheat germ (4 %, w/w) fermented with a co-culture of *Lb. plantarum* LB1 and *Lb. rossiae* LB5 prevented fungal outgrowth on bread slices to at least 28 days of storage, behaving similar to calcium propionate (0.3 %, w/w) addition (Rizzello et al., 2011). A complex synergistic system between organic acids and peptides was suggested to be responsible for mould

prevention. The sequence of an antifungal peptide was found to be encrypted in the cereal protein expansin which belongs to the plant defensin-like proteins. It was hypothesised that defensins can induce membrane permeabilization of fungi during the defence response (Picart et al., 2012).

Sourdough with propionate improves synergistic antifungal activity (Ryan et al., 2008; Zhang et al., 2010). As a result of the consumer's aversion to chemical ingredients, some studies have examined the application of natural propionic acid fermentation. *In situ*-propionate production of LAB with propionic acid bacteria (PAB) in co-fermentation is possible, but rather impracticable due to the slow or even absent growth of PAB in the sourdough system (Javanainen and Linko, 1993; Suomalainen and Mäyrä-Mäkinen, 1999). Co-culture fermentation of specific LAB strains enables another option of producing propionate naturally. Lactate is first converted into 1,2-propanediol by *Lb. buchneri* (Oude Elferink et al., 2001) and 1,2-propanediol is further converted into propionate by *Lb. diolivorans* (Krooneman et al., 2002). Zhang et al. (2010) used this cooperative metabolism of *Lb. buchneri* FUA 3252 and *Lb. diolivorans* DSM14421 for bread preservation. Although the bread prepared with 20 % sourdough achieved the longest shelf life, high amounts of propionate (10 mM/kg) and acetate (35 mM/kg) were unfavourable in terms of sensory properties (Zhang et al., 2010). Additionally, a long fermentation time of 14 days was required to ensure the formation of sufficient amounts of propionic acid.

More studies have reported prolonged bread shelf life using combined starter cultures for sourdough fermentation (Muhialdin et al., 2011; Plessas et al., 2011). The better antifungal effect was explained by higher acidification rates.

The conversion of linoic acid to an antifungal monohydroxy-octadecenoic acid was observed in sourdough fermentation by *Lb. hammesii* DSM16381 (Black et al., 2013). The sourdough has potential to be used as a biopreservative since the addition of 20 % in bread making resulted in an extended shelf life when challenged with *P. roqueforti* and *A. niger*, and environmental contamination without inoculation.

Most of the studies presented apply the starter culture in the traditional sourdough making process. However, a different approach was followed by Cizeikiene et al. (2013). After inoculation in MRS media, the single LAB cell suspension

(~$5x10^4$ CFU/cm) was sprayed on the surface of the baked wheat bread. The experimental procedure did not clarify whether the LAB cell suspension was washed or remained suspended in MRS medium. We judge the latter treatment as unacceptable for food applications.

Overall antifungal LAB-fermented sourdough serves as a great potential biopreservative ingredient to produce chemical preservative-free breads with an extended shelf life, coupled with other advantages like improved flavour and texture.

2.5.1.2 Yeast

The application of yeasts other than baker's yeast (Saccharomyces cerevisiae) with or without the combination of LAB is also suggested as a promising alternative for bread preservation (Table 2-2). For instance, the yeast *Wickerhamomyces anomalus* LCF1695 (formerly known as *Pichia anomala*) was used as mixed starter, in combination with *Lb. plantarum* 1A7, for sourdough fermentation. This combination, when added to a standard bread recipe, allowed a microbial shelf life of at least 14 days during storage at room temperature after artificial inoculum (10^2 conidia/ml) with *P. roqueforti* DPPMAF1 (Coda et al., 2011). In subsequent work by the same group another yeast strain, *Meyerozyma guilliermondii* LCF1353 (previously *Pichia guilliermondii*), was found out of 146 yeast strains to harbour marked antifungal activity toward the indicator fungus *P. roqueforti* DPPMAF1. As shown under pilot plant conditions, the bread including the sourdough fermented with a combined starter culture consisting of *M. guilliermondii* LCF1353, *W. anomalus* LCF1695 and *Lb. plantarum* 1A7 strain showed the longest shelf-life (Coda et al., 2013). High ethanol and ethyl acetate concentrations were determined in the water/salt-soluble dough extract resulting, together with proteinaceous compounds, in fungal inhibition. Bread quality parameters like specific volume and crumb hardness were not significantly different to the control breads.

The fungal outgrowth of *Penicillium paneum* KACC 44834 on white pan bread leavened with *P. anomala* SKM-T was significantly decreased in comparison to normal baker's yeast (Mo and Sung, 2014). The sensory qualities of bread were also improved by the presence of the pleasing flavour compounds phenylethyl alcohol and 2-phenylethyl

acetate, which were only produced by *P. anomal*a SKM-T. These compounds also contributed to the shelf life extension.

The antifungal activity of *W. anomalus* and *M. guilliermondii* has previously been demonstrated under silage storage conditions (Petersson and Schnürer, 1995). Nevertheless, further research on the use of these yeasts is needed to confirm the *in situ*-antifungal activity for bread biopreservation. The use of *W. anomalus* seems to be safe, because this species is also included in the QPS list, although authorised only when it is used for enzyme production (EFSA, 2012). However, the safety of the yeast *M. guilliermondii* needs to be assessed for use as a biological control agent in food.

Table 2-2 Antifungal yeasts applied in bread baking, their tested activity and related antifungal compounds.

Starter culture used	**Activity spectrum**	**Antifungal compounds in biopreservative**	**Substrate/ Application**	**Reference**
Pichia anomala SKM-T	*P. paneum*	2-phenylethyl acetate, phenylethyl alcohol	Wheat dough for white pan bread	(Mo and Sung, 2014)
Meyerozyma guilliermondii LCF1353 + *Wickerhamomyces anomalus* LCF1695 (Co-culture with *Lb. plantarum* 1A7)	*P. roqueforti* environmental moulds[a]	Antifungal peptides, ethanol, ethyl acetate	Wheat sourdough	(Coda et al., 2013)
W. anomalus LCF1695 (Co-culture with *Lb. plantarum* 1A7)	*P. roqueforti* environmental moulds[a]	Antifungal peptides, ethanol, ethyl acetate	Wheat sourdough	(Coda et al., 2011)

[a]Shelf life test without fungal spore inoculum/or exposure to bakery air.

2.5.2 Plant derive compounds

The growing interest in the application of natural ingredients with multifunctional properties in food has also led to extensive investigation of plant extracts as biopreservatives. Recently reviewed by da Cruz Cabral et al. (2013), a comprehensive summary is given on *in vitro* experiments regarding the use of plant extracts and essential oils for controlling common food spoilage fungi. Additionally the mode of action and the impact on mycotoxins is discussed. However, only a few *in situ* tests are presented, without any application on bread. It is noteworthy that we found few studies dealing with the direct addition of plant extract in bakery products. Recent developments also employ essential oils in edible coating films of active packaging and will be discussed in Section 2.5.3.

Using a simple storage test, without artificial inoculum, Wei et al. (2009) evaluated the antifungal activity of different raisin extracts and products in conventional bread. The breads made with the raisin paste and raisin water extract (7.5 %) showed the best mould-retarding properties when compared to the control containing no preservative. However, they were not significantly different to the positive control containing 0.24 % propionate. The authors related the antifungal activity to a synergistic effect between the formed Maillard products and phenolic compounds. Although bread quality parameters were not included in the study, the raisin extract seems to have potential to reduce chemical preservatives in food systems and should be further investigated.

The interest in applying antifungal proteins and peptides for bread biopreservation alone or in combination with sourdough (Coda et al., 2008; Rizzello et al., 2015, 2011, 2009), and as biocontrol agents against fungal pathogens in agriculture has increased in recent years (Yan et al., 2015). A large number of antifungal proteins have been reported from a multitude of organisms including leguminous flowering plants, non-leguminous flowering plants, gymnosperms, but also fungi, bacteria, insects and mammals (Ng, 2004). They are categorized by type and function, comprising thaumatin-like proteins, chitinases, glucanases, embryo-abundant proteins, miraculin-like proteins, cyclophilin-like proteins, allergen-like proteins, defensins, thionins, and nonspecific lipid transfer proteins (Ng, 2004). The various mechanisms of antifungal

action for a range of plant antifungal proteins were recently summarized by Yan et al. (2015). Antifungal chitinases act hydrolytically on fungal cell wall chitin leading to cell lysis (Graham and Sticklen, 1994). Defensins cause membrane permeabilization (Picart et al., 2012). A similar action was proposed for lipid transfer proteins (Selitrennikoff, 2001). It is possible to observe a combination of antifungal proteins in a single plant species (Ye et al., 2000).

Water/salt-soluble extracts of different legume flour hydrolysates (soy, lentil, pea, chick pea and faba bean) were obtained by the use of fungal proteases and tested *in vitro* against *P. roqueforti* DPPMAF1 (Rizzello et al., 2015). Pea (*Pisum sativum*) hydrolysate, the most active antifungal biopreservative, was freeze dried and used as ingredient for bread making at a level of 1.6 % (w/w). Bread made with the combination of sourdough fermented by the antifungal strain *Lb. plantarum* 1A7 and the pea flour hydrolysate had the longest shelf life. The antifungal activity was attributed to three native proteins (pea defensins 1 and 2, a nonspecific lipid transfer protein and a mixture of peptides released during hydrolysis).

Rizzello et al. (2009) also reported the use of water-soluble extract from *Amaranthus spp.* seeds during storage of gluten-free and wheat flour breads. Novel antifungal peptides (agglutinin sequences) were identified in the extract, which showed *in vitro* inhibition of a large number of spoilage fungal species isolated from bakeries (5 mg/mL). Three different types of bread were produced; control bread, bread with added amaranth water-soluble extract, and sourdough bread (*Lb. sanfranciscensis* E9) with added amaranth water-soluble extract. Bread slices were inoculated with *P. roqueforti* DPPMAF1 (10^2/mL). With the addition of the water-soluble extract to the breads the appearance of fungal mycelium was delayed by at least 7 days, being more pronounced for the sourdough breads. Furthermore, the addition of the water-soluble extract improved the quality of the gluten-free breads regarding taste and specific volume. *Lb. sanfranciscensis* is the most frequently used LAB as leavening agent for sourdough production (Gänzle, 2014). The antifungal activity of this strain was not specified by the authors. Thus, we speculate further improvement of the preservative effect when amaranth water-soluble extract would have been combined with sourdough fermented with a known antifungal LAB.

2.5.3 Protective packaging

Packaging is an important factor in extending the microbial shelf life of bakery products. Normal packed bread provides aerobic microorganisms like fungi adequate amounts of oxygen for growth. The principle of modified atmosphere packaging (MAP) revolves around reducing oxygen by replacing the headspace of the product either with carbon dioxide alone or mixtures of it and nitrogen. Generally for bakery products, the gas mixture consists of 60 % or more carbon dioxide with nitrogen acting as a filler gas. Thus, carbon dioxide is the fungistatic component slowing down the proliferation of moulds. Furthermore, it may alter the permeability of the cell membrane, reduce the internal pH of the cell and influencing certain enzyme systems and changing metabolic activities (Farber, 1991). Due to the highly porous texture of bakery products complete oxygen elimination is challenging (Hempel et al., 2013). Moreover, oxygen accumulation over time can occur even if packaging films of low oxygen permeability are used. The introduction of active packaging (AP) provided a significant improvement over MAP. AP techniques prevents bread spoilage through the use of oxygen absorbers, ethanol emitters and other antimicrobial agents which are either incorporated as a layer in the packaging or in edible films, or placed for example as sachets in the packaging. AP can be combined with the classical MAP approach. An extensive review of shelf life in packed bakery products was published by Galić et al. (2009). This review covers many studies dealing with MAP including active packaging and describes the types of material used for cereal based food packaging. Therefore, this section presents an update in research conducted within the last 5 years.

As previously mentioned, plant extract or essential oils can be used in antimicrobial films for AP of bread. Balaguer et al. (2013) found good *in vitro* antifungal activity against *P. expansum* and *A. niger* testing gliadin films containing cinnamon aldehyde. In an *in situ* test wheat bread slices were inoculated with *P. expansum* conidial suspension (10^6 spores/mL). After 30 days, fungal growth remained absent on bread slices which were packed into mono cast polypropylene bags with a piece (13 cm × 8 cm) of the antimicrobial gliadin film treated with 5 % cinnamon aldehyde at 23 °C. Cellulose-derived polymers also containing 5 % cinnamon aldehyde and

polypropylene with an organic solvent base (ethyl acetate) coated with nitrocellulose containing the cinnamon essential oil, present further materials which were used successfully for gluten-free and wheat bread shelf life extension (Gutiérrez et al., 2011; Lopes et al., 2014). The shelf life of wheat pan bread was not increased using biodegradable films based on cassava starch with cinnamon powder (Kechichian et al., 2010). The authors observed an increase in a_w of the film due to product instability. Hence, the material which supports the antimicrobial-active substance seems to be of great importance. The commission regulation (EU) No. 10/2011, also known as plastic implementation measure (PIM), controls which materials can be used for food packaging and sets specific migration limits to ensure food safety (EEC, 2011).

Otoni et al. (2014) included nano-emulsions of oregano and clove bud essential oil in edible films from methylcellulose. Their antifungal efficacy was attributed to the phenolic compounds eugenol, carvacrol and thymol. Those compounds have shown antimicrobial activity against a range of bacteria due to reduction of the proton gradient through the cell membrane. The reduced proton gradient, along with the resulting depletion of the ATP pool, kills microbial cells (Ben Arfa et al., 2006; Lambert et al., 2001). *In vitro* tests with the essential oils inhibited spore germination of *A. niger* ATCC 16404 and *Penicillium sp.* ATCC 2147 at a concentration of 20 mg/mL (Otoni et al., 2014).

The overall sensory and physicochemical properties of the bread can be influenced by aromatic compounds like cinnamon aldehyde or thymol, although present at very low levels in the final product. Although the cinnamon aroma was noticeable to a panellist at the highest concentration level (0.374 g per self-adhesive active label of 13×10 cm), no significant differences were found in cinnamon taste between control (no cinnamon aldehyde) and positive samples (Gutiérrez et al., 2011). In contrast, although antimicrobial sachets containing more than 5 % oregano essential oil reduced the growth rate of moulds on sliced bread, the content of γ-terpinene and *p*-cymene on treated bread increased throughout storage and their acceptance was reduced (Passarinho et al., 2014). Marjoram and sage essentials oil vapour treatment also showed inhibition of mould on bread slices in a model AP system (Krisch et al., 2013). However, the taste and odour was rated from strange to unacceptable.

Isothiocyanates are another group of potenial antimicrobial substances, these are hydrolysed products of sulphur containing compounds called glucosinolates, which occur naturally in cruciferous vegetables, such as broccoli, cabbage, cauliflower, kale, turnip, radish, canola, rapeseed and various mustards (Isshiki et al., 1992). Azaiez et al. (2013) demonstrated their ability in their gaseous state to inhibit the fungal growth of *Fusarium moniliforme* CECT 2987 and to reduce the levels of fumonisins produced by this strain in a bread AP model system. Allyl and phenyl isothiocyanates showed stronger activity than benzyl isothiocyanates. Allyl isothiocyanate at 500 μg/L vapor phase concentration presented the highest reduction of about 96 % fumonisin B_2 in comparison to the un-treated control. Indeed, chromatograms presented in the publication evidenced that the high peak of fumonisin B_2 present in the control sample almost disappeared in the isothiocyanate treated samples. The strongly electrophilic carbon in the isothiocyanate group (−N=C=S) easily binds to thiol and free amino groups of amino acids, peptides and proteins (Luciano and Holley, 2009). It was suggested that isothiocyanates bind the free amino group of the fumonisin, depicted in Figure 2-1. Further investigation will examine any toxicity of the products generated by the reaction of fumonisin B_2 and isothiocyanates (Azaiez et al., 2013).
To overcome problems arising from the strong aroma of highly volatile compounds, further experiments are needed to minimise the impact on product aroma, flavour and sensory attributes, while maintaining high efficacy when used in combination with other technologies. AP has a great potential in extending the shelf life of bread. Nevertheless, further improvements are needed using better biodegradable materials, to fulfil the green approach.

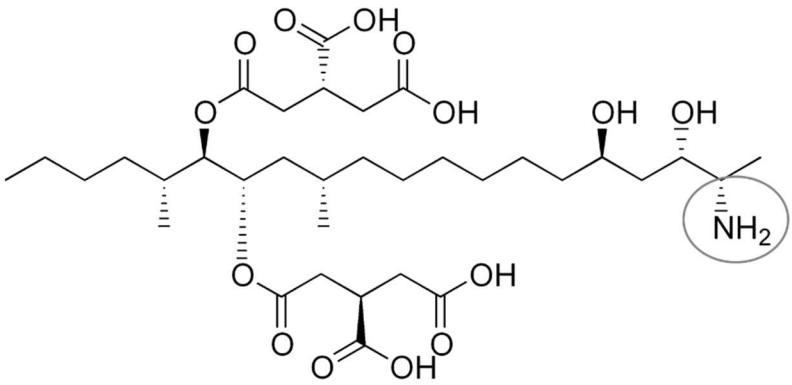


Figure 2-1 Fumonisin B2 with marked free amino group.

2.5.4 Further applications

The addition of ethanol is a traditional preservative method. Hence, from the consumer point of view, ethanol might be a more preferable preservative agent over some other chemical substances. A recently published review by Dao and Dantigny (2011) summarizes the control of food spoilage fungi by ethanol with a detailed explanation of its mode of action. In studies ethanol was used to control post-harvest fruit decay. According to an *in vitro* data based model against 12 common food spoilage moulds, the inhibition concentration of ethanol was estimated in a range between 3 to 5 % (Dantigny et al., 2005). All experiments were performed at high a_w values of 0.99 at intermediate pH of 5.8. However, for increasing the shelf life of bread concentrations between 0.2 and 12 % are reported (Dao and Dantigny, 2011). Katsinis et al. (2008) found an improved effect of sorbate and propionate on naturally contaminated products, when ethanol was added to the bread surface (0.5 % w/w). In a challenge test against *Chrysonilia sitophila* ("the red bread mould") and *Hyphopichia burtonii* ("the chalky mould") on packed and sliced bread, growth was inhibited at very low (0.8 %) and medium (2.0 %) ethanol concentrations, respectively (Berni and Scaramuzza, 2013).

Due to its low toxicity, ethanol enjoys GRAS status in the USA. In the European Union, ethanol is not included in the regulation on food additives (EEC, 2008). Thus, there is no restriction about the use of ethanol as a preservative. If added to a product, it has to be listed as "ethyl alcohol" or "ethanol" on the ingredients. The results of studies presented above suggest that ethanol could act as an effective additional

barrier to inhibit fungal growth in bakery products. Thus it represents an interesting alternative to the use of chemical preservatives and merits further research. A further application showing promise includes the addition of ethanol emitter in active packaging (Hempel et al., 2013).

One study tested the influence of 1-monoglyceride of medium chain fatty acids (MAG), 1-monocaprylin and 1-monocaprin on spoilage inhibition (*P. chrysogenum*, *P. jensenii*, *Monascus ruber*, *A. niger*) on bread (Buňková et al., 2010). The microfilm of MAG (65 mg/L) spread on the bread surface showed fungal inhibition up to 14 days storage. MAG belong to food additives in accordance with the principle of "quantum satis" addition for good manufacturing practice and commonly acts as emulsifier in breads (EEC, 2008). A mode of action was hypothesized that 1-monocaprylin destabilizes membranes by increasing membrane fluidity and the number of phase boundary defects (Hyldgaard et al., 2012).

The use of biosurfactants, in particular lipopeptides produced by microorganisms has attracted the food industry due to their wide range of functionalities including their antimicrobial activity (Campos et al., 2013). Antibacterial and antifungal lipopeptides are mostly synthesized by strains of *Bacillus subtilis* and are classified into 3 main classes; surfactin, iturin, and fengycin (Muthusamy et al., 2008). *B. subtilis* belong to the QPS *Bacillus* species, with no safety concerns being reported from their usage in food products (EFSA, 2012). Mnif et al. (2012) evaluated the influence of biosurfactant produced by *B. subtilis* SPB1 on bread quality at concentrations of 0.025, 0.05, 0.075, and 0.1 % (flour weight basis). Bread prepared with 0.075 % SPB1 biosurfactant showed higher specific volume when compared to a control containing the same amount of soya lecithin. Texture profile analysis of bread prepared with and without biosurfactant showed also decreased firmness. Microbiological analysis of the 0.075 % SPB1 biosurfactant containing bread had fungal and bacterial counts of $2 \cdot 10^3$ and $4 \cdot 10^2$ cfu/g, respectively; these counts were 1 log less than the control after 8 days of storage which was attributed to the significantly lower aw values of bread with added SPB1 biosurfactant. However, the a_w was in general low, with values below 0.8 for all the breads produced. Microbial counts do not provide sufficient data if not compared to a specific reference level. The reference values of microbial counts of

10^2 and 10^5 cfu/g for moulds and bacteria, respectively, were suggested for frozen, ready to eat, bakery wares (i.e., rolls or croissants) from the German Society for Hygiene and Microbiology (DGHM, 2006). These reference values are based on which micro-flora can be expected and are tolerated in terms of good hygiene practice. Furthermore, the sample size needs to be sufficient and appropriate representing at least a small sale unit of 50 g (DGHM, 2006). There are other antimicrobial agents derived from microorganisms showing antifungal activity. Natamycin produced by *Streptomyces natalensis* is listed as preservative E235 in the EU regulation on food additives (EEC, 2008). In Europe, it is permitted only for surface treatment for the protection of cheeses and sausages. The GRAS status in the USA allows its addition also to yoghurt (FDA, 2014).

Cherry laurel (*Prunus laurocerasus L.*) leaf extracts were suggested as novel biopreservatives after showing very low MIC (µg/mL) against a range of bread spoilage fungi (Sahan, 2011). The highest total antifungal effect was observed from ethanol and acetone extracts, but biologically active components were not analysed.

Further studies would be needed in order to confirm the *in situ* antifungal activity of MAG, antimicrobial agents and cherry laurel leaf extracts on bakery products, including assessing product safety.

2.6 Conclusion

In order to meet the needs and to fulfil the high consumer demand for additive-free bakery products, current research has focused on biopreservation to extend the shelf life of bread. Furthermore, the ineffective usage of chemical preservatives due to the development of resistance by several fungi has increased the pressure to find alternative agents.

The application of biopreservatives derived from plants or biosurfactants seem interesting alternatives to chemical preservatives. More reports are expected about the use of antifungal proteins. Antimicrobial essential oils need to be further evaluated, where progress could also lead to a better sensory profile. Protective packaging material should focus on biodegradable materials ensuring customer satisfaction.

Obviously, all data presented in Section 2.5 were obtained from different baking technologies and methodologies defining the mould-free shelf life of the resulting product. Furthermore, varying contamination levels or inoculum sizes per slice of bread, and storage conditions make an overall comparison of shelf life-extension potential between different methodologies difficult. In our opinion, mouldiness should be assessed with the first visible appearance of fungal colonies on bread and bread slices. Nevertheless, in some studies, a bread slice was considered mouldy if more than 1 % of the total surface area was covered with fungi (Ryan et al., 2011, 2008) or all the inoculated spots started to show fungal growth (Zhang et al., 2010). Thus, a standardised method for defining bread mouldiness is vital to allow meaningful comparisons to be made.

Sourdough fermented with antifungal strains serves as a high-potential biological ingredient to produce gluten-containing and gluten-free breads that meet presently consumer needs with improved nutritional value, bread quality and safety due to an extended shelf life. Despite the relatively high abundance of LAB, the antifungal potential and binding properties for mycotoxins is highly strain specific, which should be considered as a criterion for the selection of LAB for use as starter cultures in food and feed. The application of antifungal starter strains is still limited and more strains need to be investigated *in situ*. Further research is required to improve the shelf life of gluten-free bread. Research into the removal of mycotoxins in food matrices is still in its infancy. Many novel methods have been introduced with high potential for biopreservation of bakery products. The next step will be to up-scale the use of such biopreservatives in the marketplace, potentially in combination with other technologies such as intelligent packaging, which will necessitate production of such compounds at an industrial scale.

2.7 Acknowledgements

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2.8 References

Aldana, J.R., Silva, L.J.G., Pena, A., Mañes V., J., Lino, C.M., 2014. Occurrence and risk assessment of zearalenone in flours from Portuguese and Dutch markets. Food Control 45, 51–55. doi:10.1016/j.foodcont.2014.04.023

Alvarez-Jubete, L., Arendt, E.K., Gallagher, E., 2010. Nutritive value of pseudocereals and their increasing use as functional gluten-free ingredients. Trends Food Sci. Technol. 21, 106–113. doi:10.1016/j.tifs.2009.10.014

Arroyo, M., Aldred, D., Magan, N., 2005. Environmental factors and weak organic acid interactions have differential effects on control of growth and ochratoxin A production by *Penicillium verrucosum* isolates in bread. Int. J. Food Microbiol. 98, 223–31. doi:10.1016/j.ijfoodmicro.2004.07.004

Axel, C., Roecker, B., Brosnan, B., Zannini, E., Furey, A., Coffey, A., Arendt, E.K., 2015. Application of *Lactobacillus amylovorus* DSM19280 in gluten-free sourdough bread to improve the microbial shelf life. Food Microbiol. 47, 36–44. doi:10.1016/j.fm.2014.10.005

Axel, C., Zannini, E., Arendt, E.K., Waters, D.M., Czerny, M., 2014. Quantification of cyclic dipeptides from cultures of *Lactobacillus brevis* R2Δ by HRGC/MS using stable isotope dilution assay. Anal. Bioanal. Chem. 406, 2433–2444. doi:10.1007/s00216-014-7620-3

Azaiez, I., Meca, G., Manyes, L., Fernández-Franzón, M., 2013. Antifungal activity of gaseous allyl, benzyl and phenyl isothiocyanate *in vitro* and their use for fumonisins reduction in bread. Food Control 32, 428–434. doi:10.1016/j.foodcont.2013.01.020

Baek, E., Kim, H., Choi, H., Yoon, S., Kim, J., 2012. Antifungal activity of *Leuconostoc citreum* and *Weissella confusa* in rice cakes. J. Microbiol. 50, 842–8. doi:10.1007/s12275-012-2153-y

Balaguer, M.P., Lopez-Carballo, G., Catala, R., Gavara, R., Hernandez-Munoz, P., 2013. Antifungal properties of gliadin films incorporating cinnamaldehyde and application in active food packaging of bread and cheese spread foodstuffs. Int. J. Food Microbiol. 166, 369–77. doi:10.1016/j.ijfoodmicro.2013.08.012

Banu, I., Dragoi, L., Aprodu, I., 2014. From wheat to sourdough bread: a laboratory scale study on the fate of deoxynivalenol content. Qual. Assur. Saf. Crop. Foods 6, 53–60. doi:10.3920/QAS2012.0226

Belz, M.C.E., Mairinger, R., Zannini, E., Ryan, L.A.M., Cashman, K.D., Arendt, E.K., 2012. The effect of sourdough and calcium propionate on the microbial shelf-life of salt reduced bread. Appl. Microbiol. Biotechnology 96, 493–501. doi:10.1007/s00253-012-4052-x

Ben Arfa, A., Combes, S., Preziosi-Belloy, L., Gontard, N., Chalier, P., 2006. Antimicrobial activity of carvacrol related to its chemical structure. Lett. Appl. Microbiol. 43, 149–154. doi:10.1111/j.1472-765X.2006.01938.x

Berni, E., Scaramuzza, N., 2013. Effect of ethanol on growth of *Chrysonilia sitophila* ('the red bread mould') and *Hyphopichia burtonii* ('the chalky mould') in sliced bread. Lett. Appl. Microbiol. 57, 344–9. doi:10.1111/lam.12119

Black, B.A., Zannini, E., Curtis, J.M., Gänzle, M.G., 2013. Antifungal hydroxy fatty acids produced during sourdough fermentation: microbial and enzymatic pathways, and antifungal activity in bread. Appl. Environ. Microbiol. 79, 1866–73. doi:10.1128/AEM.03784-12

Błajet-Kosicka, A., Twarużek, M., Kosicki, R., Sibiorowska, E., Grajewski, J., 2014. Co-occurrence and evaluation of mycotoxins in organic and conventional rye grain and products. Food Control 38, 61–66. doi:10.1016/j.foodcont.2013.10.003

Buňková, L., Krejčí, J., Janiš, R., Kašpárková, V., Vltavská, P., Kulendová, L., Buňka, F., 2010. Influence of monoacylglycerols on growth inhibition of micromycetes *in vitro* and on bread. Eur. J. Lipid Sci. Technol. 112, 173–179. doi:10.1002/ejlt.200900070

Campos, J.M., Stamford, T.L.M., Sarubbo, L.A., de Luna, J.M., Rufino, R.D., Banat, I.M., 2013. Microbial biosurfactants as additives for food industries. Biotechnol. Prog. 29, 1097–108. doi:10.1002/btpr.1796

Choi, H., Kim, Y.-W., Hwang, I., Kim, J., Yoon, S., 2012. Evaluation of *Leuconostoc citreum* HO12 and *Weissella koreensis* HO20 isolated from kimchi as a starter culture for whole wheat sourdough. Food Chem. 134, 2208–16. doi:10.1016/j.foodchem.2012.04.047

Cizeikiene, D., Juodeikiene, G., Paskevicius, A., Bartkiene, E., 2013. Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. Food Control 31, 539–545. doi:10.1016/j.foodcont.2012.12.004

Coda, R., Cassone, A., Rizzello, C.G., Nionelli, L., Cardinali, G., Gobbetti, M., 2011. Antifungal activity of *Wickerhamomyces anomalus* and *Lactobacillus plantarum* during sourdough fermentation: identification of novel compounds and long-term effect during storage of wheat bread. Appl. Environ. Microbiol. 77, 3484–92. doi:10.1128/AEM.02669-10

Coda, R., Rizzello, C.G., Di Cagno, R., Trani, A., Cardinali, G., Gobbetti, M., 2013. Antifungal activity of *Meyerozyma guilliermondii*: identification of active compounds synthesized during dough fermentation and their effect on long-term storage of wheat bread. Food Microbiol. 33, 243–51. doi:10.1016/j.fm.2012.09.023

Coda, R., Rizzello, C.G., Nigro, F., De Angelis, M., Arnault, P., Gobbetti, M., 2008. Long-term fungal inhibitory activity of water-soluble extracts of *Phaseolus vulgaris cv. Pinto* and sourdough lactic acid bacteria during bread storage. Appl. Environ. Microbiol. 74, 7391–8. doi:10.1128/AEM.01420-08

Corsetti, A., 2013. Chapter 4 Technology of Sourdough Fermentation and Sourdough Applications, in: Gobetti, M., Gänzle, M.G. (Eds.), Handbook on Sourdough Biotechnology. Springer, pp. 85–103. doi:10.1007/978-1-4614-5425-0

Crowley, S., Mahony, J., van Sinderen, D., 2013. Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. Trends Food Sci. Technol. 33, 93–109. doi:10.1016/j.tifs.2013.07.004

Da Cruz Cabral, L., Fernández Pinto, V., Patriarca, A., 2013. Application of plant derived compounds to control fungal spoilage and mycotoxin production in foods. Int. J. Food Microbiol. 166, 1–14. doi:10.1016/j.ijfoodmicro.2013.05.026

Dal Bello, F., Clarke, C.I., Ryan, L.A.M., Ulmer, H., Schober, T.J., Ström, K., Sjögren, J., van Sinderen, D., Schnürer, J., Arendt, E.K., Strom, K., Sjogren, J., Schnurer, J., 2007. Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. J. Cereal Sci. 45, 309–318. doi:10.1016/j.jcs.2006.09.004

Dalié, D.K.D., Deschamps, A.M., Richard-Forget, F., 2010. Lactic acid bacteria – Potential for control of mould growth and mycotoxins: A review. Food Control 21, 370–380. doi:10.1016/j.foodcont.2009.07.011

Dantigny, P., Guilmart, A., Radoi, F., Bensoussan, M., Zwietering, M., 2005. Modelling the effect of ethanol on growth rate of food spoilage moulds. Int. J. Food Microbiol. 98, 261–9. doi:10.1016/j.ijfoodmicro.2004.07.008

Dao, T., Dantigny, P., 2011. Control of food spoilage fungi by ethanol. Food Control 22, 360–368. doi:10.1016/j.foodcont.2010.09.019

Demirel, R., Sariozlu, N.Y., 2014. Mycotoxigenic moulds and mycotoxins in flours consumed in Turkey. J. Sci. Food Agric. 94, 1577–84. doi:10.1002/jsfa.6460

DGHM, 2006. DGHM 9.2 TK-Backwaren [WWW Document]. Dtsch. Gesellschaft für Hyg. und Mikrobiol. , Ger. Soc. Hyg. Microbiol. URL http://www.dghm-richt-warnwerte.de/cn/bGV2ZWw9dHBsLXN1Y2hlcmdlYm5pcyZzZWFyY2hhY2Nlc3NrZXk9Q09OVEVOVCZwYWdlaWQ9Mw**.html (accessed 1.6.15)

EEC, 2006. Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union L 364, 5.

EEC, 2008. Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. Off. J. Eur. Union L 354, 16.

EEC, 2011. Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food. Off. J. Eur. Union L 012, 1.

EFSA, 2012. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update). EFSA J. 10, 3020. doi:10.2903/j.efsa.2012.3020.

Elsanhoty, R.M., Ramadan, M.F., El-Gohery, S.S., Abol-Ela, M.F., Azeke, M. a., 2013. Ability of selected microorganisms for removing aflatoxins *in vitro* and fate of aflatoxins in contaminated wheat during baladi bread baking. Food Control 33, 287–292. doi:10.1016/j.foodcont.2013.03.002

FAO, 2013. FAT Stat [WWW Document]. FAOSTAT Crop. Prod. URL http://faostat3.fao.org/browse/Q/QC/E (accessed 1.6.15)

Farber, J.M., 1991. Microbiological aspects of modified -atmophere packaging technology - a review. J. Food Prot. 54, 58–70.

Fazeli, M.R., Shahverdi, a. R., Sedaghat, B., Jamalifar, H., Samadi, N., 2004. Sourdough-isolated *Lactobacillus fermentum* as a potent anti-mould preservative of a traditional Iranian bread. Eur. Food Res. Technol. 218, 554–556. doi:10.1007/s00217-004-0898-1

FDA, 2014. GRAS Notification use of natamycin in yoghurt [WWW Document]. URL http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=517 (accessed 8.6.15)

Galić, K., Curić, D., Gabrić, D., 2009. Shelf life of packaged bakery goods--a review. Crit. Rev. Food Sci. Nutr. 49, 405–26. doi:10.1080/10408390802067878

Gänzle, M.G., 2014. Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiol. 37, 2–10. doi:10.1016/j.fm.2013.04.007

Garofalo, C., Zannini, E., Aquilanti, L., Silvestri, G., Fierro, O., Picariello, G., Clementi, F., 2012. Selection of sourdough lactobacilli with antifungal activity for use as biopreservatives in bakery products. J. Agric. Food Chem. 60, 7719–28. doi:10.1021/jf301173u

Gerez, C.L., Dallagnol, A., Ponsone, L., Chulze, S., Font de Valdez, G., 2014. Ochratoxin A production by *Aspergillus niger*: Effect of water activity and a biopreserver formulated with *Lactobacillus plantarum* CRL 778. Food Control 45, 115–119. doi:10.1016/j.foodcont.2014.04.038

Gerez, C.L., Torino, M.I., Obregozo, M.D., Font de Valdez, G., 2010. A ready-to-use antifungal starter culture improves the shelf life of packaged bread. J. Food Prot. 73, 758–62.

Gerez, C.L., Torino, M.I., Rollán, G., Font de Valdez, G., 2009. Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. Food Control 20, 144–148. doi:10.1016/j.foodcont.2008.03.005

Graham, L.S., Sticklen, M.B., 1994. Plant Chitinases. Can. J. Bot. Can. Bot. 72, 1057–1083.

Gutiérrez, B.L., Batlle, R., Andújar, S., Sánchez, C., Nerín, C., 2011. Evaluation of antimicrobial active packaging to increase shelf life of gluten free sliced bread. Packag. Technol. Sci. 24, 485–494. doi:10.1002/pts

Guynot, M.E., Ramos, a J., Sanchis, V., Marín, S., 2005. Study of benzoate, propionate, and sorbate salts as mould spoilage inhibitors on intermediate moisture bakery products of low pH (4.5-5.5). Int. J. Food Microbiol. 101, 161–8. doi:10.1016/j.ijfoodmicro.2004.11.003

Hager, A.S., Wolter, A., Czerny, M., Bez, J., Zannini, E., Arendt, E.K., 2012. Investigation of product quality, sensory profile and ultrastructure of breads made from a range of commercial gluten-free flours compared to their wheat counterparts. Eur. Food Res. Technol. 235, 333–344. doi:10.1007/s00217-012-1763-2

Hathout, A.S., Aly, S.E., 2014. Biological detoxification of mycotoxins: a review. Ann. Microbiol. 64, 905–919. doi:10.1007/s13213-014-0899-7

Hempel, A.W., O'Sullivan, M.G., Papkovsky, D.B., Kerry, J.P., 2013. Use of smart packaging technologies for monitoring and extending the shelf-life quality of modified atmosphere packaged (MAP) bread: application of intelligent oxygen sensors and active ethanol emitters. Eur. Food Res. Technol. 237, 117–124. doi:10.1007/s00217-013-1968-z

Hu, L., Koehler, P., Rychlik, M., 2013. Effect of sourdough processing and baking on the content of enniatins and beauvericin in wheat and rye bread. Eur. Food Res. Technol. 238, 581–587. doi:10.1007/s00217-013-2133-4

Hyldgaard, M., Sutherland, D.S., Sundh, M., Mygind, T., Meyer, R.L., 2012. Antimicrobial mechanism of monocaprylate. Appl. Environ. Microbiol. 78, 2957–65. doi:10.1128/AEM.07224-11

Isshiki, K., Tokuoka, K., Mori, R., Chiba, S., 1992. Preliminary examination of ellyl isothiocyanate vapor for food preservation. Biosci. Biotechnol. Biochem. 56, 1476–1477.

Javanainen, P., Linko, Y.Y., 1993. Mixed-culture pre-ferments of lactic and propionic acid bacteria for improved wheat bread shelf-life. J. Cereal Sci. 18, 75–88.

Katsinis, G., Rigas, F., Doulia, D., 2008. Synergistic effect of chemical preservatives with ethanol on the microbial shelf life of bread by factorial design. Int. J. Food Sci. Technol. 43, 208–215. doi:10.1111/j.1365-2621.2006.01386.x

Kaushik, G., 2015. Effect of processing on mycotoxin content in grains. Crit. Rev. Food Sci. Nutr. 55, 1672–1683. doi:10.1080/10408398.2012.701254

Kechichian, V., Ditchfield, C., Veiga-Santos, P., Tadini, C.C., 2010. Natural antimicrobial ingredients incorporated in biodegradable films based on cassava starch. LWT- Food Sci. Technol. 43, 1088–1094. doi:http://dx.doi.org/10.1016/j.lwt.2010.02.014

Knight, R.A., Menlove, E.M., 1961. Effect of the bread-baking process on destruction of certain mould spores. J. Sci. Food Agric. 12, 653–656. doi:10.1002/jsfa.2740121001

Krebs, H.A., Wigginst, D., Stubbs, M., 1983. Studies on the mechanism of the antifungal action of benzoate. Biochem. J. 214, 657–663.

Krisch, J., Rentskenhand, T., Horváth, G., Vágvölgyi, C., 2013. Activity of essential oils in vapor phase against bread spoilage fungi. Acta Biol. Szeged. 57, 9–12.

Krooneman, J., Faber, F., Alderkamp, A.C., Elferink, S.J.H.W.O., Driehuis, F., Cleenwerck, I., Swings, J., Gottschal, J.C., 2002. *Lactobacillus diolivorans sp . nov .,* a 1 , 2-propanediol-degrading bacterium isolated from aerobically stable maize silage. Int. J. Syst. Evol. Microbiol. 52, 639–646.

Lahtinen, S.J., Haskard, C.A., Ouwehand, A.C., Salminen, S.J., Ahokas, J.T., 2004. Binding of aflatoxin B-1 to cell wall components of *Lactobacillus rhamnosus* strain GG. Food Addit. Contam. 21, 158–164. doi:10.1080/02652030310001639521

Lambert, R.J.W., Skandamis, P.N., Coote, P.J., Nychas, G.J.E., 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. 91, 453–462. doi:10.1046/j.1365-2672.2001.01428.x

Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A., Gobbetti, M., 2000. Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. Appl. Environ. Microbiol. 66, 4084–4090. doi:10.1128/aem.66.9.4084-4090.2000

Levinskaite, L., 2012. Susceptibility of food-contaminating *Penicillium* genus fungi to some preservatives and disinfectants. Ann. Agric. Environ. Med. 19, 85–9.

Lopes, F.A., Soares, N.F.F., Lopes, C.C.P., da Silva, W.A., Baffa Júnior, J.C., Medeiro, A.E.A., 2014. Conservation of bakery products through cinnamaldehyde antimicrobial films. Packag. Technol. Sci. 27, 293–302. doi:10.1002/pts

Luciano, F.B., Holley, R.A., 2009. Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7. Int. J. Food Microbiol. 131, 240–245. doi:10.1016/j.ijfoodmicro.2009.03.005

Magan, N., Arroyo, M., Aldred, D., 2003a. 24 - Mould prevention in bread, in: Cauvain, S.P. (Ed.), Bread Making, Woodhead Publishing Series in Food Science, Technology and Nutrition. Woodhead Publishing, pp. 482–494. doi:http://dx.doi.org/10.1533/9781855737129.2.500

Magan, N., Keshri, G., Needham, R., Sneath, R., 2003b. Use of electronic nose technology for the early detection of spoilage moulds in cereal products, in: Credland, PF and Armitage, DM and Bell, CH and Cogan, PM and Highley, E. (Ed.), Advances in Stored Product Protection. CABI PUBLISHING, 875 Massachusetts Avenue, 7th Floor, Cambridge, MA 02139 USA, pp. 139–143.

Magnusson, J., Schnürer, J., 2001. *Lactobacillus coryniformis subsp. coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. Appl. Environ. Microbiol. 67, 1–5. doi:10.1128/AEM.67.1.1

Marin, S., Guynot, M.E., Neira, P., Bernado, M., Sanchis, V., Ramos, A.J., 2002. Risk assessment of the use of sub-optimal levels of weak-acid preservatives in the control of mould growth on bakery products. Int. J. Food Microbiol. 79, 203–211.

Melikoglu, M., Webb, C., 2013. Use of waste bread to produce fermentation products, in: Food Industry Wastes. Elsevier, pp. 61–76. doi:10.1016/B978-0-12-391921-2.00004-4

Mintel, 2014. Category Insight: Bread & Bread Products. mintel.com 1–6.

Mnif, I., Besbes, S., Ellouze, R., Ellouze-Chaabouni, S., Ghribi, D., 2012. Improvement of bread quality and bread shelf-life by *Bacillus subtilis* biosurfactant addition. Food Sci. Biotechnol. 21, 1105–1112. doi:10.1007/s10068-012-0144-8

Mo, E.K., Sung, C.K., 2014. Production of white pan bread leavened by *Pichia anomala* SKM-T. Food Sci. Biotechnol. 23, 431–437. doi:10.1007/s10068-014-0059-7

Moore, M.M., Dal Bello, F., Arendt, E.K., 2008. Sourdough fermented by *Lactobacillus plantarum* FST 1.7 improves the quality and shelf life of gluten-free bread. Eur. Food Res. Technol. 226, 1309–1316. doi:10.1007/s00217-007-0659-z

Moore, M.M., Schober, T.J., Dockery, P., Arendt, E.K., 2004. Textural comparisons of gluten-free and wheat-based doughs, batters, and breads. Cereal Chem. 81, 567–575. doi:10.1094/cchem.2004.81.5.567

Muhialdin, B.J., Hassan, Z., Sadon, S.K., 2011. Antifungal activity of *Lactobacillus fermentum* Te007, *Pediococcus pentosaceus* Te010, *Lactobacillus pentosus* G004, and *L. paracasi* D5 on selected foods. J. Food Sci. 76, M493–9. doi:10.1111/j.1750-3841.2011.02292.x

Muthusamy, K., Gopalakrishnan, S., Ravi, T.K., Sivachidambaram, P., 2008. Biosurfactants: Properties, commercial production and application. Curr. Sci. 94, 736–747.

Najafi, M.A., Rezaei, K., Safari, M., Razavi, S.H., 2012. Effects of several starter cultures on the anti-mold activity and sensory attributes of a traditional flat bread (Sangak) from Iran. Food Sci. Biotechnol. 21, 113–121. doi:10.1007/s10068-012-0014-4

Ng, T.B., 2004. Antifungal proteins and peptides of leguminous and non-leguminous origins. Peptides 25, 1215–1222. doi:10.1016/j.peptides.2004.03.012

Niku-Paavola, M.L., Laitila, a, Mattila-Sandholm, T., Haikara, a, 1999. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. J. Appl. Microbiol. 86, 29–35.

Oliveira, P.M., Zannini, E., Arendt, E.K., 2014. Cereal fungal infection, mycotoxins, and lactic acid bacteria mediated bioprotection: from crop farming to cereal products. Food Microbiol. 37, 78–95. doi:10.1016/j.fm.2013.06.003

Osborne, L.E., Stein, J.M., 2007. Epidemiology of *Fusarium* head blight on small-grain cereals. Int. J. Food Microbiol. 119, 103–8. doi:10.1016/j.ijfoodmicro.2007.07.032

Otoni, C.G., Pontes, S.F.O., Medeiros, E. a a, Soares, N.D.F.F., 2014. Edible films from methylcellulose and nanoemulsions of clove bud (*Syzygium aromaticum*) and oregano (*Origanum vulgare*) essential oils as shelf life extenders for sliced bread. J. Agric. Food Chem. 62, 5214–9. doi:10.1021/jf501055f

Oude Elferink, S.J.W.H., Krooneman, J., Jan, C., Spoelstra, S.F., Faber, F., Driehuis, F., 2001. Anaerobic conversion of lactic acid to acetic acid and 1, 2-propanediol by *Lactobacillus buchneri*. Anaerobic Conversion of Lactic Acid to Acetic Acid and. Appl. Environ. Microbiol. 67, 125–132. doi:10.1128/AEM.67.1.125

Passarinho, A.T.P., Dias, N.F., Camilloto, G.P., Cruz, R.S., Otoni, C.G., Moraes, A.R.F., Soares, N.D.F.F., 2014. Sliced bread preservation through oregano essential oil-containing sachet. J. Food Process Eng. 37, 53–62. doi:10.1111/jfpe.12059

Pawlowska, A.M., Zannini, E., Coffey, A., Arendt, E.K., 2012. "Green preservatives": combating fungi in the food and feed industry by applying antifungal lactic acid bacteria. Adv. Food Nutr. Res. 66, 217–238. doi:10.1016/b978-0-12-394597-6.00005-7

Pereira, V.L., Fernandes, J.O., Cunha, S.C., 2014. Mycotoxins in cereals and related foodstuffs: A review on occurrence and recent methods of analysis. Trends Food Sci. Technol. 36, 96–136. doi:10.1016/j.tifs.2014.01.005

Petersson, S., Schnürer, J., 1995. Biocontrol of mold growth in high- moisture wheat stored under airtight conditions by *Pichia anomala*, *Pichia guilliermondii*, and *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 61, 1027–1032.

Picart, P., Pirttilä, A.M., Raventos, D., Kristensen, H.-H., Sahl, H.-G., 2012. Identification of defensin-encoding genes of *Picea glauca*: characterization of PgD5, a conserved spruce defensin with strong antifungal activity. BMC Plant Biol. 12, 180. doi:10.1186/1471-2229-12-180

Plessas, S., Alexopoulos, A., Mantzourani, I., Koutinas, A., Voidarou, C., Stavropoulou, E., Bezirtzoglou, E., 2011. Application of novel starter cultures for sourdough bread production. Anaerobe 17, 486–9. doi:10.1016/j.anaerobe.2011.03.022

Reale, A., Di Renzo, T., Succi, M., Tremonte, P., Coppola, R., Sorrentino, E., 2013. Microbiological and fermentative properties of baker's yeast starter used in breadmaking. J. Food Sci. 78, M1224–31. doi:10.1111/1750-3841.12206

Rizzello, C.G., Cassone, A., Coda, R., Gobbetti, M., 2011. Antifungal activity of sourdough fermented wheat germ used as an ingredient for bread making. Food Chem. 127, 952–959. doi:10.1016/j.foodchem.2011.01.063

Rizzello, C.G., Coda, R., De Angelis, M., Di Cagno, R., Carnevali, P., Gobbetti, M., 2009. Long-term fungal inhibitory activity of water-soluble extract from *Amaranthus spp.* seeds during storage of gluten-free and wheat flour breads. Int. J. Food Microbiol. 131, 189–96. doi:10.1016/j.ijfoodmicro.2009.02.025

Rizzello, C.G., Lavecchia, A., Gramaglia, V., Gobbetti, M., 2015. Long-term fungal inhibition by *Pisum sativum* flour hydrolysate during storage of wheat flour bread. Appl. Environ. Microbiol. 81, 4195–4206. doi:10.1128/AEM.04088-14

Rizzello, C.G., Nionelli, L., Coda, R., De Angelis, M., Gobbetti, M., 2010. Effect of sourdough fermentation on stabilisation, and chemical and nutritional characteristics of wheat germ. Food Chem. 119, 1079–1089. doi:10.1016/j.foodchem.2009.08.016

Ryan, L.A.M., Dal Bello, F., Arendt, E.K., 2008. The use of sourdough fermented by antifungal LAB to reduce the amount of calcium propionate in bread. Int. J. Food Microbiol. 125, 274–278. doi:10.1016/j.ijfoodmicro.2008.04.013

Ryan, L.A.M., Dal Bello, F., Arendt, E.K., Koehler, P., 2009a. Detection and quantitation of 2,5-diketopiperazines in wheat sourdough and bread. J. Agric. Food Chem. 57, 9563–8. doi:10.1021/jf902033v

Ryan, L.A.M., Dal Bello, F., Czerny, M., Koehler, P., Arendt, E.K., 2009b. Quantification of phenyllactic acid in wheat sourdough using high resolution gas chromatography-mass spectrometry. J. Agric. Food Chem. 57, 1060–4. doi:10.1021/jf802578e

Ryan, L.A.M., Zannini, E., Dal Bello, F., Pawlowska, A., Koehler, P., Arendt, E.K., 2011. *Lactobacillus amylovoru*s DSM 19280 as a novel food-grade antifungal agent for bakery products. Int. J. Food Microbiol. 146, 276–283. doi:10.1016/j.ijfoodmicro.2011.02.036

Sahan, Y., 2011. Effect of *Prunus laurocerasus L.* (Cherry Laurel) leaf extracts on growth of bread spoilage fungi. Bulg. J. Agric. Sci. 17, 83–92.

Sciarini, L.S., Ribotta, P.D., León, A.E., Pérez, G.T., 2010. Influence of gluten-free flours and their mixtures on batter properties and bread quality. Food Bioprocess Technol. 3, 577–585. doi:10.1007/s11947-008-0098-2

Selitrennikoff, C.P., 2001. Antifungal proteins. Appl. Environ. Microbiol. 67, 2883–2894. doi:10.1128/AEM.67.7.2883-2894.2001

Škrbić, B., Živančev, J., Đurišić-Mladenović, N., Godula, M., 2012. Principal mycotoxins in wheat flour from the Serbian market: Levels and assessment of the exposure by wheat-based products. Food Control 25, 389–396. doi:10.1016/j.foodcont.2011.10.059

Stiles, M.E., 1996. Biopreservation by lactic acid bacteria. Antonie Van Leeuwenhoek 70, 331–45.

Stratford, M., Nebe-von-Caron, G., Steels, H., Novodvorska, M., Ueckert, J., Archer, D.B., 2013a. Weak-acid preservatives: pH and proton movements in the yeast *Saccharomyces cerevisiae*. Int. J. Food Microbiol. 161, 164–71. doi:10.1016/j.ijfoodmicro.2012.12.013

Stratford, M., Plumridge, A., Nebe-von-Caron, G., Archer, D.B., 2009. Inhibition of spoilage mould conidia by acetic acid and sorbic acid involves different modes of action, requiring modification of the classical weak-acid theory. Int. J. Food Microbiol. 136, 37–43. doi:10.1016/j.ijfoodmicro.2009.09.025

Stratford, M., Steels, H., Nebe-von-Caron, G., Novodvorska, M., Hayer, K., Archer, D.B., 2013b. Extreme resistance to weak-acid preservatives in the spoilage yeast *Zygosaccharomyces bailii*. Int. J. Food Microbiol. 166, 126–134. doi:10.1016/j.ijfoodmicro.2013.06.025

Suhr, K.I., Nielsen, P. V, 2004. Effect of weak acid preservatives on growth of bakery product spoilage fungi at different water activities and pH values. Int. J. Food Microbiol. 95, 67–78. doi:10.1016/j.ijfoodmicro.2004.02.004

Suomalainen, T.H., Mäyrä-Mäkinen, A.M., 1999. Propionic acid bacteria as protective cultures in fermented milks and breads. Lait 79, 165–174.

The Federation of Bakers, 2012. European Bread Market [WWW Document]. URL http://www.bakersfederation.org.uk/the-bread-industry/industry-facts/european-bread-market.html (accessed 1.6.15).

Van Sint Fiet, S., 2015. Enzyme technology secures significant freshness improvements in packaged bread. Bak. Eur. 26–28.

Varsha, K.K., Priya, S., Devendra, L., Nampoothiri, K.M., 2014. Control of spoilage fungi by protective lactic acid bacteria displaying probiotic properties. Appl. Biochem. Biotechnol. 172, 3402–13. doi:10.1007/s12010-014-0779-4

Vermeulen, N., Gänzle, M.G., Vogel, R.F., 2006. Influence of peptide supply and cosubstrates on phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM20451T and *Lactobacillus plantarum* TMW1.468. J. Agric. Food Chem. 54, 3832–3839.

Vidal, A., Marín, S., Morales, H., Ramos, a J., Sanchis, V., 2014. The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content. Food Chem. Toxicol. 68, 53–60. doi:10.1016/j.fct.2014.03.006

Vidal, A., Sanchis, V., Ramos, A.J., Marín, S., 2015. Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products. Food Chem. 178, 276–286. doi:10.1016/j.foodchem.2015.01.098

Wei, Q., Wolf-Hall, C., Hall, C. a, 2009. Application of raisin extracts as preservatives in liquid bread and bread systems. J. Food Sci. 74, M177–84. doi:10.1111/j.1750-3841.2009.01137.x

Wolter, A., Hager, A.S., Zannini, E., Czerny, M., Arendt, E.K., 2014. Impact of sourdough fermented with *Lactobacillus plantarum* FST 1.7 on baking and sensory properties of gluten-free breads. Eur. Food Res. Technol. 239, 1–12. doi:10.1007/s00217-014-2184-1

Wu, F., Groopman, J.D., Pestka, J.J., 2014. Public health impacts of foodborne mycotoxins, in: Doyle, MP and Klaenhammer, T. (Ed.), Annual Review of Food Science and Technology, Vol 5. 4139 EL Camino Way, PO Box 10139, Palo Alto, CA 94303-0897 USA, pp. 351–372. doi:10.1146/annurev-food-030713-092431

Wu, L., Wang, B., 2015. Evaluation on levels and conversion profiles of DON, 3-ADON, and 15-ADON during bread making process. Food Chem. 185, 509–516. doi:10.1016/j.foodchem.2015.03.082

Yan, J., Yuan, S., Jiang, L., Ye, X., Ng, T.B., Wu, Z., 2015. Plant antifungal proteins and their applications in agriculture. Appl. Microbiol. Biotechnol. 4961–4981. doi:10.1007/s00253-015-6654-6

Ye, X.Y., Wang, H.X., Ng, T.B., 2000. Sativin - A novel antifungal miraculin-like protein isolated from legumes of the sugar snap *Pisum sativum var. macrocarpon*. LIFE Sci. 67, 775–781. doi:10.1016/S0024-3205(00)00672-X

Zannini, E., Pontonio, E., Waters, D.M., Arendt, E.K., 2012. Applications of microbial fermentations for production of gluten-free products and perspectives. Appl. Microbiol. Biotechnol. 93, 473–485. doi:10.1007/s00253-011-3707-3

Zhang, C., Brandt, M.J., Schwab, C., Gänzle, M.G., 2010. Propionic acid production by cofermentation of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough. Food Microbiol. 27, 390–5. doi:10.1016/j.fm.2009.11.019

Chapter 3 Quantification of cyclic dipeptides from cultures of *Lactobacillus brevis* R2Δ by HRGC/MS using Stable Isotope Dilution Assay

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3.1 Abstract

Lactic acid bacteria (LAB) play an important role as natural preservatives in fermented food and beverage systems reducing the application of chemical additives. Thus, investigating their antifungal compounds, such as cyclic dipeptides, has gained prominence. Previous research has primarily focussed on isolation of these compounds. However, their precise quantification will provide further information regarding their antifungal performance in a complex system. To address this, deuterated labelled standards of the cyclic dipeptides cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Met-Pro) and cyclo(Phe-Pro) were synthesised and stable isotope dilution assays were developed enabling an accurate quantification of cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Met-Pro) and cyclo(Phe-Pro) in MRS-broth and wort. Quantitative results showed that in the *Lactobacillus brevis* R2Δ fermented MRS-broth, the concentrations of cyclo(Leu-Pro), cyclo(Pro-Pro) and cyclo(Phe-Pro) were significantly higher ($P < 0.05$), and in wort for cyclo (Leu-Pro) when compared to their controls. This indicates, that the formation of these three cyclic dipeptides is related with *Lb. brevis* R2Δ metabolism. Furthermore, this represents the first report of cyclic dipeptides quantification using stable isotope dilution assays in LAB cultures both *in vitro* and in a food system.

3.2 Introduction

Cyclic dipeptides (also known as 2,5-diketopiperazines) are low molecular weight compounds providing a multitude of bioactive properties. They are associated with activities such as anticancer, antiviral, antihyperglycemic, or act as glycosidase inhibitors, neuroprotective agents, and antifungal or antibacterial compounds, thus making them remarkable tools for industrial and clinical applications (Borthwick, 2012). As such, cyclic dipeptides have become increasingly researched, as is evident in recent literature (Borthwick, 2012; de Carvalho and Abraham, 2012; Cornacchia et al., 2012; Huang et al., 2010; Ressurreicao et al., 2011). Contrastingly, Prasad (1995) concluded that they were a relatively unexplored class of bioactive peptides at that time. Furthermore certain cyclic dipeptides have been classed as novel signal molecules in addition to acylated homoserine lactones enabling bacteria to communicate inter- and intra-species, which is known as quorum sensing (Martins and Carvalho, 2007; O'Neil et al., 2007; Wang et al., 2010). Within therapeutical practices, this offers a great opportunity in infection management through virulence attenuation by blocking cell-to-cell communication (Borthwick, 2012).

Cyclic dipeptides have also been identified in culture broths fermented with lactic acid bacteria (LAB), the class of bacteria most widely used in food fermentation (Dal Bello et al., 2007; Li et al., 2011; Magnusson et al., 2003; Niku-Paavola et al., 1999; Ryan et al., 2011; Strom et al., 2002; Yang and Chang, 2010). Many species, especially from the genus *Lactobacillus*, have been referred to the European Food Safety Authority (EFSA) for safety assessment without raising safety concerns. As a result, they have been included in the QPS (Qualified Presumption of Safety) list for authorised use in the food and feed chain within the European Union (EFSA, 2007). Nowadays, consumers are more concerned about safety and additive content in food resulting in an increasing demand for natural and "free-from" foods. As a consequence, research interest has grown in the application of LAB due to their biopreservative capacities (Pawlowska et al., 2012). The use of these bacteria can greatly contribute to an environmentally friendly, safe and ecological approach to food and feed preservation (Axel et al., 2012).

In food and beverages proline-containing cyclic dipeptides are particularly abundant (Borthwick, 2012; Chen et al., 2009, 2007; Chen et al., 2004; Gautschi et al., 1997; Ginz and Engelhardt, 2000; Ryan et al., 2009). They can be formed during food processing due to the degradation of peptides caused by high temperature or pH-value changes. The mechanism of action transforms linear peptide precursors, thus involving them in intra-molecular cyclization (Rizzi, 1989). Biosynthesis of cyclic dipeptides involves dedicated non-ribosomal peptide synthases or cyclo-peptide synthases (Gondry et al., 2009; Gruenewald et al., 2004; Schultz et al.., 2008). Proline-based cyclic dipeptides have been isolated from culture broths of different LAB (*Lactobacillus amylovorus*, *Lb. coryniformis*, *Lb. plantarum, Lb. reuteri* and *Lb. sakei,* as well as *Pediococcus pentosaceus*). These include cyclo(L-His-L-Pro), cyclo(L-Leu-L-Pro), cyclo(L-Met-L-Pro), cyclo(L-Phe–L-Pro), cyclo(L-Phe–trans-4-OH-L-Pro), cyclo(L-Pro-L-Pro) and cyclo(L-Tyr-L-Pro) and additionally, two leucine based cyclic dipeptides cyclo(Gly–Leu) and cyclo(Leu–Leu) were identified (Dal Bello et al., 2007; Li et al., 2011; Magnusson et al., 2003; Niku-Paavola et al., 1999; Ryan et al., 2011; Strom et al., 2002; Yang and Chang, 2010). The presence of these cyclic dipeptides in LAB cultures is associated with antifungal activity. LAB produce a range of secondary metabolites such as; organic acids, fatty acids, proteinaceous compounds, and a variety of other low-molecular weight compounds - providing antifungal activity (Broberg et al., 2007; Ndagano et al., 2011; Niku-Paavola et al., 1999; Ryan et al., 2011; Strom et al., 2002). However, some studies showed that the concentrations of several of these metabolites in the culture broths are much lower than their minimal inhibition concentration (MIC), which is required for non-synergistic antifungal activity. Therefore, the antifungal mode of action seems to originate from a synergistic effect of all these antifungal compounds that LAB secrete. The concentration of cyclic dipeptides in LAB fermented culture media has not been evaluated yet. Ryan et al. (2009) quantified the levels of cyclo(L-Leu-L-Pro) and cyclo(L-Phe-L-Pro) in LAB-fermented wheat dough and bread. However, the long extraction procedure is a considerable drawback of this method. Additionally it requires high volumes of solvent.

Our research presents an accurate and rapid quantification of cyclic dipeptides from LAB-fermented culture medium and an *in situ* liquid food system.

Herein, ^{2}H-labelled counterparts of four cyclic dipeptides were synthesized for the first time in order to develop stable isotope dilutions assays (SIDA) in combination with high resolution gas chromatography-low resolution mass spectrometry (HRGC/MS). SIDA has been proven as accurate quantification tools of several food ingredients, e.g., odorants, vitamins and mycotoxins (Grosch, 2001; Rychlik, 2003; Rychlik and Schieberle, 1999). Cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Met-Pro) and cyclo(Phe-Pro) were identified and quantified in both MRS-broth and wort which had been fermented with the antifungal LAB strain *Lb. brevis* R2Δ as an example. Knowledge about the concentration of these compounds contributes to further understanding of the synergistic effects, which provide the antifungal activities associated with certain LAB strains.

3.3 Material and Methods

3.3.1 Chemicals

All chemicals were purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany), unless otherwise stated. Cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Met-Pro) and cyclo(Phe-Pro) were purchased from Bachem (Weil am Rhein, Germany). Anhydrous sodium sulphate was from Merck, Darmstadt, Germany.

3.3.2 Synthesis of isotopically labelled cyclo-dipeptides

Isotopically labelled cyclo-dipeptides were not commercially available and thus were synthesised using a protium-deuterium (^1H–^2H) exchange reaction. Deuteration of the cyclic dipeptide standards was carried as described by Steinhaus and Schieberle (2005) for the deuteration of 3-methylindole. Briefly, in a self-made small autoclave (stainless steel, 80 mm, 10 mm i.d., 3 mm wall thickness) with screw cap and PTFE sealing, cyclic dipeptide (0.1 mmol) was dissolved in deuterium oxide (1.4 mL) and heated (160 °C; 168 h) in the presence of palladium on barium sulphate (5 %; 200 mg) by means of a block heater (model SBH200D, Stuart Bench Top Science Equipment, UK). After cooling, the mixture was filtered (G4 glass filter) into a vacuum flask and subsequently washed with distilled water (3 x 2 mL) and ethyl acetate (3 x 2 mL). The filtrate was then transferred into a separating funnel by rinsing the vacuum flask with distilled water (2 x 25 mL) and ethyl acetate (2 x 25 mL). After separating the organic phase, the aqueous layer was extracted with ethyl acetate (5 x 25 mL). Organic layers were combined and dried over anhydrous Na_2SO_4 (stock solution). HRGC/MS-EI analysis of the unlabelled and synthesised compounds confirmed the exchange of ^1H- by ^2H-atoms. Mass spectral data of the labelled compounds are listed below.

[$^2H_{2\text{-}10}$]-cyclo(Leu-Pro); yield: 0.0121 mmol; 12.1 %; EI-MS (m/z: intensity in %) 156 (100), 71 (49), 157 (32), 72 (32), 88 (20), 158 (17), 73 (14), 127 (13), 56 (11), 70 (11), 155 (11), 42 (10), 126 (10), 74 (10), 75 (9), 44(9), 69 (8), 43 (8).

[$^2H_{8-14}$]-cyclo(Pro-Pro); yield: 0.0111 mmol; 11.1 %; EI-MS (m/z: intensity in %) 78 (100), 208 (39), 106 (23), 77 (22), 76 (17), 44 (15), 207 (14), 152 (14), 46 (12), 74 (11), 122 (11), 58 (10), 180 (7), 48 (7), 108 (6), 134 (6), 42 (6), 124 (96), 105 (6), 120 (5).

[$^2H_{2-5}$]-cyclo(Met-Pro); yield: 0.0195 mmol; 19.5 %; EI-MS (m/z: intensity in %) 156 (100), 71 (57), 169 (24), 230 (23), 141 (18), 72 (11), 157 (10), 61 (9), 42 (8), 56 (6), 57 (6), 69 (5), 168 (5), 127 (5), 70 (4), 128 (4), 154 (4), 231 (4), 41 (3), 114 (3).

[$^2H_{7-15}$]-cyclo(Phe-Pro); yield: 0.0395 mmol; 39.7 %; EI-MS (m/z: intensity in %) 127 (100), 155 (55), 98 (42), 128 (36), 72 (27), 253 (27), 71 (20), 97 (20), 156 (20), 129 (15), 70 (12), 252 (11), 254 (10), 73 (9), 99 (9), 42 (9), 69 (7), 157 (7), 154 (6), 96 (5).

3.3.3 Standard wort production

A lab-scale (50 L) tank was used for wort production. Firstly, the malt (*Sebastian*, 2008 harvest, Cork Malting Company, Ireland) was milled with a two-roller mill (0.4 mm distance between rollers) and grist (7.2 kg) was mashed into 27 L of water at 62 °C. The following mashing regime was applied: 28.1 min at 62 °C, 25.0 min at 72 °C, and a mash off temperature of 78 °C, with a heating rate of 1 °C/min between the individual rests. Wort was boiled for 1 h and adjusted to a final extract content of 9 % (w/w) by adding hot water. Precipitates in hot wort were removed by filtration. Hot wort was transferred into a 50 L stainless steel container and cooled to 30 °C in a tempered water bath.

3.3.4 MRS-broth and wort fermentations and sample extractions

Lb. brevis R2Δ was grown in MRS-broth and separately in standard wort at 30 °C for 48 h. Subsequently, the MRS and wort cultures were centrifuged (7,500 g for 15 min at 4 °C) and the supernatants were sterile filtered (0.45 μm pore-size filter; Millipore) to prepare cell-free supernatant (CFS), which was frozen at -20 °C prior to analysis. Uninoculated MRS-broth and wort were used as controls and were processed using the same procedure as for the fermented samples.

Aliquots of the CFS and wort (1 mL) were diluted with deionised water (10 mL) and known amounts of deuterium-labelled standards were added (CFS: 6 μg for cyclo(Leu-Pro), cyclo(Met-Pro), cyclo(Phe-Pro) and 2 μg for cyclo(Pro-Pro); wort: 3 μg for cyclo(Leu-Pro), cyclo(Met-Pro), cyclo(Phe-Pro) and 2 μg for cyclo(Pro-Pro)). The samples were equilibrated for 10 min and then extracted with ethyl acetate (3 x 10 mL). Organic layers were combined, dried over anhydrous sodium sulphate and the volume reduced to 1 mL in a rotary evaporator (40 °C, 240 mbar). The extract obtained was then stored at -20 °C prior to analysis. Blank MRS-broth and wort were treated in the same way as the supernatants from culture of the antifungal strain.

3.3.5 Quantification of cyclic dipeptides by HRGC/MS

HRGC/MS analyses were performed with a DSQ™ II single quadrupole GC/MS system (Thermo Fisher Scientific) running in electron ionization mode at 70 eV of

electron energy. The GC column, an Agilent J&W DB-1701 capillary column (50 m x 0.32 mm x 0.1 μm), coated with 14 %(-cyanopropyl-phenyl)-methyl-polysiloxane, was operated with helium as a carrier gas in the split mode (split ratio 10:1). Samples were injected into a PTV (Programmable Temperature Vaporizer) injector system, which was heated to 280 °C. The oven temperature was held at 80 °C for 2 min, then raised by 10 °C/min to 280 °C and held for 10 min. The flow rate was set to 2.3 mL/min. The HRGC/MS runs were recorded using Thermo Scientific Xcalibur 2.0 software (Thermo Fisher Scientific, Hemel Hempstead, UK). Calibration curves were generated additionally by analysis of solutions containing unlabelled and labelled compounds in different ratios (1:10 to 10:1) and concentrations (0.5 – 10 μg/mL) and used to calculate the concentrations of the compounds based on their area counts using the following equation:

$$C_{Compound} = \left(\frac{A_{Compound}}{A_{IS}} \times a + b\right) \times \frac{m_{IS}}{V}$$

Where $C_{Compound}$ = concentration of appropriate cyclic dipeptide, $A_{Compound}$ = area counts for appropriate cyclic dipeptide, A_{IS} = area counts for deuterium labelled standard, a = slope from the calibration curve, b = intercept form the calibration curve, m_{IS} = amount of deuterium labelled standard, V = volume of the sample analysed.

3.3.6 Statistical analysis

The statistical analysis, using Minitab 16 software, of three independent replicates (n = 3) was performed using one-way ANOVA. Where F-values were significant, pairwise comparisons were carried out with the help of Fisher's least significant difference (LSD) procedure to determine the statistical significance between the fermented sample and unfermented control. Differences were considered significant at $P < 0.05$.

3.4 Results and Discussion

3.4.1 Deuteration of cyclic dipeptides

A prerequisite for the quantification of food components is the application of assays with high accuracy in order to obtain precise data and valuable information. Losses of the target compound and the standard during extraction and isolation prior to instrumental analysis should be consequently compensated. Thus, the target compound itself would be the "perfect" standard but a differentiation of compound and standard during analysis is of course not possible. Isotopic labelled compounds however are almost identical to unlabelled target compounds. They differ from the target compound only by the presence of elements with higher molecular weight (e.g., ^{2}H- instead of ^{1}H-atoms), which are incorporated in the unlabelled compound by synthesis. The chemical and physical properties of both compounds are almost identical and the prerequisite of compensation of losses during isolation is achieved (Grosch, 2001). The isotopic labelled standards of cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Met-Pro) and cyclo(Phe-Pro) were synthesized consequently in this study by ^{1}H-^{2}H exchange reactions in order to develop a quantification assay for the dipeptides.

Compound structures (L-isomers) are shown in Figure 3-1 (A, C, E, and G). Mass spectral data of the labelled cyclic dipeptides (MS-EI) showed the successful incorporation of 2 to 15 deuterium atoms depending on the compound, appearing as shifts of 2 to 15 mass units from the undeuterated analogues (Figure 3-1; B, D, F and H). The isotopomeric distributions were calculated on base of the area intensities of extracted ion chromatograms (Table 3-1). Thus, the ^{1}H-^{2}H exchange was not complete and multicomponent mixtures of labelled isotopomers were obtained. Efficient ^{1}H-^{2}H exchange reactions are challenging to develop (Modutlwa et al., 2010). Nonetheless, materials with narrow isotope clusters formed by ^{1}H-^{2}H exchange can be fit for use as an internal standard for MS investigations as long as the ^{2}H incorporation exceeds 99.5 % (0.5 % unlabelled reactant remaining) and a representative mass peak is present in the mixture that can be used as the reference mass of the internal standard (Derdau et al., 2009). Furthermore, at least two ^{2}H-atoms should be integrated in the standards because of the natural ^{13}C-content

resulting in a $[M^{+1}]^+$-signal of the analyte (e.g., 7.9 % for cyclo(Leu-Pro) for m/z 155, Table 3-1). Both prerequisites were fulfilled by all synthesized compounds as shown in Table 3-1, which lists the distribution of the isotopomers $^2H_0$ up to $^2H_{18}$ of each unlabelled and synthesised cyclic compounds. Regarding ^{2}H-cyclo(Pro-Pro) as an example, the mass spectrum of unlabelled compound consisted in particular of isotopomer 194 ($[M]^+$, $^2H_0$, 89.0 %) besides isotopomers 195 ($[M^{+1}]^+$, $^2H_1$, 10.0 %), 196 ($[M^{+2}]^+$, $^2H_2$, 0.9 %) and 197 ($[M^{+3}]^+$, $^2H_3$, 0.1 %), which were not detectable in ^{2}H-cyclo(Pro-Pro). A complete ^{1}H-^{2}H-exchange of all 14 hydrogen atoms and a uniform deuteration was not achieved because all isotopomers in the range 198 ($[M^{+4}]^+$, $^2H_4$) to 208 ($[M^{+14}]^+$, $^2H_{14}$) were detected. However, isotopomers 208 (54.7 %) and 207 (20.9 %, $[M^{+13}]^+$, $^2H_{13}$) were the most abundant ones and consequently chosen as target isotopomer of labelled cyclo (Pro-Pro). The $^2H_0$-isotopomer was also not determined in cyclo(Phe-Pro) but the distribution of the isotopomers was heterogeneous again (Table 3-1). The target isotopomers $^2H_{8\text{-}11}$ were selected due to their high content in the labelled cyclo(Phe-Pro). In contrast to the aforementioned labelled compounds, the $^2H_0$-isotopmer was detected in ^{2}H-cyclo(Met-Pro) (0.2 %) and ^{2}H-cyclo(Leu-Pro) (0.6 %) but this low content was accepted (Table 3-1). The target isotopomers $^2H_{2\text{-}3}$ was chosen for the same reason for both cyclo(Met-Pro) and cyclo(Leu-Pro).

Concentrations of these target labelled compounds in the stock solution were determined by HRGC/MS total ion current (TIC) quantification using a standard procedure with methyl octanoate as the internal standard (Kiefl et al., 2013; Steinhaus et al., 2003). Calibration curves were generated by analysis of mixtures containing unlabelled cyclic dipeptides (2-8 µg/mL) and methyl octanoate (1 µg/mL) in known amounts under the same experimental conditions and calibration functions were determined (Table 3-2).

The yields of the synthesised compounds were calculated based on the final and the initially used amounts of the compounds. These yields were low and ranged only between 11.1 % and 39.7 %. The losses (60 – 89 %) were caused most likely by component degradation during synthesis due to the thermal conditions and losses during liquid-liquid extraction after synthesis.

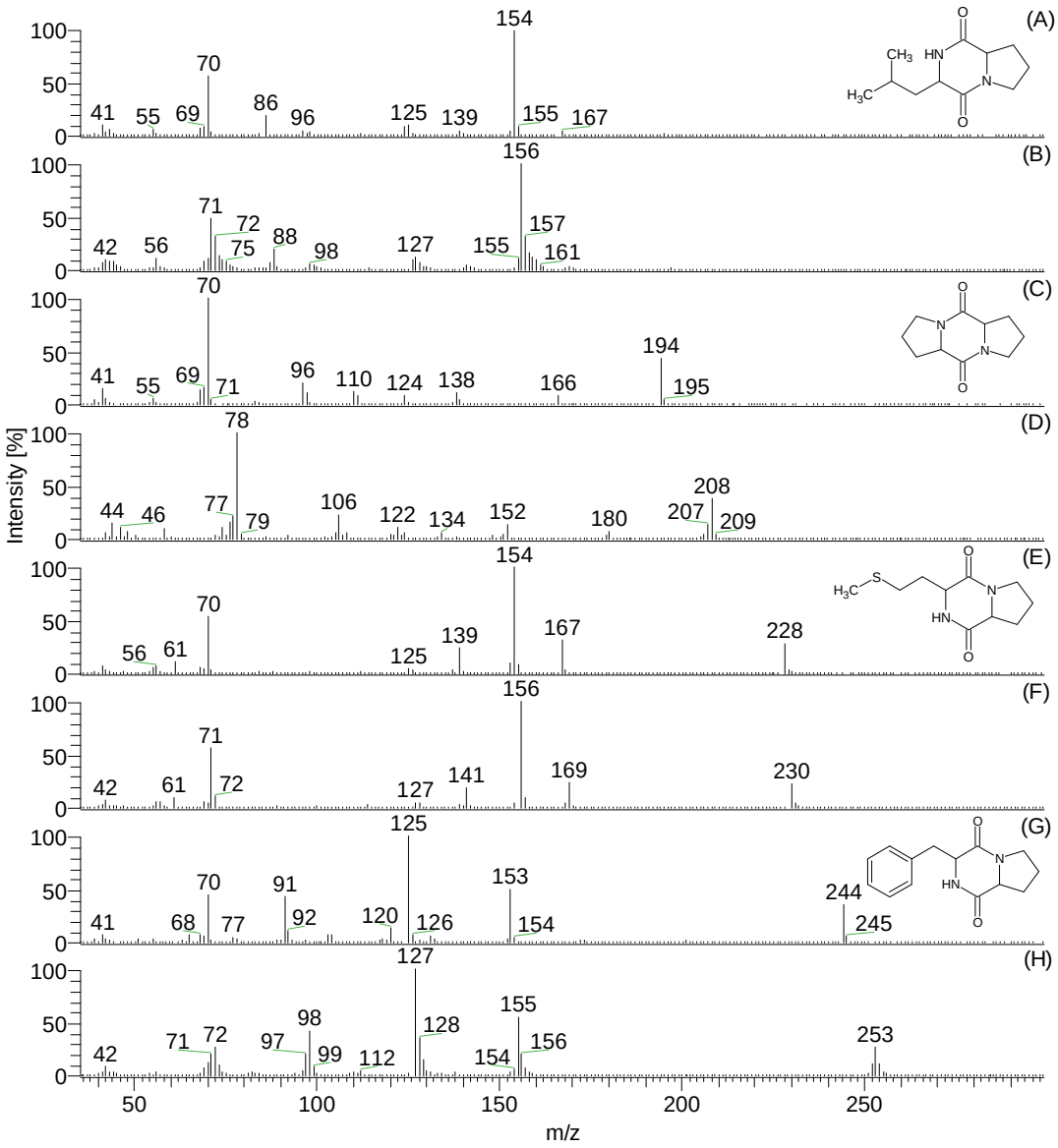


Figure 3-1 Mass spectra (MS-EI) of cyclo(Leu-Pro) (A), [²H]-cyclo(Leu-Pro) (B), cyclo(Pro-Pro) (C), [²H]-cyclo(Pro-Pro) (D), cyclo(Met-Pro) (E), [²H]-cyclo(Met-Pro) (F), cyclo(Phe-Pro) (G), [²H]-cyclo(Phe-Pro) (H).

Table 3-1 Isotopomeric distribution of unlabelled and deuterium labelled cyclic dipeptides (molecular m/z-ion of the unlabelled cyclic dipeptide in bold).

	Cyclo(Leu-Pro)			Cyclo(Pro-Pro)			Cyclo(Met-Pro)			Cyclo(Phe-Pro)		
Isotopic labelling	**m/z-ion**	**Unlabelled [%]**	**Labelled [%]**	**m/z-ion**	**Unlabelled [%]**	**Labelled [%]**	**m/z-ion**	**Unlabelled [%]**	**Labelled [%]**	**m/z-ion**	**Unlabelled [%]**	**Labelled [%]**
$^2H_0$	**154**	91.3	0.6	**194**	89	0	**228**	85.2	0.2	**244**	85.3	0
$^2H_1$	155	7.9	3.2	195	10	0	229	9.7	0	245	13.2	0
$^2H_2$	156	0.7	27.9	196	0.9	0	230	4.5	76.8	246	1.3	0
$^2H_3$	157	0	11.9	197	0.1	0	231	0.5	14	247	0.1	0
$^2H_4$	158	0	6.8	198	0	0.1	232	0.1	5.9	248	0	0
$^2H_5$	159	0	6.1	199	0	0.3	233	0	1.8	249	0	0
$^2H_6$	160	0	8	200	0	0.5	234	0	0.7	250	0	0.3
$^2H_7$	161	0	7.8	201	0	0.9	235	0	0.6	251	0	2.1
$^2H_8$	162	0	15.1	202	0	1.6	236	0	0	252	0	12.3
$^2H_9$	163	0	11.2	203	0	2.3	237	0	0	253	0	38.9
$^2H_{10}$	164	0	1.1	204	0	3.8	238	0	0	254	0	20.2
$^2H_{11}$	165	0	0.2	205	0	5.8	239	0	0	255	0	10.2
$^2H_{12}$	166	0	0	206	0	9	240	0	0	256	0	5.8
$^2H_{13}$	167	0	0	207	0	20.9	245	0	0	257	0	4.5
$^2H_{14}$	168	0	0	208	0	54.7	246	0	0	258	0	3.5
$^2H_{15}$	169	0	0	0	0	0	247	0	0	259	0	2.3
$^2H_{16}$	170	0	0	0	0	0	248	0	0	0	0	0
$^2H_{17}$	171	0	0	0	0	0	0	0	0	0	0	0
$^2H_{18}$	172	0	0	0	0	0	0	0	0	0	0	0

Table 3-2 Determination of the concentration of the labelled compounds using methyl octanoate as internal standard (1 μg/mL).

Product of synthesis	Concentration in stock solution [μg/mL]	Calibration function[a]	Coefficient of determination (R^2) value
[^{2}H]-cyclo(Leu-Pro)	15.49±0.99	y = 1.6589x - 1.5397	0.997
[^{2}H]-cyclo(Pro-Pro)	12.85±1.53	y = 1.3916x - 1.2927	0.999
[^{2}H]-cyclo(Met-Pro)	25.97±1.35	y = 0.9064x - 2.5456	0.999
[^{2}H]-cyclo(Phe-Pro)	59.10±4.21	y = 1.2211x - 0.9722	0.985

Mean values ± SD (n = 3); [a]The concentration ratio of unlabelled compound and methyl octanoate as internal standard was calculated using the function with y as ion intensity ratio of analyte and internal standard by HRGC/MS total ion current (TIC).

3.4.2 HRGC/MS cyclic dipeptide analysis method performance

A thoroughly validated high resolution HRGC/MS method was developed for the analysis of proline cyclic dipeptides. Cold injection with a PTV injector system offers many advantages compared to hot injection methods by showing greatly reduced discrimination of less volatile compounds (Poy et al., 1981). Figure 3-2 illustrates the chromatographic separation and peak shape achieved for the four target cyclic dipeptides. Peaks 1 and 2 belonging to cyclo(Leu-Pro) and cyclo(Pro-Pro) showed peak overlapping with retention indices of 2301.2 and 2302.4, respectively. However, this co-elution is offset, considering that only different and specific isotopomers of the unlabelled and labelled compounds were selected for quantification (see also Figure 3-1; A and C). Cyclo(Met-Pro) with a retention index (RI) of 2706.8 eluted separately from cyclo(Phe-Pro) which had a retention index of 2823.6. Calibration curves were generated by plotting the ratios of the area intensities of the labelled and unlabelled standards against the concentration ratios (Figure 3-3). The plots gave straight lines showing linearity in the observed range of area ratios. Coefficients of determination (R^2) values above 0.998 were obtained for standards spiked into ethyl acetate. The sensitivity of the method was expressed using the limit of detection (LOD) and the limit of quantification (LOQ). The LOD of the proposed method was determined by successive analyses of chromatographic extracts of standard solution in ethyl acetate with decreasing amounts of the analytes until a signal-to-noise ratio 3:1 was reached. The LOQ was determined considering a signal-to-noise ratio of 10:1.

The LOD achieved for the method ranged from 0.06 mg/L for cyclo(Leu-Pro), cyclo(Pro-Pro) as well as cyclo(Phe-Pro) and 0.10 mg/L for cyclo(Met-Pro) and the LOQ ranged from 0.18 to 0.3 mg/L, respectively, standard solution in ethyl acetate.

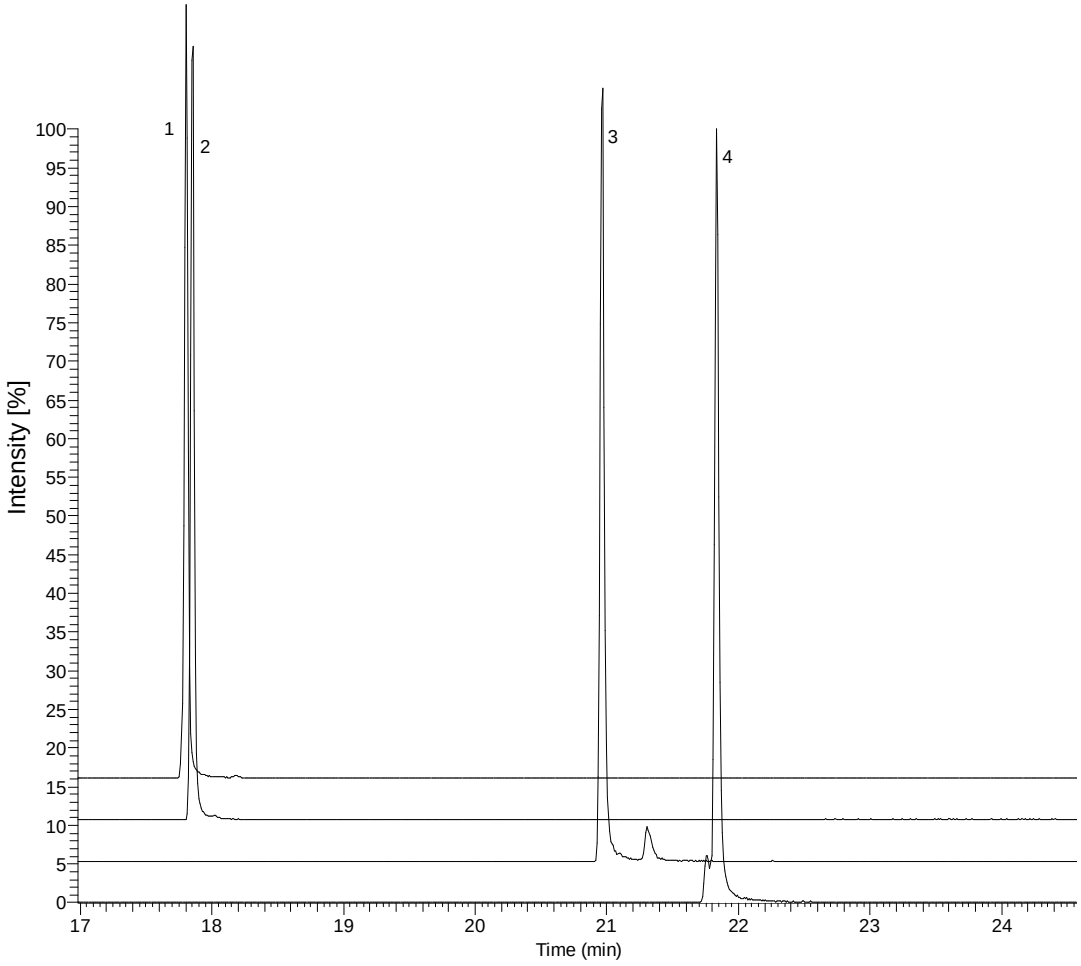


Figure 3-2 Chromatogram obtained from the cyclic dipeptide standards: 1, cyclo(Leu-Pro) at 17.80 min (RI 2301.2); 2, cyclo(Pro-Pro) at 17.86 min (RI 2302.4); 3, cyclo(Met-Pro) at 20.97 min (RI 2706.8); 4, and cyclo(Phe-Pro) at 21.83 min (RI 2823.6).

Based on the results of the isotopomeric distribution (Table 3-1), the target isotopomers of unlabelled compound and labelled standard were selected (Table 3-3) and the area intensities of the extracted ion chromatograms were determined.

Table 3-3 Selected m/z of unlabelled compounds and labelled standards and calibration functions used for the quantification of cyclic dipeptides.

Compound	Selected m/z of unlabelled compound	Internal standard[a]	Selected m/z of labelled standard	Calibration function[b]
cyclo-(Leu-Pro)	154	$^{2}H_{2-3}$	156-157	y = 0.43947x - 0.00260
cyclo-(Pro-Pro)	194	$^{2}H_{13-14}$	207-208	y = 0.69610x + 0.01919
cyclo-(Met-Pro)	228	$^{2}H_{2-3}$	230-231	y = 0.76931x - 0.03728
cyclo-(Phe-Pro)	244	$^{2}H_{8-11}$	252-255	y = 1.17399x + 0.02406

[a]Isotopic labelling of the internal standard. [b]The mass ratio of unlabelled compound and labelled standard was calculated using the function with x as ion intensity ratio of analyte and internal standard.

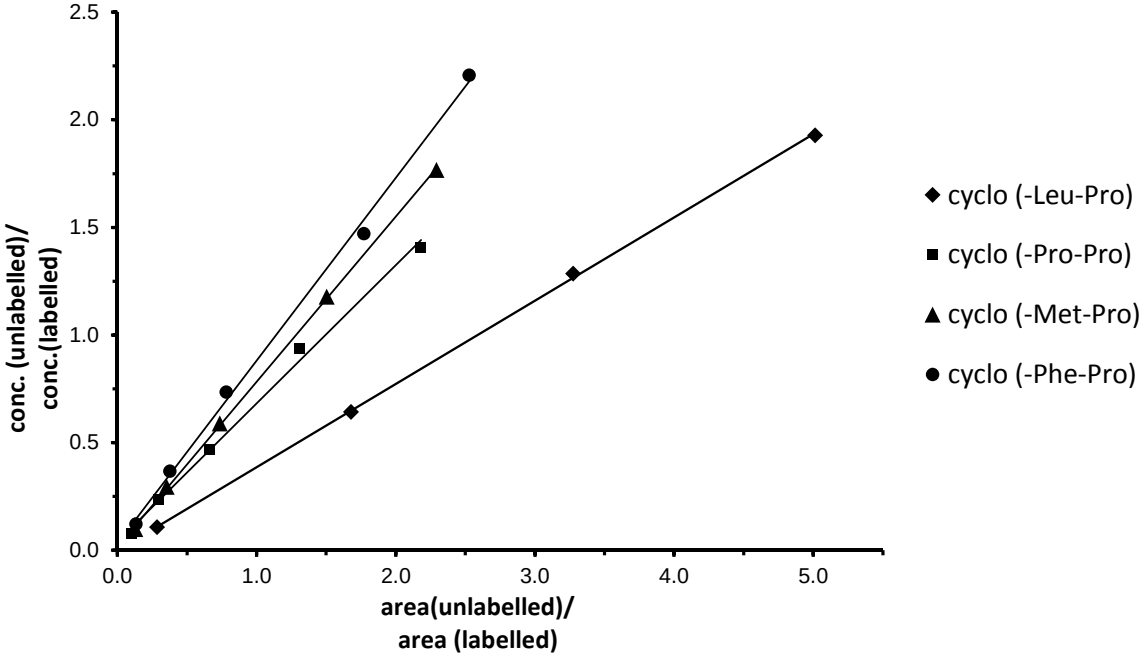


Figure 3-3 Calibration curves obtained by mass chromatography of defined labelled and unlabelled cyclic dipeptides; peak areas of the selected isotopomers of cyclic dipeptides are always related to the peak area of the isotopic labelled internal standard; R^2 values for each curve were above 0.998 (cf. Table 3-3).

3.4.3 Quantification of proline-based cyclic dipeptides in *Lb. brevis* R2Δ fermented MRS-broth and wort

Due to the biopreservation role attributed to LAB, it is useful to evaluate their production of antifungal metabolites, such as cyclic dipeptides. Previous research has primarily focussed on isolation of these compounds; however, their quantification will provide further information regarding their antifungal performance in a complex system.

The HRGC/MS method described was used to detect and quantify proline-based cyclic dipeptides present in the fermented MRS-broth and wort, which were pre-extracted with ethyl acetate. Recovery tests were conducted by spiking the cyclic dipeptides standards into unfermented MRS-broth followed by the whole extraction procedure. Recovery rates were 87 % for cyclo(Leu-Pro), 94 % for cyclo(Pro-Pro) and 98 % for cyclo(Phe-Pro). Further studies would be needed to carry out the low recovery for cyclo(Met-Pro) obtaining only 54 %. To confirm the presence of the target cyclic dipeptides in the samples, their retention indices and fragmentation patterns were compared with their counterpart standards. Figure 3-4 shows the chromatogram derived from the *Lb. brevis* R2Δ fermented MRS-broth CFS extract. The retention indices of peaks 1-4, resulting from the mass traces of the target cyclic dipeptides, were in accordance with those obtained from the standards (Figure 3-2). Furthermore, the mass spectra of these peaks (Figure 3-5) also matched the fragmentation patterns of the standards (Figure 3-1; A, C, E and G), confirming again the presence of these four cyclic dipeptides analysed in these samples. Due to the cyclo(Leu-Pro) and cyclo(Pro-Pro) co-elution, no separate mass spectrum of these individual compounds could be generated.

MRS-medium contains peptone, yeast and meat extract, which provide a source of amino acids and nitrogen for LAB metabolism and growth, but also likely consisting of cyclic dipeptides formed during manufacturer media extraction or processing through heat treatment. The same is true for wort production. Additional analyses confirmed the hypotheses of thermal generation of the four cyclic dipeptides, because the compounds were identified unequivocally in the unfermented control samples.

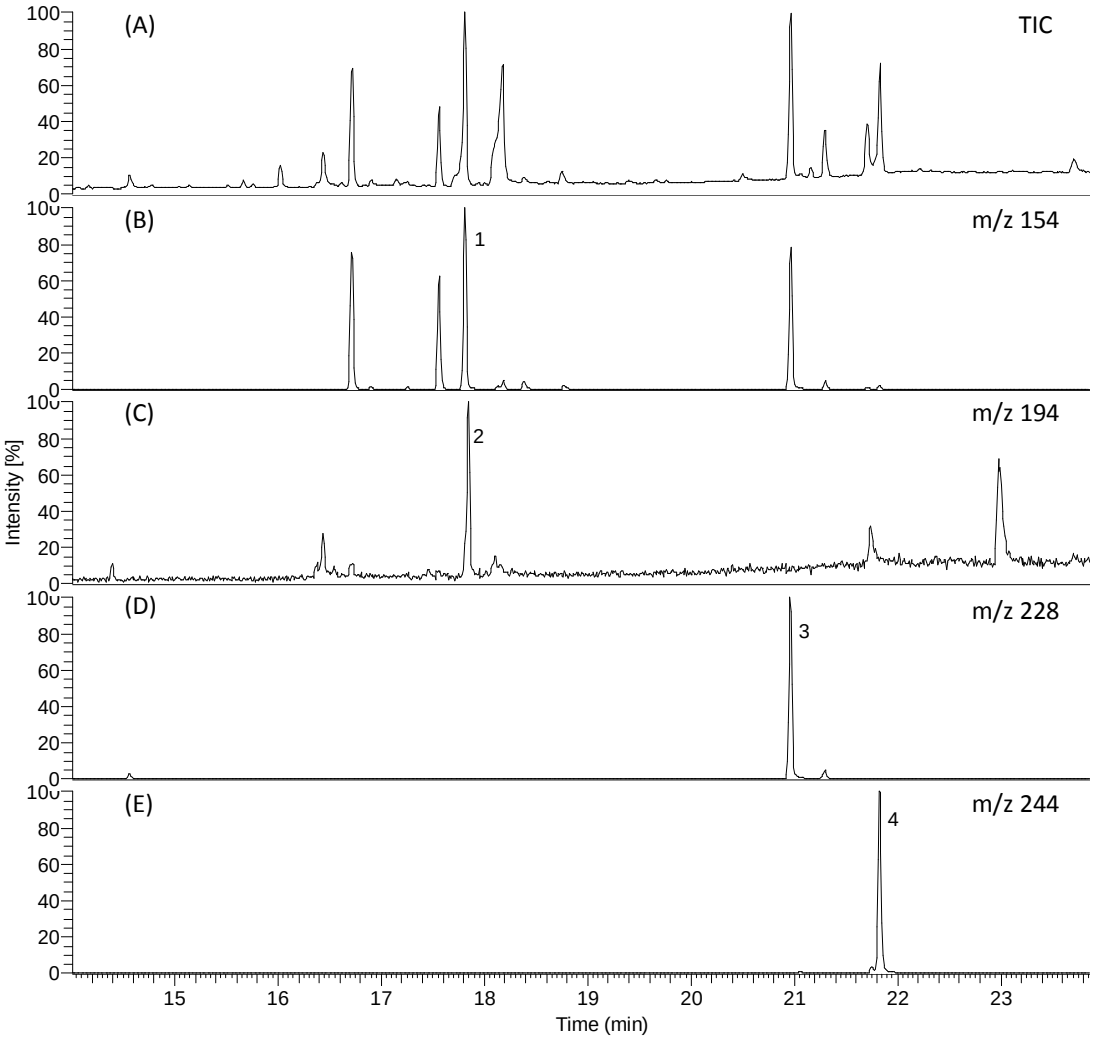


Figure 3-4 Amplified chromatogram of the *Lb. brevis* R2Δ MRS-broth CFS extract: Total ion chromatogram (A), chromatogram of m/z 154 with peak 1 for cyclo(Leu-Pro) (B), chromatogram of m/z 194 with peak 2 for cyclo(Pro-Pro) (C), chromatogram of m/z 228 with peak 3 for cyclo(Met-Pro) (D) and chromatogram of m/z 244 with peak 4 for cyclo(Phe-Pro) (E).

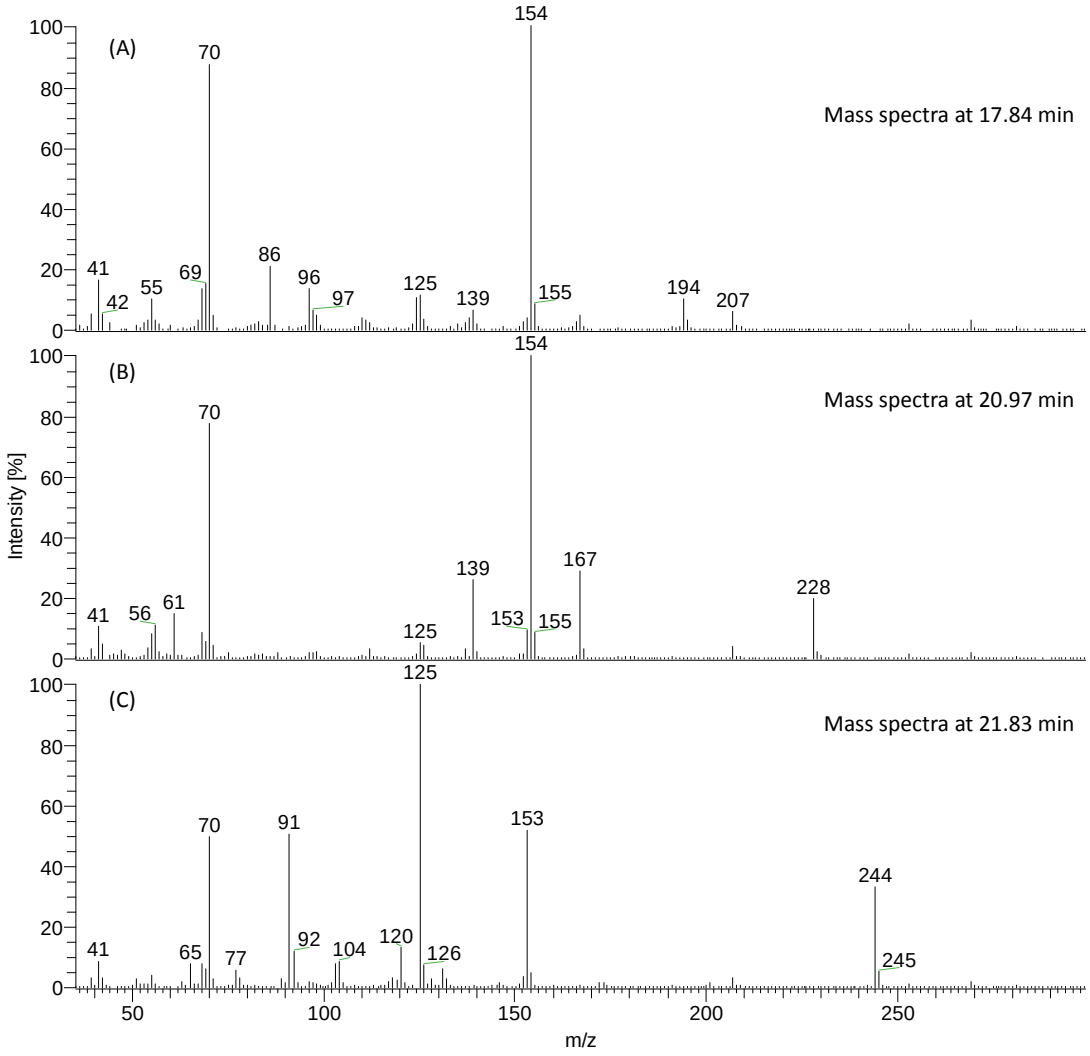


Figure 3-5 Mass spectra obtained from the extract of MRS-broth fermented with *Lb. brevis* R2Δ: mass spectrum at 17.84 min (RI 2302) resulted from peak 1 + 2 (A), mass spectrum at 20.97 min (RI 2706.8) resulted from peak 3 (B) and mass spectrum at 21.83 min (RI 2823.6) resulted from peak 4 (C).

In order to determine the metabolic ability of *Lb. brevis* R2Δ to generate the dipeptides, the components were quantified in cell-free supernatants of fermented MRS-medium and wort as well in the unfermented control samples. Table 3-4 summarizes the quantification results of the four proline-based cyclic dipeptides.

Table 3-4 Concentration of cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Met-Pro) and cyclo(Phe-Pro) in cultures of *Lb. brevis* R2Δ, acid treated broth and in unfermented controls.

Sample	Cyclo (Leu-Pro) [mg/L]	Cyclo (Pro-Pro) [mg/L]	Cyclo (Met-Pro) [mg/L]	Cyclo (Phe-Pro) [mg/L]
Wort - *Lb. brevis* R2Δ	0.60 ± 0.01^{A}	< 0.06*	< 0.1*	0.64 ± 0.03^{A}
Wort – unfermented	0.55 ± 0.01^{B}	< 0.06*	< 0.1*	0.64 ± 0.03^{A}
MRS-broth - *Lb. brevis* R2Δ	8.13 ± 0.07^{a}	0.63 ± 0.04^{a}	14.20 ± 0.28^{a}	11.85 ± 0.24^{a}
MRS-broth – acidified	7.77 ± 0.11^{b}	0.61 ± 0.03^{a}	13.60 ± 0.09^{b}	11.21 ± 0.50^{a}
MRS-broth - unfermented	6.81 ± 0.13^{c}	0.52 ± 0.03^{b}	14.28 ± 0.14^{a}	9.81 ± 0.16^{b}

Mean values ± SD (n = 3); analysis performed by one-way ANOVA and pairwise comparisons using Fisher's LSD procedure; mean values within columns labelled with a common upper case letter from the wort samples are not significantly different ($P < 0.05$); mean values within columns labelled with a common lower case letter from the broth samples are not significantly different ($P < 0.05$);* concentration lower than limit of detection.

In fermented wort, only cyclo(Leu-Pro) and cyclo(Phe-Pro) were quantified above their LODs in low concentration levels of 0.60 and 0.64 mg/L, respectively. In the wort control, the same concentration of cyclo(Phe-Pro) was determined. Although cyclo(Leu-Pro) was present in the control at 0.55 mg/L, statistical evaluation demonstrated a significant difference ($P < 0.05$). Furthermore, statistically significant differences in concentration levels could be observed between fermented and unfermented MRS-broth samples. The concentration of cyclo(Leu-Pro) was 8.1 mg/L in the fermented broth, 1.3 mg/L higher than in the unfermented control ($P < 0.01$). The concentration of cyclo(Phe-Pro) increased from 9.8 to 11.8 mg/L ($P < 0.01$) and from 0.52 to 0.63 mg/L ($P < 0.05$) for cyclo(Pro-Pro) when fermented with *Lb. brevis* R2Δ. The highest concentration measured was for cyclo(Met-Pro) which was determined at a level of 14.2 mg/L in fermented and control MRS-broth. To

summarize, concentrations of cyclo (Leu-Pro), cyclo(Pro-Pro) and cyclo(Phe-Pro) were significantly higher in fermented MRS-broth ($P < 0.05$). However, a significant generation in wort was only found for cyclo(Leu-Pro). In general, wort had a lower content of cyclic dipeptides.

The results indicate that there is a connection between the formation of these three cyclic dipeptides and *Lb. brevis* R2Δ fermentation, although the determined increases induced by *Lb. brevis* R2Δ amounted only to about 20 % in MRS broth and 10 % in wort. The protein content in wort is about 0.5 % and therefore lower than in MRS-broth, which contains about 2 % protein. Consequently, MRS-broth provided a better substrate for production of cyclic dipeptides than wort. As such, the authors speculate that LAB fermentation of protein-rich foods could be better substrates to enhance the production of anti-fungal cyclic dipeptides, for example cheeses and fermented meats.

Cyclic dipeptides have been already isolated from cultures of different antifungal LAB (Dal Bello et al., 2007; Li et al., 2011; Magnusson et al., 2003; Niku-Paavola et al., 1999; Ryan et al., 2011; Strom et al., 2002; Yang and Chang, 2010). Strom et al. (2002) assumed, that their production might be a secondary effect related to quorum sensing or other unknown mechanisms. Their production in relation to *Lb. brevis* growth has not previously been described in the literature. The protein metabolism of LAB and their corresponding enzymes are well studied (Gänzle et al., 2007; Kunji et al., 1996; Liu et al., 2010). However, there is no report of non-ribosomal peptide synthases or cyclo-peptide synthases activities in LAB. Wang et al. (2010) determined cyclo(L-Phe–L-Pro) in the supernatants of *Burkholderia cepacia* CF-66 cultures at 3.5 mg/L. Additionally, this cyclic dipeptide was detected at levels of 15.7 mg/L in *Propionibacterium jensenii* in fermented modified SL broth (contains yeast extract) but 14.7 mg/L was measured in the unfermented control (Lind et al., 2007). Thus, the authors concluded that cyclo(L-Phe-L-Pro) was not produced by propionibacteria. Our results showed that the unfermented MRS media, and wort to a lesser degree, includes peptides partially composed of cyclic dipeptides. In the present study the concentrations of cyclo(Phe-Pro), cyclo(Leu-Pro) and cyclo(Pro-Pro) increased significantly by about 20 % upon fermentation using MRS-broth. Ryan et al. (2009)

examined the levels of cyclo(L-Leu-L-Pro) and cyclo(L-Phe-L-Pro) in wheat sourdough and bread fermented with *Lb. plantarum* FST1.7. The levels of these cyclic dipeptides increased significantly from approximately 0.001 in the control to 0.035 (cyclo(L-Leu-L-Pro)) and 0.025 mg/kg (cyclo(L-Phe-L-Pro)), respectively for both biologically and chemically acidified dough samples, after 48 h of incubation. In our study, acid-treated (lactic: acetic acids, 4:1) MRS-media also increased the cyclic dipeptide concentration (Table 3-4). The acids were spiked into the broth prior incubation (37 °C) and the concentration of cyclic dipeptides was measured after 48 h. Wheat flour contains proteolytic enzymes which show optimal activity around pH 4 (Bleukx et al., 1997; Kawamura and Yonezawa, 1982). Thus the source of proteolytic enzymes to provide linear dipeptides as precursors in sourdough fermentation seems to be regardless, whether they originate from the flour itself or the metabolic activity of the starter culture. The same applies for sourdough fermentation to the pH reduction for acidic pH cyclization of linear dipeptides, whether it is biologically or chemically. This might explain, why Ryan et al. (2009) could not find any differences between biologically and chemically acidified sourdough. In our study, the concentration of cyclo(Leu-Pro), cyclo(Pro-Pro) and cyclo(Phe-Pro) tended to be higher in MRS-broth fermented with *Lb. brevis* R2Δ and the amount found for cyclo(Leu-Pro) was even significantly higher for biologically acidified broth compared to chemically acidified and unfermented control (Table 3-4). MRS-broth as a growth media for LAB does not provide enzyme activity. Conclusively, acidification of MRS-broth alone without proteolytic enzymes does not provide as many precursor as with LAB for the subsequent cyclization, thus supporting the hypothesis that *Lb. brevis* R2Δ metabolism influences their formation in MRS-broth, but as a side effect. MIC values for the single cyclic dipeptides against different fungi reported in the literature are ranged between 6-50 g/L (Ryan et al., 2011; Strom et al., 2002; Yan et al., 2004). These concentrations are above the threshold for bitter taste (Gautschi et al., 1997), conflicting their application as antifungal agents in food controversially. Only one study reports lower MIC between 2 and 128 mg/L (Kumar et al., 2013). Compared with the other reports demonstrating 1000 fold higher MIC's, the latter result is questionable. Nevertheless, concentrations of cyclic dipeptides investigated in

Lb. brevis R2Δ supernatants in this study are much below the MIC necessary to inhibit many of the fungi reported, as an individual antifungal compound. Thus, the antifungal activity of the cyclic dipeptides in *Lb. brevis* R2Δ supernatants plays a minor role. Other produced compounds might be more responsible for its antifungal action. Additionally, the synergistic effect among antifungal compounds produced during LAB fermentation (i.e., organic acids) play a key role in retarding mould growth. Several studies have previously reported the promising antifungal activity of some *Lb. brevis* strains (Falguni et al., 2010; Gerez et al., 2009; Mauch et al., 2010). Mauch et al. (2010) presented the strong antifungal activity of *Lb. brevis* PS1, which was the most active strain against *Fusarium* sp. However, heating and proteolytic treatments of the inoculated supernatants showed reduced activity, thus it was concluded that larger proteinaceous compounds worked synergistically with organic acids as the primary antifungal agents present. A similar finding was also reported for *Lb. brevis* NCDC02 (Falguni et al., 2010). However, synergistic effects can play a crucial role in the inhibition of undesirable microorganisms. Strong activity against anaerobic Gram-negative and Gram-positive bacteria has previously been reported for combined formulations of cyclic dipeptides (Rhee, 2004). Thus, it would be interesting to study cyclic dipeptide synergistic effects in relation to antifungal activity in their naturally occurring levels in food systems. Furthermore, the stereochemistry of cyclic dipeptides also plays an important role in their inhibitory activities. For example, it has been reported that the trans-isomers, cyclo(L-Leu-D-Pro) or cyclo(D-Leu-L-Pro), exhibited lower inhibition than the enantiomers cyclo(L-Leu-L-Pro) or cyclo(D-Leu-D-Pro) (Yan et al., 2004).

3.5 Conclusion

The present study was designed to use an accurate HRGC/MS method for the identification of cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Met-Pro) and cyclo(Phe-Pro) and quantification of the compounds by SIDAs using their deuterated counterparts. Samples analysed were LAB fermented MRS-broth growth media and wort. Thus, the method offers additional applications for analyses of cyclic dipeptides in other liquid matrices, including beverages. To compare the concentration of the target compounds in fermented and control samples, the antifungal strain *Lb. brevis* R2Δ was chosen as an example. The concentrations of cyclo(Leu-Pro), cyclo(Pro-Pro), and cyclo(Phe-Pro) increased moderately by fermentation activity of the microorganism most likely as a secondary side effect resulting from acid-induced cyclization of precursor formed linear dipeptides. However, the concentrations found are 1000 fold lower than the MICs indicating a minor significance of cyclic dipeptides in the antifungal action of *Lb. brevis* R2Δ. Further studies should focus on the analyses of cyclic dipeptides and other antifungal compounds to ascertain their synergistic effects using a range of LAB strains. The novel application of deuterated cyclic dipeptides for SIDA reported here can potentially be used to mass screen LAB fermentates for their cyclic dipeptide production profiles or even to design a custom-built synergistic LAB antifungal profile adjunct for use in a range of food and beverage systems.

3.6 Acknowledgements

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3.7 References

Axel C, Zannini E, Coffey A, Guo JH, Waters DM, Arendt EK (2012) Ecofriendly control of potato late blight causative agent and the potential role of lactic acid bacteria: a review. Applied Microbiology and Biotechnology 96 (1):37-48. Doi: 10.1007/s00253-012-4282-y

Bleukx W, Roels SP, Delcour JA (1997) On the presence and activities of proteolytic enzymes in vital wheat gluten. Journal of Cereal Science 26 (2):183-193. doi:10.1006/jcrs.1997.0123

Borthwick AD (2012) 2,5-Diketopiperazines: synthesis, reactions, medicinal chemistry, and bioactive natural products. Chemical Reviews 112 (7):3641-3716. doi:10.1021/cr200398y

Broberg A, Jacobsson K, Strom K, Schnurer J (2007) Metabolite profiles of lactic acid bacteria in grass silage. Applied and Environmental Microbiology 73 (17):5547-5552. doi:10.1128/aem.02939-06

de Carvalho MP, Abraham WR (2012) Antimicrobial and biofilm inhibiting diketopiperazines. Current Medicinal Chemistry 19 (21):3564-3577

Chen MZ, Da Costa NC, Dewis M, Reiber L, Trinnaman L (2007) AGFD 60-2, 5-Diketopiperazines (cyclic dipeptides) in various foods: identification, synthesis, and sensory evaluation. Abstracts of Papers of the American Chemical Society 234

Chen MZ, Dewis ML, Kraut K, Merritt D, Reiber L, Trinnaman L, Da Costa NC (2009) 2, 5-Diketopiperazines (cyclic dipeptides) in beef: identification, synthesis, and sensory evaluation. Journal of Food Science 74 (2):C100-C105. doi:10.1111/j.1750-3841.2009.01062.x

Chen YH, Liou SE, Chen CC (2004) Two-step mass spectrometric approach for the identification of diketopiperazines in chicken essence. European Food Research and Technology 218 (6):589-597. doi:10.1007/s00217-004-0901-x

Cornacchia C, Cacciatore I, Baldassarre L, Mollica A, Feliciani F, Pinnen F (2012) 2,5-Diketopiperazines as neuroprotective agents. Mini-Reviews in Medicinal Chemistry 12 (1):2-12

Dal Bello F, Clarke CI, Ryan LAM, Ulmer H, Schober TJ, Strom K, Sjogren J, van Sinderen D, Schnurer J, Arendt EK (2007) Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. Journal of Cereal Science 45 (3):309-318. doi:10.1016/j.jcs.2006.09.004

Derdau V, Atzrodt J, Zimmermann J, Kroll C, Bruckner F (2009) Hydrogen-deuterium exchange reactions of aromatic compounds and heterocycles by NaBD4-activated rhodium, platinum and palladium catalysts. Chemistry-a European Journal 15 (40):10397-10404. doi:10.1002/chem.200901107

Falguni P, Shilpa V, Mann B (2010) Production of proteinaceous antifungal substances from *Lactobacillus brevis* NCDC 02. International Journal of Dairy Technology 63 (1):70-76. doi:10.1111/j.1471-0307.2009.00553.x

Gänzle MG, Vermeulen N, Vogel RF (2007) Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiology 24 (2):128-138. doi:10.1016/j.fm.2006.07.006

Gautschi M, Schmid JP, Peppard TL, Ryan TP, Tuorto RM, Yang XG (1997) Chemical characterization of diketopiperazines in beer. Journal of Agricultural and Food Chemistry 45 (8):3183-3189. doi:10.1021/jf9700992

Gerez CL, Torino MI, Rollan G, de Valdez GF (2009) Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. Food Control 20 (2):144-148. doi:10.1016/j.foodcont.2008.03.005

Ginz M, Engelhardt UH (2000) Identification of proline-based diketopiperazines in roasted coffee. Journal of Agricultural and Food Chemistry 48 (8):3528-3532. doi:10.1021/jf991256v

Gondry M, Sauguet L, Belin P, Thai R, Amouroux R, Tellier C, Tuphile K, Jacquet M, Braud S, Courcon M, Masson C, Dubois S, Lautru S, Lecoq A, Hashimoto S-i, Genet R, Pernodet J-L (2009) Cyclodipeptide synthases are a family of tRNA-dependent peptide bond-forming enzymes. Nature Chemical Biology 5 (6):414-420. doi:10.1038/nchembio.175

Grosch W (2001) Evaluation of the key odorants of foods by dilution experiments, aroma models and omission. Chemical Senses 26 (5):533-545. doi:10.1093/chemse/26.5.533

Gruenewald S, Mootz HD, Stehmeier P, Stachelhaus T (2004) *In vivo* production of artificial nonribosomal peptide products in the heterologous host *Escherichia coli*. Applied and Environmental Microbiology 70 (6):3282-3291. doi:10.1128/aem.70.6.3282-3291.2004

Huang RM, Zhou XF, Xu TH, Yang XW, Liu YH (2010) Diketopiperazines from marine organisms. Chemistry & Biodiversity 7 (12):2809-2829. doi:10.1002/cbdv.200900211

Kawamura Y, Yonezawa D (1982) Wheat-flour proteases and their action on gluten proteins in dilute acetic-acid. Agricultural and Biological Chemistry 46 (3):767-773

Kiefl J, Pollner G, Schieberle P (2013) Sensomics analysis of key hazelnut odorants (*Corylus avellana L. 'Tonda Gentile'*) using comprehensive two-dimensional gas chromatography in combination with time-of-flight mass spectrometry (GCxGC-TOF-MS). Journal of Agricultural and Food Chemistry 61 (22):5226-5235. doi:10.1021/jf400807w

Kumar N, Mohandas C, Nambisan B, Kumar DRS, Lankalapalli RS (2013) Isolation of proline-based cyclic dipeptides from *Bacillus sp* N strain associated with rhabitid entomopathogenic nematode and its antimicrobial properties. World Journal of Microbiology and Biotechnology 29 (2):355-364. doi:10.1007/s11274-012-1189-9

Kunji ERS, Mierau I, Hagting A, Poolman B, Konings WN (1996) The proteolytic systems of lactic acid bacteria. Antonie Van Leeuwenhoek, International Journal of General and Molecular Microbiology 70 (2-4):187-221. doi:10.1007/bf00395933

Li J, Wang W, Xu SX, Magarvey NA, McCormick JK (2011) *Lactobacillus reuteri*-produced cyclic dipeptides quench agr-mediated expression of toxic shock syndrome toxin-1 in staphylococci. Proceedings of the National Academy of

Sciences of the United States of America 108 (8):3360-3365. doi:10.1073/pnas.1017431108

Lind H, Sjogren J, Gohil S, Kenne L, Schnurer J, Broberg A (2007) Antifungal compounds from cultures of dairy *propionibacteria* type strains. Fems Microbiology Letters 271 (2):310-315. doi:10.1111/j.1574-6968.2007.00730.x

Liu MJ, Bayjanov JR, Renckens B, Nauta A, Siezen RJ (2010) The proteolytic system of lactic acid bacteria revisited: a genomic comparison. Bmc Genomics 11. doi:10.1186/1471-2164-11-36

Magnusson J, Strom K, Roos S, Sjogren J, Schnurer J (2003) Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. Fems Microbiology Letters 219 (1):129-135. doi:10.1016/s0378-1097(02)01207-7

Martins MB, Carvalho I (2007) Diketopiperazines: biological activity and synthesis. Tetrahedron 63 (40):9923-9932. doi:10.1016/j.tet.2007.04.105

Mauch A, Dal Bello F, Coffey A, Arendt EK (2010) The use of *Lactobacillus brevis* PS1 to *in vitro* inhibit the outgrowth of *Fusarium culmorum* and other common *Fusarium* species found on barley. International Journal of Food Microbioly 141 (1-2):116-121. doi:10.1016/j.ijfoodmicro.2010.05.002

Modutlwa N, Maegawa T, Monguchi Y, Sajiki H (2010) Synthesis of deuterium-labelled drugs by hydrogen-deuterium (H-D) exchange using heterogeneous catalysis. Journal of Labelled Compounds & Radiopharmaceuticals 53 (11-12):686-692. doi:10.1002/jlcr.1848

Ndagano D, Lamoureux T, Dortu C, Vandermoten S, Thonart P (2011) Antifungal activity of 2 lactic acid bacteria of the *Weissella* genus isolated from food. Journal of Food Science 76 (6):M305-M311. doi:10.1111/j.1750-3841.2011.02257.x

Niku-Paavola ML, Laitila A, Mattila-Sandholm T, Haikara A (1999) New types of antimicrobial compounds produced by *Lactobacillus plantarum*. Journal of Applied Microbiology 86 (1):29-35. doi:10.1046/j.1365-2672.1999.00632.x

O'Neill JC, Lin Q, Geske GD, Carlson B, Blackwell HE (2007) Evaluation of 2,5-diketopiperazines as quorum sensing modulators. Abstracts of Papers of the American Chemical Society 233

Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. (2007). The EFSA Journal 587:1-16

Pawlowska AM, Zannini E, Coffey A, Arendt EK (2012) "Green preservatives": combating fungi in the food and feed industry by applying antifungal lactic acid bacteria. Advances in Food and Nutrition Research 66:217-238. doi:10.1016/b978-0-12-394597-6.00005-7

Poy F, Visani S, Terrosi F (1981) Automatic injection in high-resolution gas-chromatography- a programmed temperature vaporizer as a general-purpose injection system. Journal of Chromatography 217 (NOV):81-90. doi:10.1016/s0021-9673(00)88063-2

Prasad C (1995) Bioactive cyclic dipeptides. Peptides 16 (1):151-164. doi:10.1016/0196-9781(94)00017-z

Ressurreicao ASM, Delatouche R, Gennari C, Piarulli U (2011) Bifunctional 2,5-diketopiperazines as rigid three-dimensional scaffolds in receptors and peptidomimetics. European Journal Organic Chemestry (2):217-228. doi:10.1002/ejoc.201001330

Rhee KH (2004) Cyclic dipeptides exhibit synergistic, broad spectrum antimicrobial effects and have anti-mutagenic properties. International Journal of Antimicrobial Agents 24 (5):423-427. doi:10.1016/j.ijantimicag.2004.05.005

Rizzi GP (1989) Heat-induced flavor formation from peptides. Acs Symposium Series 409:172-181

Ryan LAM, Zannini E, Dal Bello F, Pawlowska A, Koehler P, Arendt EK (2011) *Lactobacillus amylovorus* DSM 19280 as a novel food-grade antifungal agent for bakery products. International Journal of Food Microbioly 146 (3):276-283. doi:10.1016/j.ijfoodmicro.2011.02.036

Ryan LAM, Dal Bello F, Arendt EK, Koehler P (2009) Detection and quantitation of 2,5-diketopiperazines in wheat sourdough and bread. Journal of Agricultural and Food Chemistry 57 (20):9563-9568. doi:10.1021/jf902033v

Rychlik M, Schieberle P (1999) Quantification of the mycotoxin patulin by a stable isotope dilution assay. Journal of Agricultural and Food Chemistry 47 (9):3749-3755. doi:10.1021/jf990198a

Rychlik M (2003) Simultaneous analysis of folic acid and pantothenic acid in foods enriched with vitamins by stable isotope dilution assays. Analytica Chimica Acta 495 (1-2):133-141. doi:10.1016/j.aca.2003.08.020

Schultz AW, Oh D-C, Carney JR, Williamson RT, Udwary DW, Jensen PR, Gould SJ, Fenical W, Moore BS (2008) Biosynthesis and structures of cyclomarins and cyclomarazines, prenylated cyclic peptides of marine actinobacterial origin. Journal of the American Chemical Society 130 (13):4507-4516. doi:10.1021/ja711188x

Steinhaus M, Fritsch HT, Schieberle P (2003) Quantitation of (R)- and (S)-linalool in beer using solid phase microextraction (SPME) in combination with a stable isotope dilution assay (SIDA). Journal of Agricultural and Food Chemistry 51 (24):7100-7105. doi:10.1021/jf0347057

Steinhaus M, Schieberle P (2005) Role of the fermentation process in off-odorant formation in white pepper: On-site trial in Thailand. Journal of Agricultural and Food Chemistry 53 (15):6056-6060. doi:10.1021/jf050604s

Strom K, Sjogren J, Broberg A, Schnurer J (2002) *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Applied and Environmental Microbiology 68 (9):4322-4327. doi:10.1128/aem.68.9.4322-4327.2002

Wang J-H, Quan C-S, Qi X-H, Li X, Fan S-D (2010) Determination of diketopiperazines of *Burkholderia cepacia* CF-66 by gas chromatography-mass spectrometry. Analytical and Bioanalytical Chemistry 396 (5):1773-1779. doi:10.1007/s00216-009-3379-3

Yan PS, Song Y, Sakuno E, Nakajima H, Nakagawa H, Yabe K (2004) Cyclo(L-leucyl-L-prolyl) produced by *Achromobacter xylosoxidans* inhibits aflatoxin production by *Aspergillus parasiticus*. Applied and Environmental Microbiology 70 (12):7466-7473. doi:10.1128/aem.7012.7466-7473.2004

Yang EJ, Chang HC (2010) Purification of a new antifungal compound produced by *Lactobacillus plantarum* AF1 isolated from kimchi. International Journal of Food Microbioly 139 (1-2):56-63. doi:10.1016/j.ijfoodmicro.2010.02.012

Chapter 4 Application of *Lactobacillus amylovorus* DSM19280 in gluten-free sourdough bread to improve the microbial shelf life

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4.1 Abstract

The present study investigated the antifungal activity of *Lactobacillus amylovorus* DSM19280 as a starter culture for gluten-free quinoa sourdough bread under pilot-plant conditions to extend the microbial shelf life. Challenge tests against environmental moulds were conducted and a negative control with non-antifungal strain, *Lb. amylovorus* DSM20531^T, as well as a chemically acidified and a non-acidified control were included. Organic acid production, antifungal metabolites, carbohydrates changes during fermentation and bread quality were compared to wheat counterparts. The application of quinoa sourdough fermented with the antifungal *Lb. amylovorus* DSM19280 extended the mould free shelf life by 4 days compared to the non-acidified control. No significant difference in lactic acid production was found between the lactobacilli strains. HPLC-UV/DAD was used to quantify antifungal compounds. The concentration of 4-hydroxyphenyllactic acid, phloretic acid, 3-phenyllactic acid and hydroferulic acid were significantly higher ($P<0.01$) in the quinoa sourdough fermented with the antifungal *Lb. amylovorus* DSM19280 when compared to the non-antifungal strain, thus indicating their contribution to the antifungal activity. Evaluation of bread characteristics such as specific volume or crumb hardness, revealed that the addition of *Lb. amylovorus* fermented sourdough also improved gluten-free bread quality. In conclusion, the combination of quinoa flour fermented with the antifungal *Lb. amylovorus* DSM19280 serves a great potential biopreservative ingredient to produce gluten-free breads with an improved nutritional value, better bread quality and higher safety due to an extended shelf life, and therefore meeting consumer needs for good quality and preservatives-free food products.

4.2 Introduction

Interest in the application of alternative gluten-free crops for the production of cereal-based foods is growing constantly, mainly due to the rising diagnosis of coeliac disease. As a result, the trend for ethnic and ancient grains has increasingly attracted bakery industries as well as consumers worldwide. Due to its high nutritional value, the pseudo-cereal quinoa as such holds exceptional promise for utilization in food and beverages (Arendt and Zannini, 2013). It is rich in proteins and essential amino acids. The lipid fraction contains high amounts of polyunsaturated fatty acids and in comparison to other grains, quinoa is found to be rich in the vitamins A, E and folate. During the last years, novel products appeared on the market labelled with proposed healthier and more natural features compared to their classic wheat counterparts. However, gluten-free bread production results in major challenges for bakers and cereal technologists. Complex recipe formulations, using different starches, proteins, gums and hydrocolloids, are necessary to mimic the structure-building and water binding properties of gluten (Zannini et al., 2012). Although progress in gluten-free bread formulations have been achieved so far, the inclusion of the above mentioned ingredients/additives presents several disadvantages such as excessive prices (Moroni et al., 2009), other allergic reactions (Ortolani and Pastorello, 2006) and predominantly, the use of additives does not match the actual consumers' requirements for natural products (Zannini et al., 2012). Furthermore, consumers' concerns about safety and additive contents in food has received much attention over the last years. In turn, such minimal processed food without chemical preservatives should be still of high-quality and have an extended shelf life (Pawlowska et al., 2012). The shelf life of bread is determined by both staling behaviour and microbial deterioration. The latter one is a serious and expensive problem implicated with an estimated loss of the world's bread production of 5 to 10 % (Pitt and Hocking, 2009). Apart from the unpleasant sight of visible mould growth, fungi are also responsible for off-flavour formation, the production of mycotoxins as well as other allergenic compounds, which might be produced even before fungal outgrowth is visible. Furthermore, due to higher water contents and also higher water availability, gluten-free breads are more susceptible for fungal spoilage (Hager et al., 2012a).

The "free-from" trend leads the bakery industry to reconsider traditional preservation methods and replace chemical preservatives with natural alternatives to guarantee a clean label. One of the most established food biopreservation method is fermentation, a process based on the growth of microorganisms in foods. Among bakery products the microorganisms most widely used are lactic acid bacteria (LAB) applied as starter cultures for sourdough. A further benefit from sourdough LAB, in particular those belonging to the genus *Lactobacillus*, is that many species have been referred to the European Food Safety Authority (EFSA) for safety assessment without raising safety concerns. As a result, they have been included in the QPS (Qualified Presumption of Safety) list for authorised use in the food and feed chain within the European Union (EFSA, 2012). This promotes many lactobacilli starter cultures to be categorised as biological agents excluded for labelling by the EU regulation on food additives (EEC, 2008). The same applies to the U.S., where they enjoy the generally regarded as safe (GRAS) status regulated by the U.S. Food and Drug Administration.

Formerly, it was believed that the organic acids produced by LAB, particularly lactic and acetic acid, were the main agents responsible for antifungal activity due to the pH decrease. Besides organic acids, a range of other secondary metabolites produced by LAB has been identified additionally as the source of the antifungal activity. A comprehensive overview about individual substances, their possible antifungal mechanism and some applications of antifungal acting LAB was recently reviewed by Crowley et al. (2013). Antifungal compounds also include fatty acids, acids with phenyl groups, proteinaceous compounds and a variety of other low-molecular weight compounds. In recent years, remarkable effort has been directed to research the antifungal activity of LAB in order to reduce fungal spoilage of foods and extend shelf life. However, many of them were studied under laboratory conditions. Various applications in food *in situ* systems exist, but particular research in gluten-free sourdough bread systems is still limited (Moore et al., 2008). In fact, neither the suitability of antifungal strains as starter cultures for the fermentation of gluten-free flour nor the quality of the final breads have been completely evaluated to date.

The aim of the present study was to apply antifungal LAB fermented sourdough as natural preservative in gluten-free bread to inhibit spoilage and consequently improve

safety and quality. Therefore, the antifungal activity of *Lactobacillus amylovorus* DSM19280 was investigated in a simple gluten-free bread system, particularly in quinoa sourdough bread, under pilot-plant conditions. Bread characteristics such as specific volume and textural and visual crumb properties were evaluated and compared to their wheat counterpart. In addition, the flours used were fully characterised for their chemical composition. Sourdough was further analysed for pH value, total titratable acidity (TTA) as well as metabolite profile.

4.3 Materials and Methods

4.3.1 Materials

The flours used in this study were quinoa flour (Irish Independent Health Food Ltd, Ballyvourney, Ireland, moisture 10.7 %) and wheat flour (baker's flour, Odlums, Dublin, Ireland, moisture 13.5 %). Dry yeast was supplied by Puratos, Groot-Bijgaarden, Belgium; sugar from Siucra, Dublin, Ireland and salt from Glacia British Salt Limited, Cheshire, UK.

Chemicals and analytical standards were mainly purchased from Sigma Aldrich (Dublin, Ireland). The antifungal compound 3-phenyllactic acid was acquired from BaChem (Weil am Rhein, Germany). All analytical standards had a purity of ≥ 95 %.

4.3.2 Cultures, media and growth conditions

The patented antifungal strain *Lb. amylovorus* DSM19280 (Arendt et al., 2009) was obtained from the culture collection of UCC (School of Food and Nutritional Science, University College Cork, Cork, Ireland) and *Lb. amylovorus* DSM20531^T, provided from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was preliminarily evaluated for its *in vitro* antifungal activity (data not shown) and then chosen as a negative control.

The lactobacilli cultures stored in 35 % glycerol stock solution at -80 °C were routinely refreshed on de Man-Rogosa-Sharpe agar (MRS) 5 agar (Meroth et al., 2003). For optimal growth condition after the dormant state, sugars (maltose, glucose and fructose) were autoclaved separately to avoid Maillard reaction and a sterile filtered vitamin mixture (cobalamin, folic acid, nicotinic acid amide, pantothenic acid, pyridoxal phosphate, and thiamine, 0.2 g/L each) was added to the agar after sterilisation. For sourdough cell counts commercial MRS agar was used (CM0361, Oxoid, Basingstoke, Hampshire, England). Both MRS5 and MRS agar were dyed with 0.05 g/L bromocresol green (Sigma-Aldrich, Steinheim, Germany). Lactobacilli were grown at 30 °C for 48 h under anaerobic conditions.

4.3.3 Starch hydrolysis test

To test the amylolytic activity of the strains, starch agar was prepared containing 3 g/L meat extract, 10 g/L soluble starch as well as 12 g/L agar. After 48 h incubation time using 10 µL of a 16 h culture, the plate surface was flooded with Gram iodine solution to make starch hydrolysis visible by a colourless zone surrounding colonies.

4.3.4 Flour characterisation

For compositional analysis, crude fat, protein, moisture and ash content of the flours were determined according to the AACC (American Association of Cereal Chemists) methods 30-10, 46-12, 44-15A and 08-01, respectively. Protein content was calculated with a conversion factor of 6.25 for quinoa and 5.83 for wheat. Analysis of total amino acids was carried out according to the EU regulation Nr. 152/09 after hydrolysis with 6 M hydrochloric acid for 23 hours (EEC, 2009). The amino acids were separated by ion exchange chromatography and determined by reaction with ninhydrin using photometric detection at 570 nm (440 nm for proline). Methionine, cysteine as well as tryptophan were not analysed. Total and damaged starch levels as well as amylose/amylopectin ratio were determined using enzyme kits (K-TSTA, K-SDAM, K-AMYL). The endogenous activity of α- and β-amylase were also analysed enzymatically (K-CERA, K-BETA3). All enzyme kits were supplied by Megazyme, Bray, Ireland.

4.3.5 Sourdough Type II preparation and analysis

Sourdough preparation was performed as described by Moore et al. (2008) with some modifications. Single colonies were taken from MRS5 agar plates, pre-inoculated in 10 mL MRS5 broth at 30 °C for 24 h and subcultured (1 %) in 45 mL of MRS5 broth at 30 °C for 16 h. Cells were harvested by centrifugation (5000 rpm, 10 min), washed once and resuspended in 45 mL sterile tap water. The starter cultures were added to the sourdough to an initial inoculum size of 7 log cfu/g dough. Sourdoughs were prepared with an equal weight of flour and sterile tap water, dough yield (DY) of 200. The doughs were fermented at 30 °C for 48 h (stirring thoroughly after 24 h). LAB cell counts were determined at 0 h and 48 h of fermentation. The identity of starter

cultures was confirmed by colony morphology and metabolic patterns (Wolter et al., 2014a). Total titratable acidity (TTA) and pH values of the fermented sourdoughs were measured using standard procedure (Arbeitsgemeinschaft Getreideforschung e.V., Detmold, Germany, 1994).

In addition, the effect the organic acids, lactic and acetic acid, on bread shelf life was evaluated. Therefore, a chemically acidified (1.4 % with a mixture of lactic and acetic acid (4:1, v/v) control sourdough (CA) with a DY of 200 was produced and to exclude acidification by naturally occurring LAB, chloramphenicol and erythromycin (60 and 200 µg/g dough, respectively) were added to the dough, which was then fermented at 30 °C for 24 h (Ryan et al., 2008).

4.3.6 Metabolite analysis in sourdough

An Agilent 1260 high performance liquid chromatography system equipped with a refractive index detector (RID) and an ultra violet-diode array detector (UV/DAD) was used to quantify carbohydrates (0.125-2.5 mM), organic acids (2-32 mM) as well as antifungal compounds (5-50 ppm). Standard calibration curves were prepared with 5 different concentrations and measured in duplicates always at the beginning and end of a sample set. Calibration curves showed good linearity with correlation coefficients of ≥ 0.999 for all compounds. For sugar and acid analyses, freeze-dried sourdough samples and flour were extracted with distilled water, clarified with Carrez I and Carrez II (1:5 w/v) and syringed filtered (0.2 µm pore size filter). Sugars were quantified over the RID (35 °C) by elution of the extract from a Hi-Plex H column (300 x 7.7 mm, 8 µm; Agilent, Cork, Ireland), equipped with a guard column (50 x 7.7 mm, 8 µm; Agilent, Cork, Ireland), using water at a flow rate of 0.6 mL/min at 25 °C. Setting the UV/DAD at 210 nm, lactic acid in the sourdough was determined after elution with 0.004 M sulphuric acid at 65 °C from the same column and a flow of 0.5 mL/min. Injection volumes were 20 µL.

The analysis of 15 known antifungal compounds was carried out according to Brosnan et al. (2014). The sample (2 g) was mixed with 10 mL water and 10 mL ethyl acetate containing 1 % formic acid. The mixture was added to 4 g magnesium sulphate and 1 g of sodium chloride shaken for 1 min and centrifuged for 10 min (3,000 rpm).

The organic solvent supernatant was removed and added to a dispersive solid phase extraction (SPE) Kit (Cat #: 5982- 4956, 15 mL, Agilent, Dublin) and shaken for 1 min. The SPE tube was then centrifuged for 10 min (3,000 rpm). The solvent was decanted into a new 15 mL tube to which dimethyl sulfoxide (100 µL) was added. This was dried under reduced pressure in a vacuum centrifuge (Scanvag, Scanspeed) at 45 °C at 500 rpm, reconstituted to 1 mL with water/acetonitrile (90/10; 900 µL) and syringed filtered (0.2 µm pore size filter), into a HPLC amber vials (1.5 mL capacity). 10 µL was injected onto the HPLC- UV/DAD system. A gradient flow with water and acetonitrile (B) (both containing 0.1 % formic acid) was performed to ensure separation of compounds (0 min – 5 % B; 5 min – 10 % B; 15 min – 15 % B; 35 min – 40 % B; 45 min – 95 %; 50 min – 95 % B) on a Gemini C18 column (150 x 2.0 mm, 5 µm; Phenomenex, Macclesfield, UK) equipped with a C18 guard column (4 x 3 mm, Phenomenex, Macclesfield, UK) at a flow rate of 0.2 mL/min kept at a temperature of 30 °C. Compounds were quantified at a wavelength of 210 nm. For peak identification UV spectra were recorded between 190 and 400 nm. Recovery tests were conducted by spiking the antifungal standards into the CA control samples (15 ppm/g sourdough) followed by the whole extraction procedure assessing the extraction quality.

4.3.7 Bread preparation

The bread-making procedure was carried out according to Hager et al. (2012a). For sourdough breads, 20 % of the flour was fermented. Yeast and sugar were dissolved in the water (35 °C) and activated for 10 min in a proofer (KOMA Sunriser, Roermond, the Netherlands) set to 30 °C at a relative humidity (RH) of 85 %. This suspension was added to the mixed dry ingredients along with the sourdough. Mixing was carried out using the batter attachment of a Kenwood chef classic (1 min at step 1; scrape down; 1.5 min at step 2). The batter was scaled to 400 g into baking tins (15 x 9.5 x 7 cm). After a 30 min proofing time (35 °C; 85 % RH) the breads were baked (45 min at 190 °C; top and bottom heat). The deck oven (MIWE condo, Arnstein, Germany) was previously steamed with 0.3 L of water. The same recipe was used for the wheat control, except water levels were 63 %. Fermentation and baking parameter

were performed according to Ryan et al. (2008). After 2 h cooling down at ambient temperature, bread loaves were analysed subsequently and heat sealed in plastic bags and stored at room temperature for further evaluations. Three loaves per batch were used for shelf life monitoring.

4.3.8 Bread evaluation

Breads were characterised as previously described by Hager et al. (2012a). For determination of bake loss, each loaf was weighed before and after baking procedure. Loaf-specific volume was analysed using a Volscan Profiler (Stable Micro Systems, UK). For the evaluation of the physical crumb texture, four bread slices (25 mm thickness) were taken from the centre of each loaf. Texture profile analysis (TPA) was performed 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. The applied settings were a test speed of 5 mm/s with a force of 0.98 N to compress the middle of the breadcrumb to 50 % of its initial height. Calculated by the TPA software the hardness of three loaves of each batch were analysed on day 0, day 2 and day 5 of storage. Evaluation of crumb grain was carried out by using a C-cell Bread Imaging System (Calibre Control International Ltd., UK). To describe the structure of bread slices the following parameters of digital image analysis were chosen: slice area (total area of a product slice), number of cells (number of discrete cells detected within the slice), area of cells (the total area of cells as a percentage of the total slice area), porosity (ratio pore area/slice area), cell volume, wall thickness and slice brightness (mean grey level of pixels, value 0 – 255). TTA and pH values of the bread crumb were measured in the same way as described for sourdoughs.

4.3.9 Shelf life

The shelf life of the breads was monitored using the mould environmental challenge method developed by Dal Bello et al. (2007). Each loaf was sliced transversely using a wooden slice box to attain equal slices of 20 mm thickness. Slices were put on a sterile metal wrack, exposed to the bakery environment for 5 min on each side and then packed separately into a sterile plastic bag and heat sealed. To ensure comparable

aerobic conditions in each bag two filter pipette tips were inserted. The bags were examined for mould growth during a 14-day storage period at an average temperature of 20 ± 2 °C. Non acidified bread and CA bread, were also challenged and used as controls. Mould growth was evaluated based on the percentage of the total surface area of each slice where fungal outgrowth occurred. Mouldy slices were visibly rated as "<10 % mouldy", "10–24 % mouldy", "25-49 % mouldy" and ">50 % mouldy" (Figure 4-1). No visible moulds was evaluated as "mould free". In total a series of 12 slices per batch was prepared in duplicate (3 loafs, 4 central slices each).

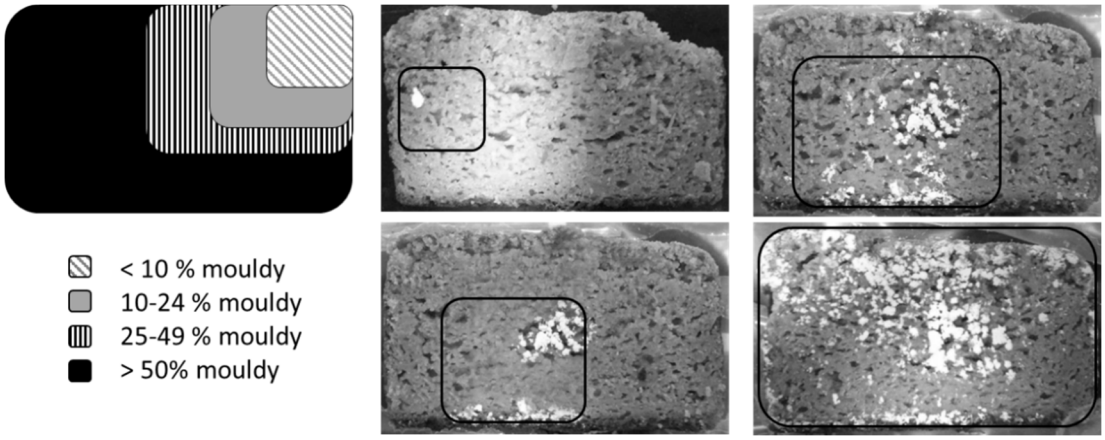


Figure 4-1 Evaluation of fungal outgrowth on quinoa bread slices.

4.3.10 Statistical analysis

Analysis for flour characterisation was done in triplicates. Sourdough extractions were carried out from two independent fermentations, extracting each sample twice. Baking trials were carried out using 3 independent replicates (n = 3). Therefore 3 samples of each batch were analysed with the exception of texture profile analysis where 12 samples of each batch were analysed. Statistical analysis was performed using Minitab 16 software. Data were checked for outliers 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's *post hoc* test to describe the statistical significance between the antifungal, non-antifungal and non-acidified control.

4.4 Results and Discussion

4.4.1 Flour composition and fermentation

Table 4-1 shows the detailed chemical composition of the quinoa flour in comparison to the wheat flour used in this study. The results are comparable to other flour compositions reported (Arendt and Zannini, 2013; Elgeti et al., 2014; Hager et al., 2012b).

Investigations of quinoa sourdough fermentation are still rare and inconsistent values for the carbohydrates levels in quinoa flour have been reported (Elgeti et al., 2014; Wolter et al., 2014a). In our study, sucrose and glucose were quantified at levels of 46 and 53 mmol/kg, respectively (Table 4-2). The two *Lb. amylovorus* strains grew well in the quinoa sourdough system. The initial inoculation level from 7 log cfu/g sourdough increased up to 2 log units after 48 h fermentation (data not shown). The high amount of damaged starch present in the quinoa flour used (Table 4-1) might have contributed to enzyme hydrolysis during sourdough fermentation providing substrates for bacterial metabolism. Additionally, different amylolytic activities in flour and microorganisms may also have contributed to this variation. The quinoa flour used exhibited no beta-amylase activity, whereas high concentration was measured in the wheat flour (Table 4-1).

Table 4-1 Quinoa and wheat flour composition (values based on fresh weight of samples)[a].

Composition	Quinoa flour	Wheat flour
Total starch [%]	51.36±0.20	67.14±0.18
Amylose [%]	15.80±0.42	20.23±0.34
Damaged starch [%]	13.28±0.15	6.48±0.07
Alpha amylase [U/g]	0.21±0.02	0.14±0.02
Beta amylase [U/g]	ND	17.36±1.18
Protein [%]	14.20±0.03	11.5±0.01
Alanine [%]	0.659	0.274
Aspartic acid [%]	1.288	0.470
Arginine [%]	1.253	0.389
Glutamic acid [%]	2.154	3.221
Glycine [%]	0.805	0.357
Hystidine[b] [%]	0.405	0.240
Isoleucine[b] [%]	0.618	0.398
Leucine[b] [%]	1.023	0.675
Lysine[b] [%]	0.856	0.243
Phenylalanine[b] [%]	0.645	0.432
Proline [%]	0.695	1.082
Serine [%]	0.696	0.466
Threonine[b] [%]	0.716	0.286
Tyrosine [%]	0.414	0.278
Valine[b] [%]	0.718	0.494
Water [%]	10.66±0.05	13.45±0.06
Fat [%]	6.04±0.06	1.08±0.02
Ash [%]	2.82±0.00	1.92±0.02

[a] Mean values ± standard deviation (n = 3), [b] essential amino acids; ND means not detected.

During sourdough fermentation beta-amylase degrades starch into maltose units (Gänzle, 2014). Accordingly, high amounts of maltose were measured in wheat sourdough and no maltose was detectable in the quinoa sourdough (Table 4-2).

Noticeable is the high accumulation of glucose in the quinoa sourdough after fermentation. Starch degradation in substrates with low amylase activity depend on extracellular amylases of lactic acid bacteria and amylolytic strains (Gänzle, 2014). Extracellular amylase activity was characterized in several lactobacilli, including *Lb. amylovorus* (Gänzle and Follador, 2012). In an *in vitro* starch digestibility agar plate test the antifungal *Lb. amylovorus* DSM19280 showed excellent amylolytic activity, visible as a colourless zone surrounding the colony (Figure 4-2 a). A negative example is presented in (Figure 4-2 b).

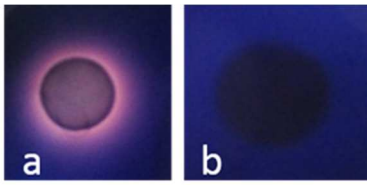


Figure 4-2 *In-vitro* starch digestibility agar plate test, a) positive *Lb. amylovorus* 19280; b) negative *Lb. brevis* R2Δ.

Recently, Ryan et al. (2011) evaluated *Lb. amylovorus* DSM19280 and *Lb. amylovorus* DSM20531^T for the ability to ferment and compete in a wheat sourdough system. However, this study has shown that both lactobacilli seem to be also competitive in a quinoa sourdough system, since uniform colony morphology on agar plates were observed with absence of contamination. As expected, the higher buffering capacity of the quinoa flour due to the higher mineral content resulted in greater lactic acid production with corresponding higher TTA values compared to the wheat sourdough (Table 4-2). Overall, there were no significant differences for the amount of produced lactic acid between the antifungal and non-antifungal lactobacilli.

Table 4-2 Carbohydrate levels, lactate formation, TTA and pH in sourdoughs made from quinoa and wheat flour (DY 200) after 48 h fermentation[a].

Metabolites[mmol/kg flour]	Fructose	Glucose	Sucrose	Maltose	Raffinose	Lactate	TTA [mL]	pH
Quinoa unfermented	ND	52±6	46±6	ND	ND	n.d.	3.4±0.1	6.2±0.0
Quinoa *Lb. amylovorus* DSM19280	47±1	333±15	ND	ND	ND	473±21	35.7±0.9	3.9±0.0
Quinoa *Lb. amylovorus* DSM20531^T	20±1	295±6	ND	ND	ND	503±19	35.5±0.5	3.9±0.0
Wheat unfermented	ND	ND	42±8	ND	5±1	n.d.	2.4±0.0	6.2±0.0
Wheat *Lb. amylovorus* DSM19280	27±1	14±2	ND	176±8	ND	368±11	16.3±1.2	3.8±0.0
Wheat *Lb. amylovorus* DSM20531^T	7±0	16±1	ND	161±10	ND	379±21	14.3±0.0	3.9±0.0

[a] Shown results are average values and standard deviations of two independent fermentations, each sample extracted twice; ND/n.d. means not detected/determined.

4.4.2 Microbial bread shelf life

In recent years, interest in food biopreservation has dramatically increased and too little attention has been given to establish biological methods to improve the microbial shelf life of gluten-free bread. Although Moore et al. (2008) has conducted the most comprehensive study to prolong the shelf life of gluten-free bread by the addition of sourdough to date, it must, however, be critically stated that they achieved only one more mould free day using a *Lb. plantarum* strain in a gluten-free flour mixture. The same strain failed in the study by Wolter et al. (2014a). In the present research, the known antifungal activity of *Lb. amylovorus* DSM19280 was investigated to prevent mould in 100 % quinoa sourdough breads. This strain has already proven to be active against a range of different moulds occurring in food (Belz et al., 2012; Lynch et al., 2014; Ryan et al., 2011; Waters et al., 2012).

Figure 4-3 represents an overview of the shelf life and mould growth characteristics for the biologically acidified breads as well as for the control breads. From a consumer's point of view, first visible mould on bread slices was considered to be a critical point of shelf life evaluation. With the addition of sourdough fermented with the antifungal *Lb. amylovorus* DSM19280 the mould free shelf life was extended for 4 days compared to the non-acidified control where first moulds were visible after 2 days. A prolongation of 2 days was achieved with addition of *Lb. amylovorus* DSM20531^T fermented and CA sourdough. In average bread slices stayed longer mould free in biologically acidified samples. At the end of the storage time, the control bread slices were completely mouldy. In contrast to Ryan et al. (2011), the negative control *Lb. amylovorus* DSM20531^T as well as the CA control did show a low activity against environmental moulds. This delay of mould growth is likely associated with the presence of organic acids (Lynch et al., 2014). The TTA values between these in the sourdough breads were similar (Table 4-3). Overall, the use of the antifungal strain resulted in the longest mould free shelf-life.

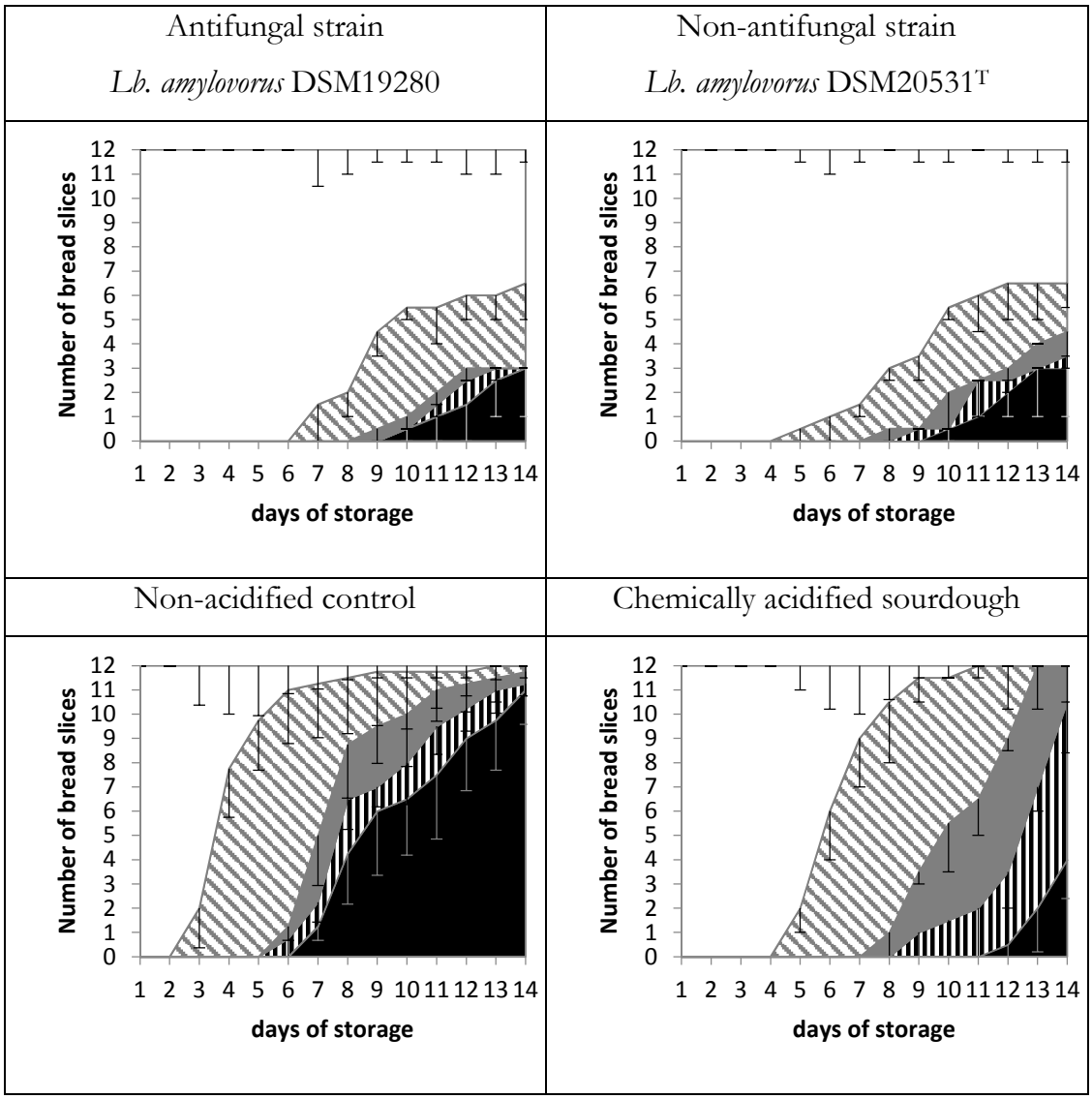


Figure 4-3 Shelf life of quinoa bread against environmental moulds during a 14-day storage period. Bread spoilage is indicated as percentage of the total surface area of each of the 12 slices where fungal outgrowth occurred: Mould free slices (white area), <10 % mouldy (grey diagonally striped area), 10–24 % mouldy (grey area), 25-49 % mouldy (black horizontally striped area) and >50 % mouldy (black area). Mean values are shown (n = 2), error bars indicate standard deviations.

Table 4-3 pH and TTA values of quinoa sourdoughs breads[a].

Sample	pH	TTA [mL]
Quinoa unfermented	5.95±0.01a	6.6±0.5c
Quinoa chemically acidified	5.40±0.01b	9.9±0.1b
Quinoa *Lb. amylovorus* DSM19280	5.01±0.03c	11.2±0.7a
Quinoa *Lb. amylovorus* DSM20531^T	5.03±0.01c	10.8±0.9ab

[a] Mean values ± standard deviation (n = 3). Values in one column followed by the same letter are not significantly different ($P < 0.05$).

Apart from lactic acid, Ryan et al. (2011) identified 15 antifungal compounds and Lynch et al. (2014) quantified six including three further compounds present in the MRS cell free supernatants of *Lb. amylovorus* DSM19280. The minimal inhibition concentration (MIC) vary appreciably between those compounds and fungi tested ranging from 0.1 mg/mL up to 200 mg/mL (Aziz et al., 1998; Broberg et al., 2007; Ryan et al., 2011). However, studies have shown that the amounts of several of those antifungal metabolites detected in culture broths were much lower (ppm range) than their MIC (Axel et al., 2014; Brosnan et al., 2014; Ndagano et al., 2011). Hence, a complex synergistic mechanism seems to be required for antifungal activity. The production of antifungal compounds is substrate specific (Vermeulen et al., 2006). Therefore, it is reasonable to quantify antifungal compounds present in sourdoughs. Previous sourdough research has mainly focused on the evaluation of 3-phenyllactic acid. The concentration of this metabolite ranged in wheat sourdough from 16 to 33 ppm (Ryan et al., 2009; Vermeulen et al., 2006). The production of 3-phenyllactic acid and hydroxy-phenyllactic acid in inoculated quinoa slurries (DY 300), was 46 and 64 ppm, approximately 2.6 times higher than in the wheat samples (Dallagnol et al., 2013). Phenyl and substituted phenyl derivatives occur also naturally in plants (Gómez-Caravaca et al., 2011). In our study, the analysis of 15 antifungal phenyl and substituted phenyl derivates should provide more insight where the preservative effect in the sourdough originates. Figure 4-4 shows the chromatograms of extracted quinoa sourdough samples fermented with the antifungal *Lb. amylovorus* DSM19280 strain in comparison with the CA control and a standard mix. In the LAB fermented

quinoa samples, 4-hydroxyphenyllactic acid, 4-hydroxybenzoic acid, vanillic acid, phloretic acid, 3-phenyllactic acid and hydroferulic acid were clearly identified. 4-Hydroxybenzoic acid and vanillic acid are naturally occurring compounds, since they were also present in the CA quinoa control dough and quinoa flour (data not shown) and reported by literature mentioned earlier. The antifungal compounds phloretic and hydroferulic acid are, to the authors' knowledge, newly identified compounds in quinoa sourdough. With 4-hydroxyphenyllactic acid, 3-phenyllactic acid and hydroferulic acid, only 3 compounds were found in the fermented wheat sourdoughs, whereas none of them was detectable in the CA wheat control and wheat flour (data not shown).

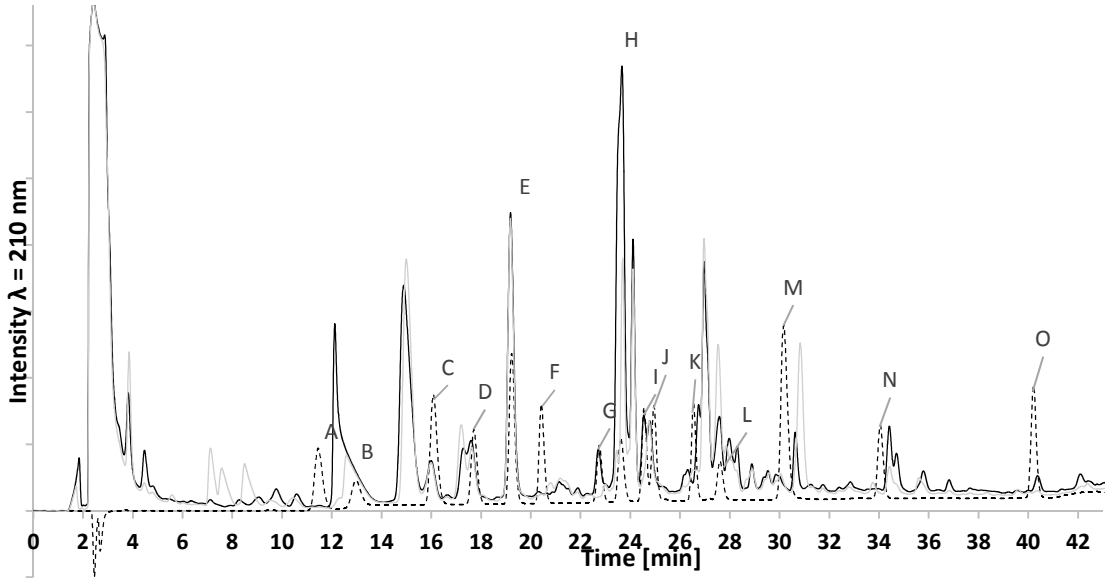


Figure 4-4 Chromatographic profile of a 15 µg/mL standard mix (dashed line); freeze dried quinoa sourdough samples, fermented with *Lb. amylovorus* DSM19280 (black line) and chemical acidified control (grey line) extracted according to Brosnan et al. (2014). Compounds listed in Table 4-4.

The recovery rates varied greatly between 23 and 127 % for quinoa and between 63 and 187 % for wheat (Table 4-4). Relative standard deviation values ranged between 1.0-21.8 % and 1.8-9.9 %, respectively. Due to matrix effects further studies are needed to increase low recoveries, as found for example for benzoic acid obtaining only 23 % in the quinoa matrix. Due to these difficulties encountered, the presents of more compounds in small concentrations overlayed by matrix is likely. Additionally,

mass spectrometry of the sample extracts is needed in order to confirm the identity of the compounds. Nevertheless, the quantification results are presented in Table 4-4 whereas average values were related to their recovery rates. In the quinoa sourdough fermented with the antifungal strain the concentration of the antifungal compounds were significantly higher ($P < 0.01$) when compared to the non-antifungal strain (Table 4-4). The higher concentration of these compounds might have contributed to the longer inhibition of mould in the antifungal quinoa sourdough bread. In contrast, the antifungal wheat sourdough contained less compounds. This confirms, that the production is substrate and strain dependent.

Table 4-4 Concentration of antifungal compounds (ppm)[a].

	Quinoa sourdough fermented with *Lb. amylovorus*			Wheat sourdough fermented with *Lb. amylovorus*		
Compound name	**DSM19280**	**DSM20531^T**	**Recovery [%][c]**	**DSM19280**	**DSM20531^T**	**Recovery [%][c]**
A Catechol	ND	ND	63.5	ND	ND	91.4
B 4-Hydroxyphenyllactic acid	51.1±4.0A	25.6±3.7B	75.7	31.4±3.7b	51.3±10.2a	N/D
C 4-Hydroxybenzoic acid	10.6±0.4[b]	8.7±0.6[b]	66.7	ND	ND	130.1
D Hydrocaffeic acid	ND	ND	122.4	ND	ND	130.8
E Vanillic acid	33.9±1.9[b]	25.5±2.3[b]	88.8	ND	ND	125.4
F Caffeic acid	ND	ND	87.3	ND	ND	186.9
G Phloretic acid	15.1±1.3A	8.9±0.8B	109.6	ND	ND	134.7
H 3-Phenyllactic acid	142.2±7.8A	77.6±7.0B	60.2	35.6±2.4b	40.7±3.4a	155.2
I Hydroferulic acid	12.4±0.8A	8.6±0.8B	102.5	14.7±2.7a	14.5±1.2a	123.7
J *p*-Coumaric acid	ND	ND	99.3	ND	ND	122.0
K Ferulic acid	ND	ND	127.2	ND	ND	142.8
L Benzoic acid	ND	ND	22.5	ND	ND	147.9
M Salicylic acid	ND	ND	47.7	ND	ND	95.7
N Hydrocinnamic acid	ND	ND	120.7	ND	ND	100.6
O Methylcinnamic acid	ND	ND	52.0	ND	ND	63.5
Total concentration	190.6	112.3		107.8	132.4	

[a] Shown results are average values (related to the recoveries) and standard deviations of two independent fermentations, each freeze dried sourdough sample extracted twice. Values in one row followed by the same upper case letter are not significantly different ($p<0.01$). Values in one row followed by the same lower case letter are not significantly different ($P<0.05$). [b] Naturally present. [c] average results from three extractions. ND means concentration not determined/detected.

4.4.3 Bread properties

Sourdough addition to gluten-free bread is reported to better keep freshness over storage (Moore et al., 2007; Wolter et al., 2014b). The same applies for the wheat system (Ryan et al., 2011). As expected, the crumb of the quinoa breads was much firmer than for the wheat breads (Figure 4-5). Comparing the crumb hardness of the sourdough breads (quinoa and wheat) with the non-acidified controls, the obtained results clearly highlight that the addition of sourdough led to a softer crumb for fresh breads (day 0) as well as stored breads (day 2 and day 5). Due to the lack of the gluten network gluten-free breads are generally more prone to stale in comparison to wheat breads (Sciarini et al., 2010). A more rapid onset of staling in gluten-free breads appears with high starch contents (Hager et al., 2012a). The low starch content of quinoa flour seems to have a significant influence in its low rate of staling, which can be even improved by the addition of sourdough.

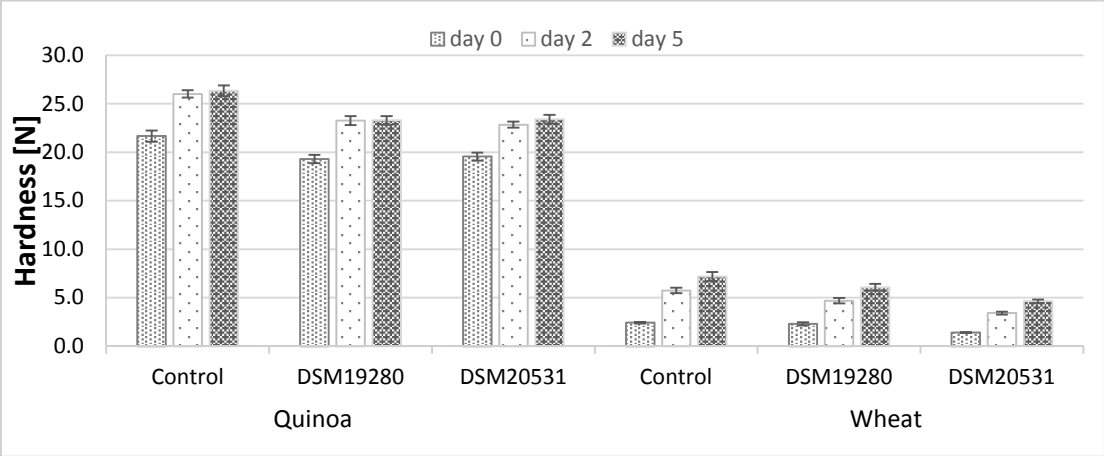


Figure 4-5 Crumb hardness for quinoa and wheat sourdough breads and their controls; mean values ± confidence interval ($\alpha = 0.05$).

To achieve good quality gluten-free breads with high volume and improved texture, industrial bakeries apply complex recipes and additives. However, these interferences by different matrix components were excluded, since a basic bread recipe was used. Generally, the addition of sourdough to gluten-free breads does not have a significant influence on specific volume (Moore et al., 2007; Wolter et al., 2014b). However, the addition of sourdough in our study yielded in a significant higher specific loaf volume

when compared to the non-acidified breads (Table 4-5). Nevertheless, this increase amounted only to about 6 %, making it rather negligible. Amongst other aspects, the influence of sourdough to either decrease or increase the specific volume of bread has been described as strain-dependent (Katina et al., 2006).

Digital image analysis was used for a quantitative description of the crumb grain (Table 4-5). A similar trend was observed for all quinoa breads, with no distinct differences in slice area, number of cells, porosity, cell volume, and slice brightness. Nevertheless, for mechanical strength of the bread grain, the surrounding matrix referred as cell walls are the most significant contributors (Scanlon and Zghal, 2001). The analysis showed that the cell wall for the non-acidified quinoa control was significantly thinner than for both biologically acidified breads. Consequently, the addition of sourdough resulted in quinoa breads with a higher mechanical stability. In disagreement to the study of Wolter et al. (2014b) the application of sourdough did not generally lead to an increase in the cell volumes and accordingly a more open crumb structure and lower values for crumb brightness. These differences could be correlated as also strain dependent.

Table 4-5 Bread and crumb characteristics of sourdough breads and control breads made from quinoa and wheat flour[a].

	Sample	Specific volume [mL/g]	Slice area [mm²]	Number of cells	Porosity (area of cells) [%]	cell volume [mm³]	Wall thickness [mm]	Slice brightness [%]
Wheat	DSM19280	4.01±0.10b	7797±325b	4220±219b	53.4±0.3b	6.2±0.6b	0.45±0.01a	104±2b
	DSM20531^T	4.45±0.06a	8555±177a	4343±178b	54.8±0.3a	6.9±0.3ab	0.46±0.01a	106±2ab
	Control	4.05±0.06b	7976±308b	4810±242a	53.3±0.6b	7.4±0.5a	0.45±0.01a	110±2a
Quinoa	DSM19280	1.12±0.01A	2763±140A	2410±177A	47.61±0.74A	5.0±0.5A	0.41±0.01A	66.8±2.6A
	DSM20531^T	1.11±0.01A	2796±144A	2496±173A	47.39±0.65A	4.7±0.4A	0.40±0.01A	67.7±2.6A
	Control	1.06±0.01B	2654±137A	2473±157A	48.07±0.42A	4.5±0.3A	0.39±0.01B	65.5±2.8A

[a] Values given as mean ± confidence interval (α =0.05). Values in one column followed by the same upper case or lower case letter are not significantly different (P<0.05).

4.5 Conclusion

The mould free shelf life of the quinoa breads increased for 4 days in comparison to the non-acidified control when the sourdough fermented with *Lb. amylovorus* DSM19280 was incorporated in the bread recipe. Quinoa breads containing *Lb. amylovorus* fermented sourdough also showed improved bread quality and reduced bread staling. Consequently, the known antifungal *Lb. amylovorus* DSM19280 is a suitable starter culture for quinoa fermentations. Quinoa flour is rich in essential amino acid and high in proteins making the sourdough a promising biopreservative ingredient for gluten-free baked goods.

HPLC-UV/DAD analysis of the sourdough extracts revealed the presents of antifungal compounds. The concentration of some of those compounds was higher in the antifungal active quinoa sourdough than in the non-antifungal fermentation product. In contrast to the quinoa sourdough, the antifungal wheat sourdough contained less compounds than the non-antifungal. This indicates that the production of these metabolites in the sourdough is strain and substrate specific and may contribute to the synergistic biopreservative effect, but not exclusively. Considering the addition of sourdough at 20 % flour based to the bread recipe and taking bake loss into account, the average concentration of these compounds in the antifungal quinoa bread would only amount about 30 ppm. More compounds might be required concomitantly to achieve the increase of the shelf life in the sourdough breads fermented with antifungal strains. Hence, further analysis and improved extraction procedures of the sourdough samples are vital to research and clarify which other components are present and might act synergistically.

4.6 Acknowledgements

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4.7 References

Arbeitsgemeinschaft Getreideforschung e.V., 1994. Standard-Methoden für Getreide, Mehl und Brot. Verlag Moritz Schäfer Detmold, Ger. vol. 7.

Arendt, E.K., Dal Bello, F., Ryan, L.A.M., 2009. Increasing the shelf life of bakery and patisserie products by using the antifungal *Lactobacillus amylovorus* DSM 19280. Patent: WO/2009/14

Arendt, E.K., Zannini, E., 2013. 12. Quinoa, in: Cereal Grains for the Food and Beverage Industries. Woodhead Publishing Limited Cambridge, UK, pp. 409–438.

Axel, C., Zannini, E., Arendt, E.K., Waters, D.M., Czerny, M., 2014. Quantification of cyclic dipeptides from cultures of *Lactobacillus brevis* R2Δ by HRGC/MS using stable isotope dilution assay. Analytical and Bioanalytical Chemistry. 406, 2433–2444. doi:10.1007/s00216-014-7620-3

Aziz, N.H., Farag, S.E., Mousa, L.A.A., Abo-Zaid, M.A., 1998. Comparative antibacterial and antifungal effects of some phenolic compounds. Microbios 93, 43–54.

Belz, M.C.E., Mairinger, R., Zannini, E., Ryan, L. A. M., Cashman, K.D., Arendt, E.K., 2012. The effect of sourdough and calcium propionate on the microbial shelf-life of salt reduced bread. Applied Microbiology and Biotechnology 96, 493–501. doi:10.1007/s00253-012-4052-x

Broberg, A., Jacobsson, K., Ström, K., Schnürer, J., 2007. Metabolite profiles of lactic acid bacteria in grass silage. Applied and Environmental Microbiology 73, 5547–52. doi:10.1128/AEM.02939-06

Brosnan, B., Coffey, A., Arendt, E.K., Furey, A., 2014. The QuEChERS approach in a novel application for the identification of antifungal Compounds produced by lactic acid bacteria cultures. Talanta. doi:10.1016/j.talanta.2014.05.006

Crowley, S., Mahony, J., van Sinderen, D., 2013. Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. Trends in Food Science and Technology 33, 93–109. doi:10.1016/j.tifs.2013.07.004

Dal Bello, F., Clarke, C.I., Ryan, L.A.M., Ulmer, H., Schober, T.J., Ström, K., Sjögren, J., van Sinderen, D., Schnürer, J., Arendt, E.K., Strom, K., Sjogren, J., Schnurer,

J., 2007. Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. Journal of Cereal Science 45, 309–318. doi:10.1016/j.jcs.2006.09.004

Dallagnol, A.M., Pescuma, M., De Valdez, G.F., Rollán, G., 2013. Fermentation of quinoa and wheat slurries by *Lactobacillus plantarum* CRL 778: proteolytic activity. Applied Microbiology and Biotechnology 97, 3129–40. doi:10.1007/s00253-012-4520-3

EEC, 2008. Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. Official Journal of the European Union L 354, 31.

EEC, 2009. Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. Official Journal of the European Union L 054, 26.

EFSA, 2012. Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update). EFSA Journal 10(12)3020. 1–84. doi:10.2903/j.efsa.2012.3020.

Elgeti, D., Nordlohne, S.D., Foste, M., Besl, M., Linden, M.H., Heinz, V., Jekle, M., Becker, T., 2014. Volume and texture improvement of gluten-free bread using quinoa white flour. Journal of Cereal Science 59, 41–47. doi:10.1016/j.jcs.2013.10.010

Gänzle, M.G., 2014. Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiology 37, 2–10. doi:10.1016/j.fm.2013.04.007

Gänzle, M.G., Follador, R., 2012. Metabolism of oligosaccharides and starch in lactobacilli: a review. Frontiers in Microbiology 3, 340. doi:10.3389/fmicb.2012.00340

Gómez-Caravaca, A.M., Segura-Carretero, A., Fernández-Gutiérrez, A., Caboni, M.F., 2011. Simultaneous determination of phenolic compounds and saponins in quinoa (*Chenopodium quinoa Willd*) by a liquid chromatography-diode array detection-electrospray ionization-time-of-flight mass spectrometry methodology. Journal of Agricultural and Food Chemistry 59, 10815–25. doi:10.1021/jf202224j

Hager, A.S., Wolter, A., Czerny, M., Bez, J., Zannini, E., Arendt, E.K., 2012a. Investigation of product quality, sensory profile and ultrastructure of breads made from a range of commercial gluten-free flours compared to their wheat counterparts. European Food Research and Technology 235, 333–344. doi:10.1007/s00217-012-1763-2

Hager, A.S., Wolter, A., Jacob, F., Zannini, E., Arendt, E.K., 2012b. Nutritional properties and ultra-structure of commercial gluten free flours from different botanical sources compared to wheat flours. Journal of Cereal Science 56, 239–247. doi:10.1016/j.jcs.2012.06.005

Katina, K., Salmenkallio-Marttila, M., Partanen, R., Forssell, P., Autio, K., 2006. Effects of sourdough and enzymes on staling of high-fibre wheat bread. LWT-Food Science and Technology 39, 479–491. doi:10.1016/j.lwt.2005.03.013

Lynch, K.M., Pawlowska, A.M., Brosnan, B., Coffey, A., Zannini, E., Furey, A., McSweeney, P.L.H., Waters, D.M., Arendt, E.K., 2014. Application of *Lactobacillus amylovorus* as an antifungal adjunct to extend the shelf-life of Cheddar cheese. International Dairy Journal 34, 167–173. doi:10.1016/j.idairyj.2013.07.017

Meroth, C.B., Walter, J., Hertel, C., Brandt, J., Hammes, W.P., Brandt, M.J., 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69, 475–482. doi:10.1128/aem.69.1.475-482.2003

Moore, M.M., Dal Bello, F., Arendt, E.K., 2008. Sourdough fermented by *Lactobacillus plantarum* FST 1.7 improves the quality and shelf life of gluten-free bread. European Food Research and Technology 226, 1309–1316. doi:10.1007/s00217-007-0659-z

Moore, M.M., Juga, B., Schober, T.J., Arendt, E.K., 2007. Effect of lactic acid bacteria on properties of gluten-free sourdoughs, batters, and quality and ultrastructure of gluten-free bread. Cereal Chemistry 84, 357–364. doi:10.1094/cchem-84-4-0357

Moroni, A. V, Dal Bello, F., Arendt, E.K., 2009. Sourdough in gluten-free bread-making: an ancient technology to solve a novel issue? Food Microbiology 26, 676–84. doi:10.1016/j.fm.2009.07.001

Ndagano, D., Lamoureux, T., Dortu, C., Vandermoten, S., Thonart, P., 2011. Antifungal activity of 2 lactic acid bacteria of the *Weissella* genus isolated from food. Journal of Food Science 76, M305–311. doi:10.1111/j.1750-3841.2011.02257.x

Ortolani, C., Pastorello, E.A., 2006. Food allergies and food intolerances. Best Practice & Research Clinical Gastroenterology 20, 467–483. doi:10.1016/j.bpg.2005.11.010

Pawlowska, A.M., Zannini, E., Coffey, A., Arendt, E.K., 2012. "Green preservatives": combating fungi in the food and feed industry by applying antifungal lactic acid bacteria. Advances in Food and Nutrition Research 66, 217–238.

Pitt, J.I., Hocking, A.D., 2009. Fungi and Food Spoilage, 3rd ed. Springer.

Ryan, L.A.M., Dal Bello, F., Arendt, E.K., 2008. The use of sourdough fermented by antifungal LAB to reduce the amount of calcium propionate in bread. International Journal of Food Microbiology 125, 274–8. doi:10.1016/j.ijfoodmicro.2008.04.013

Ryan, L.A.M., Dal Bello, F., Czerny, M., Koehler, P., Arendt, E.K., 2009. Quantification of phenyllactic acid in wheat sourdough using high resolution gas chromatography-mass spectrometry. Journal of Agricultural and Food Chemistry 57, 1060–4. doi:10.1021/jf802578e

Ryan, L.A.M., Zannini, E., Dal Bello, F., Pawlowska, A., Koehler, P., Arendt, E.K., 2011. *Lactobacillus amylovorus* DSM 19280 as a novel food-grade antifungal agent for bakery products. International Journal of Food Microbiology 146, 276–283. doi:10.1016/j.ijfoodmicro.2011.02.036

Scanlon, M.G., Zghal, M.C., 2001. Bread properties and crumb structure. Food Research International 34, 841–864. doi:10.1016/s0963-9969(01)00109-0

Sciarini, L.S., Ribotta, P.D., León, A.E., Pérez, G.T., 2010. Influence of gluten-free flours and their mixtures on batter properties and bread quality. Food and Bioprocess Technology 3, 577–585. doi:10.1007/s11947-008-0098-2

Vermeulen, N., Gänzle, M.G., Vogel, R.F., 2006. Influence of peptide supply and cosubstrates on phenylalanine metabolism of *Lactobacillus sanfranciscensis*

DSM20451T and *Lactobacillus plantarum* TMW1.468. Journal of Agricultural and Food Chemistry 54, 3832–3839.

Waters, D.M., Parlet, C., Moroni, A., Arendt, E.K., 2012. Identification of the fungal microflora of coffee beans from different origins and evaluation of different decontamination concepts, in: 24th International Conference on Coffee Science, ASIC Costa Rica 24th. pp. 145–152.

Wolter, A., Hager, A.S., Zannini, E., Czerny, M., Arendt, E.K., 2014a. Impact of sourdough fermented with *Lactobacillus plantarum* FST 1.7 on baking and sensory properties of gluten-free breads. European Food Research and Technology 239, 1–12. doi:10.1007/s00217-014-2184-1

Wolter, A., Hager, A.S., Zannini, E., Czerny, M., Arendt, E.K., 2014b. Influence of dextran-producing *Weissella* cibaria on baking properties and sensory profile of gluten-free and wheat breads. International Journal of Food Microbiology 172, 83–91. doi:10.1016/j.ijfoodmicro.2013.11.015

Zannini, E., Pontonio, E., Waters, D.M., Arendt, E.K., 2012. Applications of microbial fermentations for production of gluten-free products and perspectives. Applied Microbiology and Biotechnology 93, 473–485. doi:10.1007/s00253-011-3707-3

Chapter 5 Antifungal carboxylic acids produced by *Lactobacillus reuteri* R29 during sourdough fermentation: Substrate dependency and *in situ* antifungal activity in bread

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5.1 Abstract

The main objective of this study was to investigate the sourdough fermentation of quinoa, rice and wheat flour using the antifungal strain *Lactobacillus reuteri* R29 as starter culture. In addition to the levels of all major compounds (starch, protein, water, fat, mineral) also amino acid profile and flour enzyme activity were determined. The impact of the fermentation substrate was evaluated in terms of metabolic activity, acidification pattern and quantity of antifungal compounds. Furthermore, the sourdough was applied *in situ* as biopreservative to prolong the shelf life of bread using challenge tests against environmental moulds. *Lb. reuteri* R29 was able to grow highly competitively in all sourdough systems. However, cell growth was most favoured in the wheat system ($P < 0.01$). Lactic acid production and TTA values were lowest in rice sourdough followed by wheat and quinoa. The sourdough fermentation of different flour substrates using the antifungal strain *Lb. reuteri* R29 generated a complex and significantly different profile of antifungal compounds. Extracted quinoa sourdough detected the greatest number of antifungal compounds ($n = 11$) at a much higher concentration than what was detected from rice ($n = 8$) and wheat ($n = 9$) sourdough. Among them, 3-phenyllactic acid and 2-hydroxyisocaproic acid were present at a significant concentration. This was correlated with the superior protein content of quinoa flour and its high protease activity. With the addition of *Lb. reuteri* R29 inoculated sourdough the shelf life was extended by 2 days for quinoa (+ 100 %) and rice bread (+ 67 %). A prolongation of 3 days (+ 75 %) was achieved for the corresponding wheat sourdough bread. The findings of this study showed that *Lb. reuteri* R29 is a suitable bio-protective starter for the successful production of antifungal active sourdough using wheat or gluten-free flours as substrate.

5.2 Introduction

Highly processed food containing artificial preservatives are increasingly rejected by the consumers who increasingly prefer natural and wholesome food products. Nevertheless, microbial safety is required to be preserved within the product's shelf life besides appearance, taste and texture quality aspects. This is a challenge for the food industry, which has led to the revival of food fermentations and biopreservation techniques. Among bakery products the most economically and ecologically friendly method is the use of lactic acid bacteria (LAB) applied as starter culture for sourdough (Zannini et al., 2012). Due to that many species of LAB, (especially from the genus *Lactobacillus*) enjoy GRAS (Generally Regarded As Safe) and QPS (Qualified Presumption of Safety) status, their application is authorised for the safe use in the food and feed chain. The application of LAB fermented sourdough in bread products offers improved flavour, texture and nutritional properties as well as safety due to extended shelf life meeting presently consumer needs (Arendt et al., 2007; Ryan et al., 2011). This also applies for gluten-free bread (Axel et al., 2015a). Due to the rising diagnosis of coeliac disease, an autoimmune disorder of the small intestine associated with a permanent intolerance to gluten and gluten-like proteins, an increasing interest exists in the development of high quality gluten-free products (Moroni et al., 2009).

Bread spoilage is caused predominantly by fungal spores. LAB are known to produce a range of active compounds inhibiting fungal growth. This activity is believed to result from a synergistic effect between pH decrease and the antifungal metabolites produced by LAB (Cortés-Zavaleta et al., 2014; Ndagano et al., 2011). These include low molecular mass compounds such as carboxylic acids; phenyl and substituted phenyl derivates (3-phenyllactic, 4-hydroxyphenyllactic or benzoic acid), cyclic dipeptides, hydroxy fatty acids or antifungal peptides. The production of antifungal compounds depends on the microbial growth and metabolic activity of each strain which are further influenced by endogenous factors such as flour carbohydrates, enzymes and microbial interaction (Axel et al., 2015a; Meroth et al., 2003; Van der Meulen et al., 2007). Accordingly, the evaluation is highly complex. Sourdough containing naturally produced antifungal compounds can replace chemical

preservatives in bread products guaranteeing a clean label which can be claimed with "no preservatives" or "natural".

In the last decades, considerable effort has been directed to screen the antifungal activity of LAB in order to find suitable starter cultures for biopreservation. This content was recently reviewed by a number of studies (Crowley et al., 2013; Dalié et al., 2010; Oliveira et al., 2014) and in terms of bread shelf life (Axel et al., 2015b).

However, the application of antifungal starter strains for sourdough fermentation is still limited and more strains should be investigated *in situ*, research is further needed to improve the shelf life of gluten-free breads (Axel et al., 2015b).

Little attention has been directed to determine the concentration of antifungal compounds formed by LAB during different sourdough fermentations. A few studies report the extraction of antifungal carboxylic acids from the sourdough matrix (Axel et al., 2015a; Dallagnol et al., 2013; Ryan et al., 2009). Disadvantages encountered were either long extraction procedures, usage of large amounts of solvents or high matrix effects and partially low recoveries. With the method developed by Brosnan et al. (2015) a multi-compound (20) UHPLC-MS/MS method was introduced offering shorter analysis time and good recoveries for the determination of *in situ* formed compounds from sourdough.

The main focus of this study addresses the investigation of sourdough fermentation with antifungal potential to prolong the shelf life of bread. Therefore, three different flours (quinoa, rice and wheat) were selected as fermentation substrate and fully characterised. *Lactobacillus reuteri* R29 was chosen as the antifungal starter culture. The impact of the substrate on the quantity of antifungal carboxylic acid produced during LAB sourdough fermentation was evaluated applying the method of Brosnan et al. (2015) and related to LAB metabolism and substrate features. Finally, the biopreservatives were applied in sourdough breads and the shelf life was evaluated using a mould environmental challenge test, which was compared to non-acidified controls.

5.3 Material and Methods

5.3.1 Materials

The flours used in this study were quinoa flour (Irish Independent Health Food Ltd, Ballyvourney, Ireland), white rice flour (Doves Farm Foods Ltd, Berkshire, UK) and wheat flour (baker's flour, Odlums, Dublin, Ireland), dry yeast (Puratos, Groot-Bijgaarden, Belgium), sugar (Siucra, Dublin, Ireland) and salt (Glacia British Salt Limited, Cheshire, UK).

Chemicals and analytical standards were mainly purchased from Sigma Aldrich (Dublin, Ireland). The antifungal compound 3-phenyllactic acid was acquired from BaChem (Weil am Rhein, Germany). All analytical standards had a purity of ≥ 95 %.

5.3.2 Flour characterisation

For compositional analysis, crude fat, protein, moisture and ash content of the flours were determined according to the AACC (American Association of Cereal Chemists) methods 30-10, 46-12, 44-15A and 08-01, respectively. Protein contents were calculated with a protein factor of 6.25, except for wheat flour where 5.83 was used. Analysis of total amino acids was carried out according to the EU regulation Nr. 152/09 after hydrolysis with 6 M hydrochloric acid for 23 hours (EEC, 2009). The amino acids were separated by ion exchange chromatography and determined by reaction with ninhydrin using photometric detection at 570 nm (440 nm for proline). Methionine, cysteine as well as tryptophan were not analysed. Total and damaged starch levels as well as amylose/amylopectin ratio were determined using enzyme kits (K-TSTA, K-SDAM, K-AMYL). The endogenous activity of α- and β-amylase as well as amyloglucosidase were also analysed enzymatically (K-CERA, K-BETA3, R-AMGR3, Megazyme, Bray, Ireland). The activity of endo-proteinase was analysed spectrophotometrically using haemoglobin as substrate (Brijs et al., 1999).

5.3.3 Sourdough Type II preparation

Lb. reuteri R29 was obtained from the culture collection of UCC (School of Food and Nutritional Science, University College Cork, Cork, Ireland). This strain was shown to have antifungal activity *in vitro* and in *in situ* malting trials (Oliveira et al., 2015a,

2015b). The strain was stored in a 35 % glycerol stock at -80 °C and routinely maintained on deMan-Rogosa (MRS) agar (48 h at 37 °C). For the preparation of working cultures, single colonies were picked from the agar plates, cultured in MRS broth (10 mL) at 37 °C for 24 h, and sub-cultured (1 %) for 16 h. Cells were harvested by centrifugation (5000 rpm, 10 min), washed, re-suspended in sterile water and added to the sourdough to an initial cell count of approximately 10^7 cfu/g dough. Sourdoughs were prepared with an equal weight of flour and water; dough yield (DY) of 200. The doughs were fermented at 37 °C for 48 h. LAB cell counts were determined at 0 h and 48 h of fermentation for determining the cell growth. The identity of starter cultures was confirmed by colony morphology and metabolic patterns (Wolter et al., 2014a). Fermentation microbiota were dominated by the starter culture since uniform colony morphology on agar plates were observed with absence of contaminations. Fermentations were carried out in duplicate independent experiments.

5.3.4 Sourdough analysis

Total titratable acidity (TTA) and pH values of the fermented sourdoughs were measured using a standard procedure (Arbeitsgemeinschaft Getreideforschung e.V., 1994). An Agilent 1260 high performance liquid chromatography system equipped a Hi-Plex H column (300 x 7.7 mm, 8 μm; Agilent, Cork, Ireland) and an appropriate guard column (50 x 7.7 mm, 8 μm; Agilent, Cork, Ireland) was used to quantify sugars (0.125-2.5 mM) and organic acids (2-32 mM) from extracted freeze dried sourdough and flour samples. Samples preparation was done according to Wolter et al. (2014b). Concentrations of fructose, glucose, maltose, raffinose and sucrose in flour and sourdough samples were analysed using a refractive index detector. Lactate and acetate acid were detected using a diode array detector (λ = 210 nm). Samples for sugar and acid determination were eluted with water or 0.004 M sulphuric acid at a flow rate of 0.6 mL/min and 25 °C or 65 °C, respectively.

The analysis of 20 known antifungal compounds was carried out using a Waters Quattro premier XE UPLC system (Brosnan et al., 2015). The LC-MS/MS method was operated in negative electrospray ionisation mode as described by Brosnan et al.

(2015) to detect the antifungal compounds. QuEChERS method (quick, easy, cheap, effective, rugged and safe) was applied to extract *in situ* antifungal compounds from the different sourdough matrices (Brosnan et al., 2015).

5.3.5 *In situ* antifungal activity

Lb. reuteri R29 fermented sourdoughs (20 % flour based) were incorporated into simple bread recipes and breads were baked as previously described by (Hager et al. 2012a). Water levels used were 95, 120 and 63 % for quinoa, rice and wheat breads, respectively which were determined through preliminary baking trials for the gluten-free flours and through farinograph measurements for the wheat flour (Hager et al. (2012a). Control breads were prepared accordingly. Bread loaves were cooled at room temperature for 2 h. Three loaves per replicate were sliced transversely in a sterile manner to obtain uniform slices of 20 mm thickness (4 slices per bread). Each side of the slice was exposed to the air for 5 min, packed in a plastic bag and heat sealed. Ensuring comparable aerobic conditions in each bag two filter pipette tips were inserted. The bags were examined for mould growth during a 14-day storage period at an average temperature of 20 ± 2 °C. Mould growth was evaluated based on the percentage of the total surface area of each slice where fungal outgrowth occurred (Axel et al., 2015a).

5.3.6 Statistical analysis

Analysis for flour characterisation was done in triplicates. Sourdough extractions for sugars and acids were carried out from two independent fermentations, extracting each sample twice and for antifungal compounds, each sample was extracted three times. *In situ*-shelf life tests were done in duplicates. 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's *post hoc* test to describe the statistical significance between the antifungal, non-antifungal and non-acidified control.

5.4 Results and Discussion

The main objective of this study was to investigate the sourdough fermentation of quinoa, rice and wheat flour using the antifungal strain *Lb. reuteri* R29 as starter culture.

5.4.1 Flour analysis

Table 5-1 shows the detailed chemical composition of the flours used in this study. The results are comparable to other flour compositions reported (Arendt and Zannini, 2013; Hager et al., 2012b). However, the composition of the fermentation substrate including enzyme activities is important in order to understand how it influences the fermentation pathways, secondary systems for energy production as well as the formation of antifungal compounds. Thus, the following sections focus on the characterisation of the sourdoughs derived from the known metabolic pathways of *Lb. reuteri* and flour components.

Table 5-1 Quinoa, rice and wheat flour composition (values based on fresh weight of samples)[a].

Composition	Quinoa flour[b]	Rice flour	Wheat flour[b]
Total starch [%]	51.36±0.20	75.21±0.28	67.14±0.18
Amylose [%]	15.80±0.42	27.65±0.90	20.23±0.34
Damaged starch [%]	13.28±0.15	0.74±0.02	6.48±0.07
Alpha amylase [U/g]	0.21±0.02	0.01±0.00	0.14±0.02
Beta amylase [U/g]	ND	0.02±0.00	17.36±1.18
Amyloglucosidase [U/g]	3.78±0.05	9.67±0.14	ND
Protease [U/g]	21.97±2.15	10.54±1.79	4.13±0.49
Protein [%]	14.20±0.03	6.37±0.05	11.5±0.01
Alanine [%]	0.659	0.385	0.274
Arginine [%]	1.253	0.557	0.389
Aspartic acid [%]	1.288	0.697	0.470
Glutamic acid [%]	2.154	1.353	3.221
Glycine [%]	0.805	0.360	0.357
Hystidine[c] [%]	0.405	0.178	0.240
Isoleucine[c] [%]	0.618	0.325	0.398
Leucine[c] [%]	1.023	0.615	0.675
Lysine[c] [%]	0.856	0.278	0.243
Phenylalanine[c] [%]	0.645	0.440	0.432
Proline [%]	0.695	0.337	1.082
Serine [%]	0.696	0.432	0.466
Threonine[c] [%]	0.716	0.319	0.286
Tyrosine [%]	0.414	0.230	0.278
Valine[c] [%]	0.718	0.430	0.494
Water [%]	10.66±0.05	13.90±0.06	13.45±0.06
Fat [%]	6.04±0.06	0.71±0.02	1.08±0.02
Ash [%]	2.82±0.00	0.40±0.01	1.92±0.02

[a] Mean values ± standard deviation (n = 3), [b] see also Table 4-1, [c] essential amino acids, ND means not detected.

5.4.2 Characterization of the antifungal LAB sourdough

The obligately heterofermentative *Lb. reuteri* is highly adapted to the sourdough (wheat and rye) environment (Hüfner et al., 2008). However, the antifungal strain used in this study *Lb. reuteri* R29 was isolated from an acidic niche (human source) providing the strain an easy adaptability to acidic conditions (Oliveira et al., 2015a). In fact, *Lb. reuteri* is known for its acid tolerance due to the arginine deiminase pathway which produces intracellular produced ammonia (Gänzle et al., 2007). The antifungal strain *Lb. reuteri* R29 was inoculated into the three different sourdough matrices. Growth ability, acidification pattern and the production of antifungal metabolites were examined.

5.4.2.1 Carbohydrate metabolism

The initial concentration of fermentable carbohydrates in quinoa, rice and wheat flours were relatively low (Table 5-2). Accordingly, starch-degradation during sourdough fermentation is crucial. Depending on the activity of the starch degrading enzymes in the flours, different fermentable sugars are produced. Maltose is a major carbon source in wheat sourdoughs which remains available throughout fermentation (Sekwati-Monang et al., 2012). The wheat flour used in this study exhibited a very high β-amylase activity (Table 5-1). Accordingly, maltose was the only disaccharide detected in wheat sourdoughs, but also an increased glucose level was measured when compared to the unfermented wheat flour (Table 5-2). *Lb. reuteri* metabolizes maltose by choice via maltose phosphorylase and β-phosphoglucomutase, where glucose-6-phosphate is preferentially metabolized and simultaneously glucose is released (Gänzle and Follador, 2012) which explains the higher residual concentration of glucose in the wheat sourdough after fermentation. In contrast, due to the absence or minor activity of β-amylase analysed in the gluten-free flours (Table 5-1), maltose could not be detected in rice and quinoa sourdoughs (Table 5-2). Therefore, glucose was the dominant carbon source in these gluten-free sourdoughs. Amyloglucosidase was found to contribute mainly to the production of fermentable glucose in rice and quinoa flour (Elgeti et al., 2014; Kishio and Aoyagi, 2014). This enzyme catalyses the hydrolysis of terminal 1,4-linked glucose residues successively from the non-reducing

ends of maltooligo- and polysaccharides with release of glucose. In our study, rice flour exhibited the highest activity of amyloglucosidase followed by quinoa flour; whereas no amyloglucosidase activity could be detected in wheat flour (Table 5-1). Heterofermentative metabolism of glucose occurs via the pentose phosphate way (Gänzle et al., 2007). *Lb. reuteri* started to metabolize fructose when maltose was depleted using it as carbon source, since no formation of mannitol occurred (Stolz et al., 1995). Consumption of fructose in our study seemed not to be favoured by the antifungal *Lb. reuteri* R29 in quinoa and wheat sourdoughs. No fructose was detected in the fermented rice sourdough. The analytical profile index (API) test showed that *Lb. reuteri* R29 was able *in vitro* to ferment maltose, glucose and sucrose (data not shown).

Table 5-2 Initial and residual sugars [mmol/kg flour] from flours and sourdoughs (SD) fermented with *Lb. reuteri* R29[a].

Sample		Sugar [mmol/kg flour]				
		Fructose	**Glucose**	**Sucrose**	**Maltose**	**Raffinose**
Quinoa	Flour[b]	8±2	52±6	46±6	ND	ND
	SD	73±10	54±14	ND	ND	ND
Rice	Flour	ND	ND	26±3	ND	ND
	SD	ND	153±2	ND	ND	ND
Wheat	Flour[b]	ND	ND	42±8	ND	5±1
	SD	42±4	145±14	ND	47±9	ND

[a] Shown results are average values and standard deviations of two independent fermentations, each sample extracted twice; [b] see also Table 4-2, ND means not detected.

5.4.2.2 Impact of flour substrate on LAB viability and acidification rate

The pH value is the most used control parameter during sourdough fermentation technology (Capuani et al., 2012). In fact, the drop in pH is an essential step. The acid production causes an increase in the proteases and amylases activity of the flour which induces protein and starch degradation providing more substrate ensuring further LAB growth. Moreover, it leads to shorter dough mixing times, dough softening and

a reduction of staling in the final product (Arendt et al., 2007). *Lb. reuteri* R29 was able to grow highly competitively in all sourdough systems. However, the cell growth (Δlog cfu/g) was highest in wheat flour, followed by rice and quinoa; $P < 0.01$ (Figure 5-1). This favoured cell growth in the wheat sourdough system is likely associated with the high amylase activity in the wheat flour and *Lb. reuteri*'s preference of metabolising maltose. Phosphorolysis of maltose does not expend ATP for generation of glucose-6-phosphate and is energetically more favourable than hydrolysis (Stolz et al., 1996).

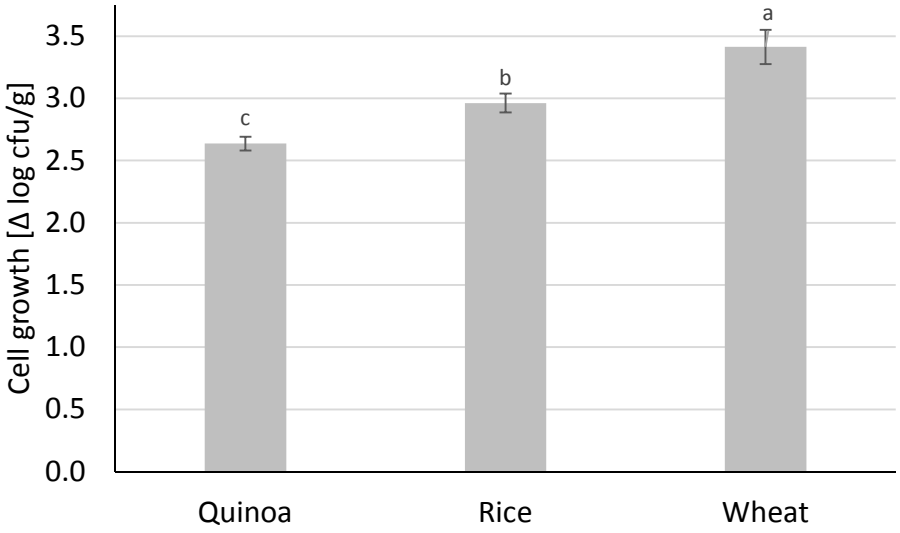


Figure 5-1 Cell growth after sourdough fermentation (48 h) with *Lb. reuteri* R29 using different substrates; mean values ± confidence interval ($\alpha = 0.05$), different lower case letters indicate statistical difference ($P < 0.01$).

The nature of the flour also has an effect on the acidification rate of the sourdough. In particular, a high mineral content increases the buffering capacity of the sourdough system enabling an enhanced lactic acid production without altering the final pH (Gänzle et al., 1998). As a result of the production process for white rice flour, the mineral rich bran layer is removed from the grain. Therefore, white rice flour is generally inferior in its mineral content and does not significantly contribute to mineral nutrition for most essential elements, when compared to other grain counterparts (Arendt and Zannini, 2013). The white rice flour used in this study had the lowest mineral content of only 0.4 % (Table 5-1). Accordingly, lactic acid

production and TTA value were lowest in rice sourdough after fermentation, nevertheless the lowest pH value of 3.6 (Table 5-3). The highest mineral content was measured for quinoa flour (Table 5-1), where fermentation resulted in the greatest production of lactic acid with corresponding highest TTA when compared to rice and wheat (Table 5-3).

Acetate content in sourdoughs depends on the availability of substrates used as electron acceptors by the lactobacilli (Hammes et al., 1996). Potential electron acceptors are citrate, fructose, malate or fumerate. Wheat flour fermentation resulted in the lowest acetate level, followed by rice and quinoa sourdough (Table 5-3).

Table 5-3 Values for pH, total titratable acid (TTA) and organic acids (lactate and acetate) of flours and sourdough (SD) after fermentation using *Lb. reuteri* R29[a].

Sample		pH	TTA [mL]	Lactate [mmol/kg]	Acetate [mmol/kg]
Quinoa	Flour[b]	6.2±0.0	3.4±0.1	n.d.	n.d.
	SD	4.2±0.03	34.66±2.03	505±6	184±9
Rice	Flour	6.46±0.01	1.55±0.1	n.d.	n.d.
	SD	3.56±0.02	14.98±1.02	251±12	64±11
Wheat	Flour[b]	6.20±0.01	2.4±0.0	n.d.	n.d.
	SD	3.81±0.02	18.7±0.2	406±27	49±5

[a] Shown results are average values and standard deviations of two independent fermentations, each sample extracted twice; [b] see also Table 4-2, n.d. means not determined.

5.4.2.3 Influence of flour substrate on the quantity of antifungal compounds

Antifungal carboxylic acids were analysed according to Brosnan et al. (2015). This method is thoroughly validated and recoveries of the compounds in the different sourdough matrices were found to range from 60.3 to 104.3 %.

The results are presented in Table 5-4. The sourdough fermentation of different flour substrates using the antifungal strain *Lb. reuteri* R29 generated a complex and significantly different profile of antifungal compounds. Extracted quinoa sourdough detected the greatest number of antifungal compounds (n = 11) at a much higher

concentration than what was detected from rice (n = 8) and wheat (n = 9) sourdough. Chromatograms of the identified antifungal compounds from the sourdoughs fermented with the antifungal *Lb. reuteri* R29 are depicted in Figure 5-2.

Table 5-4 Antifungal compounds [ppm] analysed in freeze dried quinoa, rice and wheat sourdoughs fermented with *Lb. reuteri* R29[a].

	Substrate/ Antifungal compounds	Quinoa	Rice	Wheat
A	Catechol	ND	ND	ND
B	4-Hydroxyphenyllactic acid	30.7±7.7	73.2±20.0	ND
C	4-Hydroxybenzoic acid	ND	1.4±0.1	ND
D	Hydrocaffeic acid	ND	0.1±0.0	ND
E	2-Hydroxyisocaproic acid	651.9±3.8	648.2±44.8	360.0±0.6
F	Vanillic acid	133.6±2.1	9.4±0.2	6.9±0.2
G	Caffeic acid	1.1±0.1	1.8±0.1	2.0±0.1
H	3-Phenyllactic acid	422.1±11.1	85.4±8.4	194.1±3.5
I	Phloretic acid	ND	ND	1.9±0.0
J	Hydroferulic acid	0.6±0.1	ND	ND
K	Benzoic acid	1.6±0.3	ND	ND
L	*p*-Coumaric acid	10.4±4.2	ND	0.3±0.0
M	Ferulic acid	21.9±2.4	ND	12.9±0.1
N	Salicylic acid	7.0±1.1	3.0±0.4	3.3±0.0
O	Azelaic acid	28.9±4.1	3.7±0.2	7.2±3.9
P	Methylcinnamic acid	ND	ND	ND
Q	Hydrocinnamic acid	ND	ND	ND
R	2-Hydroxydodecanoic acid	ND	ND	ND
S	β-Hydroxylauric acid	ND	ND	ND
T	Hydroxymyristic acid	ND	ND	ND
	Total concentration	1309.8	826.0	588.6

[a] Shown results are average values ± standard deviations of two independent fermentations, each sample extracted three times; ND means not detected.

This confirms previous research, that the production of antifungal compounds is substrate specific due to the different initial composition of the substrates (Axel et al., 2015a; Oliveira et al., 2015a; Vermeulen et al., 2006). Lactobacilli can generate energy through different metabolic pathways. The main antifungal compounds found in the quinoa sourdough, 2-hydroxyisocaproic acid and 3-phenyllactic acid, in

concentrations of 625 and 422 ppm, respectively, originate from the LAB amino acid metabolism. LAB need multiple amino acids meeting their nutritional requirements. As such, protein degradation poses another essential step during sourdough fermentation. However, many lactobacilli do not possess extracellular proteinase activity (Pepe et al., 2003). Thus, flour proteases initiated protein degradation contributes greatly for the peptide supply to be uptaken by LAB transport systems into the cell during sourdough fermentation (Gänzle et al., 2008). Intracellular, phenylalanine is converted by a transamination reaction to phenylpyruvic acid. Phenylpyruvic acid is further reduced to 3-phenyllactic acid by a dehydrogenase. 2-Hydroxyisocaproic acid is a by-product of the leucine pathway. The quinoa flour used had a superior protein content, which was almost double that of rice flour; in contrast, starch content was lowest in quinoa flour (Table 5-1). Quinoa (*Chenopodium quinoa Willd.*) is a so-called pseudo-cereal, which is dicotyledonous, that is different to the monocotyledonous true cereal grains like wheat and rice. As already mentioned before, its high mineral content also allowed the quinoa sourdough a higher buffering capacity enabling the production of higher acids contents (Table 5-1, Table 5-3). Moreover, due to the highest protease activity of the flour (Table 5-1), protein degradation during fermentation was expected to be also higher in quinoa sourdough fermentation providing more peptides to uptake for LAB amino acids metabolism. The need to gain energy out of the higher amino acid metabolism might be associated to compensate the low starch content of the quinoa flour. Although rice flour had a lower protein content (6.4 %) than wheat flour (11.5 %), its protease activity was more than double as high as the wheat counterpart (Table 5-1). This could explain higher concentration of the antifungal compounds 2-hydroxyisocaproic acid and 4-hydroxyphenyllactic acid (Table 5-4).

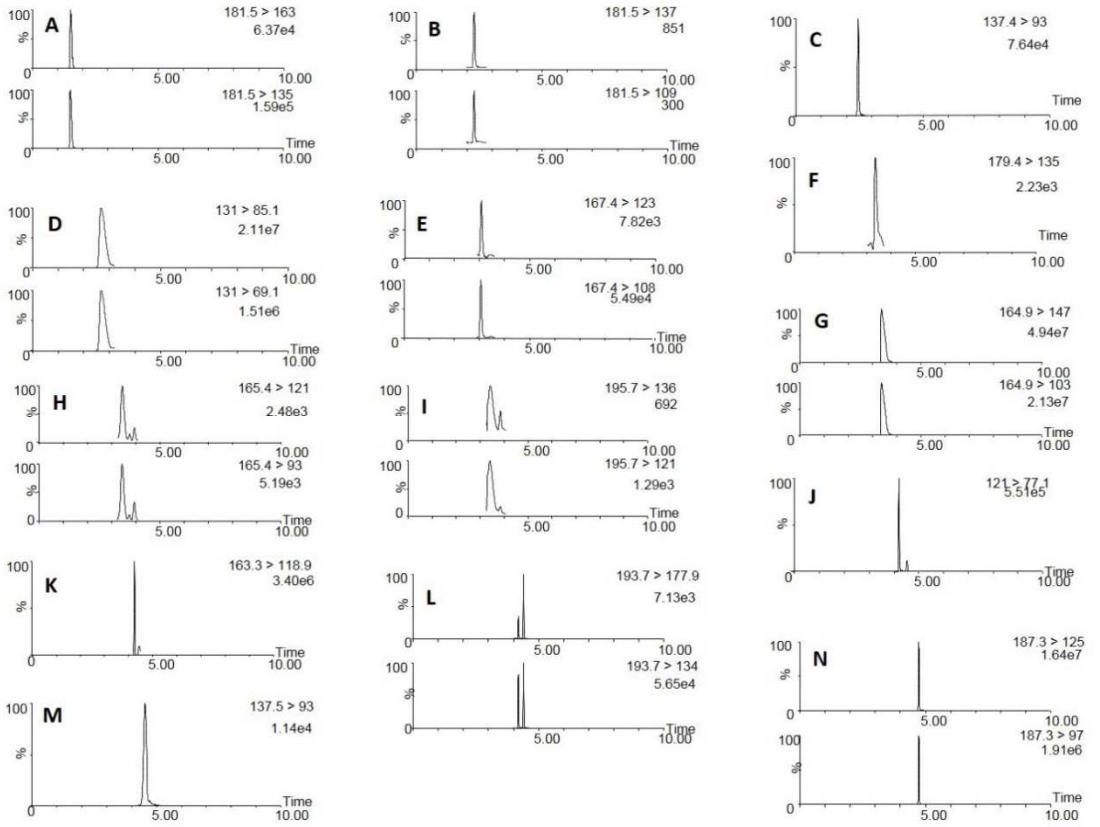


Figure 5-2 Chromatograms with identified compounds in samples of quinoa, rice and wheat sourdough doughs fermented with *Lb. reuteri* R29; A) 4-hydroxyphenyllactic acid, B) hydrocaffeic acid, C) 4-hydroxybenzoic acid, D 2-hydroxyisocaproic acid, E) vanillic acid, F) caffeic acid, G) 3-phenyllactic acid, H) phloretic acid, I) hydroferulic acid, J) benzoic acid, K) *p*-coumaric acid, L) ferulic acid, M) salicylic acid, N) azelaic acid; chromatograms from quinoa sample mainly with B and C from rice and H from wheat sample.

The antifungal activity of 3-phenyllactic acid has been recently investigated and MIC between 2,500 to 10,000 ppm are reported (Broberg et al., 2007; Ryan et al., 2011; Ström et al., 2002). Sakko et al. (2014) demonstrated antifungal efficacy for 2-hydroxyisocaproic acid against *Aspergillus sp.* with MIC values greater than 18,000 ppm. Concentrations found in the sourdoughs were much lower.

Another group of antifungal compounds belong to phenolic acids which include derivatives of either cinnamic acid (*p*-coumaric, caffeic, ferulic acids) or benzoic acid (4-hydroxybenzoic, vanillic acids). They are commonly present in insoluble bound forms, typically components of more complex cross-linking polymers, particularly arabinoxylans (Li et al., 2008; Vogel, 2008) providing antioxidant and antimicrobial

activities (Viswanath et al., 2009). Their location is mainly concentrated in the bran fractions of the grains (Adom et al., 2005). Thus, they are present at lower levels in white flours. Ferulate and *p*-coumarate are also present in significant quantities in the primary cell walls in one dicot order, the *Caryophyllales*, which includes crops like quinoa. Hager et al. (2012b) found the lowest polyphenol content in wheat and rice flour which were five times lower than in quinoa flour. During sourdough fermentation free phenolics are released by cereal enzymes or chemical reactions which can be catalysed by LAB (Gänzle, 2014). Repeatedly, the composition of the quinoa flour favoured the formation of antifungal compounds. In the quinoa sourdough samples, concentration of caffeic, *p*-coumaric, ferulic and vanillic acid reached the highest total amount of 167 ppm, whereas in the rice sourdough *p*-coumaric and ferulic could not be detected (Table 5-4). Further metabolism of these free phenolics is possible due to the activity of strain-specific phenolic acid decarboxylases and cinnamic acid reductases (Svensson et al., 2010). As a result hydrocaffeic acid, hydroferulic acid and phloretic acid can be formed. Caffeic acid, *p*-coumaric acid, and ferulic acid were not metabolized by *Lb. reuteri* FUA3168 (Svensson et al., 2010). In our study, *Lb. reuteri* R29-fermented quinoa sourdough with 0.6 ppm, had a very low level of hydroferulic acid, with 1.9 ppm a low level of phloretic acid in wheat sourdough and hydrocaffeic acid was only present at a very low level in the fermented rice flour (Table 5-4). These results indicate a low decarboxylation and reduction rate with *Lb. reuteri* R29.

5.4.3 *In situ* application of sourdough biopreservative

The *Lb. reuteri* R29-fermented quinoa, rice and wheat sourdoughs, containing varying levels of antifungal compounds, were used as biopreservatives and incorporated into simple bread recipes. Figure 5-3 represents an overview of the shelf life and mould growth characteristics for the biologically acidified breads as well as for the control breads. In the control breads, the mould free shelf life was shortest in quinoa bread (2 days), followed by rice (3 days) and wheat (4 days). These differences in shelf life when comparing the control breads are mainly associated with the different raw materials and water levels used in the production process. The optimal water level has

to be adjusted for each bread recipe (Hager et al., 2012a). In general, gluten-free breads contain more water in the recipe. Accordingly, gluten-free breads result in higher water activities. Thus, they are more susceptible for microbial spoilage. With the addition of *Lb. reuteri* R29 inoculated sourdough the shelf life was extended by 2 days for quinoa and rice bread. Although there were less antifungal carboxylic acids present in the wheat sourdough, a prolongation of 3 days was achieved for the corresponding sourdough bread (Figure 5-3). The presented results indicate, that the preservation effect of sourdough in the final bread product seems to be much more complex. Using a different antifungal strain, *Lb. amylovorus* DSM19280, Axel et al. (2015a) and Ryan et al. (2011) have demonstrated a longer shelf life extension in quinoa and wheat bread, respectively. Less concentration of antifungal carboxylic acids were reported than in this present study. It was concluded, that *Lb. amylovorus* DSM19280 might produce more compounds concomitantly contributing to the increase of the shelf life in those sourdough breads (Axel et al., 2015a).

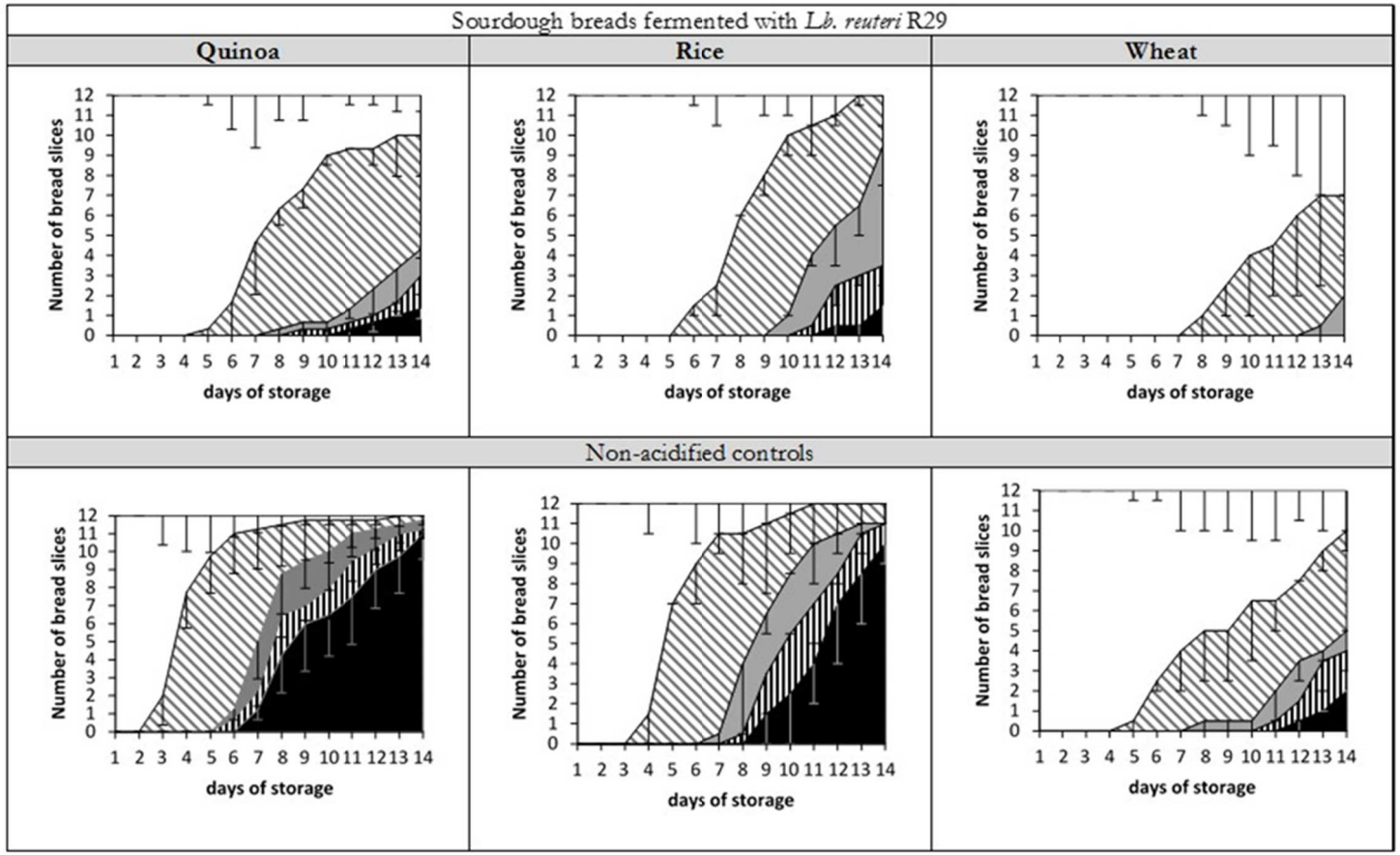


Figure 5-3 Shelf life of quinoa, rice and wheat control breads and sourdough breads fermented with *Lb. reuteri* R29 after challenging against environmental moulds during a 14-day storage period. Bread spoilage is indicated as percentage of the total surface area of each of the 12 slices where fungal outgrowth occurred: Mould free slices (white area), < 10 % mouldy (grey diagonally striped area), 10–24 % mouldy (grey area), 25-49 % mouldy (black horizontally striped area) and > 50 % mouldy (black area). Mean values are shown (n = 2), error bars indicate standard deviations.

5.5 Conclusion

The findings of this study showed that *Lb. reuteri* R29 is a competitive bio-protective starter for the successful production of antifungal active sourdough using wheat and gluten-free flours as substrate.

The origin of the antifungal carboxylic acids studied here, can be well explained by means of flour composition, enzyme activity and LAB metabolic activity during fermentation. The chemical composition of quinoa flour was superior for the production of antifungal carboxylic acids. 3-Phenyllactic acid and 2-hydroxyisocaproic acid were found in highest concentration in all three substrates. As found for rice and wheat sourdough, a lower protein content in the substrate resulted in an inferior production of antifungal compounds. In contrast, a higher protease activity during fermentation (rice) might compensate this lack of protein which can give rise to more amino acid or peptide availability and concomitantly higher antifungal compounds.

However, LAB sourdough fermentation of different flour substrates generates a complex and significantly different profile of antifungal compounds. This might be customized in evaluating more substrates (i.e., sorghum, rye or buckwheat), additional enzyme treatments in sourdough fermentation and possible flour blending for the development of safe cereal-based biopreservatives. Furthermore, it would be interesting to investigate the application of the high in antifungal compounds quinoa sourdough in other gluten-free and wheat breads.

Nevertheless, more research needs to be conducted, evidencing the contribution of antifungal carboxylic acids in sourdough bread in terms of their antifungal activity and concentration. Therefore, the impact of those compounds has to be studied more fundamentally and different species should be included to also compare species depended antifungal profiles.

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5.7 References

Adom, K.K., Sorrells, M.E., Liu, R.H., 2005. Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. Journal of Agricultural and Food Chemistry 53, 2297–22306.

Arbeitsgemeinschaft Getreideforschung e.V., 1994. Standard-Methoden für Getreide, Mehl und Brot. Verlag Moritz Schäfer: Detmold, Germany, vol. 7.

Arendt, E.K., Ryan, L.A.M., Dal Bello, F., 2007. Impact of sourdough on the texture of bread. Food Microbiology 24, 165–174. doi:10.1016/j.fm.2006.07.011

Arendt, E.K., Zannini, E., 2013. Cereal Grains For the Food and Beverage Industries. Woodhead Publishing.

Axel, C., Roecker, B., Brosnan, B., Zannini, E., Furey, A., Coffey, A., Arendt, E.K., 2015a. Application of *Lactobacillus amylovorus* DSM19280 in gluten-free sourdough bread to improve the microbial shelf life. Food Microbiology 47, 36–44. doi:10.1016/j.fm.2014.10.005

Axel, C., Zannini, E., Arendt, E.K., 2015b. Mould spoilage of bread and its biopreservation: A review of current strategies for bread shelf life extension. Critical Reviews in Food Science and Nutrition. (Submitted manuscript)

Brijs, K., Bleukx, W., Delcour, J.A., 1999. Proteolytic activities in dormant rye (*Secale cereale L.*) grain. Journal of Agricultural and Food Chemistry 47, 3572–3578.

Broberg, A., Jacobsson, K., Ström, K., Schnürer, J., 2007. Metabolite profiles of lactic acid bacteria in grass Silage. Applied and Environmental Microbiology 73, 5547–5552. doi:10.1128/aem.02939-06

Brosnan, B., Axel, C., Coffey, A., Arendt, E.K., Furey, A., 2015. Development of an UHPLC-MS/MS method for the detection of antifungal compounds in lactic acid bacteria fermented sourdough. Food Chemistry (Manuscript for submission)

Capuani, A., Behr, J., Vogel, R.F., 2012. Influence of lactic acid bacteria on the oxidation–reduction potential of buckwheat (*Fagopyrum esculentum Moench*) sourdoughs. European Food Research and Technology 235, 1063–1069. doi:10.1007/s00217-012-1834-4

Cortés-Zavaleta, O., López-Malo, a, Hernández-Mendoza, a, García, H.S., 2014. Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid

production. International Journal of Food Microbiology 173, 30–5. doi:10.1016/j.ijfoodmicro.2013.12.016

Crowley, S., Mahony, J., van Sinderen, D., 2013. Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. Trends in Food Science & Technology 33, 93–109. doi:10.1016/j.tifs.2013.07.004

Dalié, D.K.D., Deschamps, a. M., Richard-Forget, F., 2010. Lactic acid bacteria – Potential for control of mould growth and mycotoxins: A review. Food Control 21, 370–380. doi:10.1016/j.foodcont.2009.07.011

Dallagnol, A.M., Pescuma, M., De Valdez, G.F., Rollán, G., 2013. Fermentation of quinoa and wheat slurries by *Lactobacillus plantarum* CRL 778: proteolytic activity. Applied Microbiology and Biotechnology 97, 3129–40. doi:10.1007/s00253-012-4520-3

EEC, 2009. Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. Official Journal of the European Union L 054, 26.

Elgeti, D., Nordlohne, S.D., Foste, M., Besl, M., Linden, M.H., Heinz, V., Jekle, M., Becker, T., 2014. Volume and texture improvement of gluten-free bread using quinoa white flour. Journal of Cereal Science 59, 41–47. doi:10.1016/j.jcs.2013.10.010

Gänzle, M.G., 2014. Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiology 37, 2–10. doi:10.1016/j.fm.2013.04.007

Gänzle, M.G., Ehrmann, M., Hammes, W.P., 1998. Sourdough Fermentation. Applied and Environmental Microbiology 64, 2616–2623.

Gänzle, M.G., Follador, R., 2012. Metabolism of oligosaccharides and starch in lactobacilli: a review. Frontiers in Microbiology 3, 340. doi:10.3389/fmicb.2012.00340

Gänzle, M.G., Loponen, J., Gobbetti, M., 2008. Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality. Trends in Food Science & Technology 19, 513–521. doi:10.1016/j.tifs.2008.04.002

Gänzle, M.G., Vermeulen, N., Vogel, R.F., 2007. Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiology 24, 128–138. doi:10.1016/j.fm.2006.07.006

Hager, A.S., Wolter, A., Czerny, M., Bez, J., Zannini, E., Arendt, E.K., 2012a. Investigation of product quality, sensory profile and ultrastructure of breads made from a range of commercial gluten-free flours compared to their wheat counterparts. European Food Research and Technology 235, 333–344. doi:10.1007/s00217-012-1763-2

Hager, A.S., Wolter, A., Jacob, F., Zannini, E., Arendt, E.K., 2012b. Nutritional properties and ultra-structure of commercial gluten free flours from different botanical sources compared to wheat flours. Journal of Cereal Science 56, 239–247. doi:10.1016/j.jcs.2012.06.005

Hammes, W.P., Stolz, P., Gänzle, M.G., 1996. Metabolism of lactobacilli in traditional sourdoughs. Advances in Food Sciences 18, 176–184.

Hüfner, E., Britton, R. a, Roos, S., Jonsson, H., Hertel, C., 2008. Global transcriptional response of *Lactobacillus reuteri* to the sourdough environment. Systematic and applied microbiology 31, 323–38. doi:10.1016/j.syapm.2008.06.005

Kishio, S., Aoyagi, Y., 2014. Cultivar- and region-specific differences in the starch-degrading enzymes produced during rice soaking. Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi 61, 232–243. doi:10.3136/nskkk.61.232

Li, L., Shewry, P.R., Ward, J.L., 2008. Phenolic acids in wheat varieties in the HEALTHGRAIN Diversity Screen. Journal of Agricultural and Food Chemistry 56, 9732–9. doi:10.1021/jf801069s

Meroth, C.B., Walter, J., Hertel, C., Brandt, J., Hammes, W.P., Brandt, M.J., 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69, 475–482. doi:10.1128/aem.69.1.475-482.2003

Moroni, A. V, Dal Bello, F., Arendt, E.K., 2009. Sourdough in gluten-free bread-making: an ancient technology to solve a novel issue? Food Microbiology 26, 676–84. doi:10.1016/j.fm.2009.07.001

Ndagano, D., Lamoureux, T., Dortu, C., Vandermoten, S., Thonart, P., 2011. Antifungal activity of 2 lactic acid bacteria of the *Weissella* genus isolated from food. Journal of Food Science 76, M305–311. doi:10.1111/j.1750-3841.2011.02257.x

Oliveira, P.M., Brosnan, B., Furey, A., Coffey, A., Zannini, E., Arendt, E.K., 2015a. Lactic acid bacteria bioprotection applied to the malting process. Part I: Strain characterization and identification of antifungal compounds. Food Control 51, 433–443. doi:10.1016/j.foodcont.2014.07.00

Oliveira, P.M., Brosnan, B., Jacob, F., Furey, A., Coffey, A., Zannini, E., Arendt, E.K., 2015b. Lactic acid bacteria bioprotection applied to the malting process. Part II: Substrate impact and mycotoxin reduction. Food Control. doi:10.1016/j.foodcont.2014.11.011

Oliveira, P.M., Zannini, E., Arendt, E.K., 2014. Cereal fungal infection, mycotoxins, and lactic acid bacteria mediated bioprotection: from crop farming to cereal products. Food Microbiology 37, 78–95. doi:10.1016/j.fm.2013.06.003

Pepe, O., Villani, F., Oliviero, D., Greco, T., Coppola, S., 2003. Effect of proteolytic starter cultures as leavening agents of pizza dough. International Journal of Food Microbiology 84, 319–326. doi:10.1016/S0168-1605(02)00473-7

Ryan, L.A.M., Dal Bello, F., Czerny, M., Koehler, P., Arendt, E.K., 2009. Quantification of phenyllactic acid in wheat sourdough using high resolution gas chromatography-mass spectrometry. Journal of Agricultural and Food Chemistry 57, 1060–4. doi:10.1021/jf802578e

Ryan, L.A.M., Zannini, E., Dal Bello, F., Pawlowska, A., Koehler, P., Arendt, E.K., 2011. *Lactobacillus amylovorus* DSM 19280 as a novel food-grade antifungal agent for bakery products. International Journal of Food Microbiology 146, 276–283. doi:10.1016/j.ijfoodmicro.2011.02.036

Sakko, M., Moore, C., Novak-Frazer, L., Rautemaa, V., Sorsa, T., Hietala, P., Järvinen, a, Bowyer, P., Tjäderhane, L., Rautemaa, R., 2014. 2-Hydroxyisocaproic acid is

fungicidal for *Candida* and *Aspergillus* species. Mycoses 57, 214–21. doi:10.1111/myc.12145

Sekwati-Monang, B., Valcheva, R., Gänzle, M.G., 2012. Microbial ecology of sorghum sourdoughs: effect of substrate supply and phenolic compounds on composition of fermentation microbiota. International Journal of Food Microbiology 159, 240–246. doi:10.1016/j.ijfoodmicro.2012.09.013

Stolz, P., Hammes, W.P., Vogel, F., 1996. Maltose-phosphorylase and hexokinase activity of lactobacilli from traditionally prepared sourdoughs. Advances in Food Sciences 18, 1–6.

Stolz, P., Vogel, R.F., Hammes, W.P., 1995. Utilization of electron acceptors by lactobacilli isolated from sourdough II. *Lactobacillus pontis*, *L. reuteri*, *L. amylovorus*, and *L. fermentum*. Zeitschrift fur Lebensmittel-Untersuchung und -Forschung 201, 402–410. doi:10.1007/BF01192742

Ström, K., Sjögren, J., Broberg, A., Schnürer, J., 2002. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic Acid. Applied and Environmental Microbiology 68, 4322–4327. doi:10.1128/AEM.68.9.4322

Svensson, L., Sekwati-Monang, B., Lutz, D.L., Schieber, A., Gänzle, M.G., 2010. Phenolic acids and flavonoids in non-fermented and fermented red sorghum (*Sorghum bicolor (L.) Moench*). Journal of Agricultural and Food Chemistry 58, 9214–20. doi:10.1021/jf101504v

Van der Meulen, R., Scheirlinck, I., Van Schoor, A., Huys, G., Vancanneyt, M., Vandamme, P., De Vuyst, L., 2007. Population dynamics and metabolite target analysis of lactic acid bacteria during laboratory fermentations of wheat and spelt sourdoughs. Applied and Environmental Microbiology 73, 4741–50. doi:10.1128/AEM.00315-07

Vermeulen, N., Gänzle, M.G., Vogel, R.F., 2006. Influence of peptide supply and cosubstrates on phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM20451T and *Lactobacillus plantarum* TMW1.468. Journal of Agricultural and Food Chemistry 54, 3832–3839.

Viswanath, V., Urooj, A., Malleshi, N.G., 2009. Evaluation of antioxidant and antimicrobial properties of finger millet polyphenols (*Eleusine coracana*). Food Chemistry 114, 340–346. doi:10.1016/j.foodchem.2008.09.053

Vogel, J., 2008. Unique aspects of the grass cell wall. Current Opinion in Plant Biology 11, 301–7. doi:10.1016/j.pbi.2008.03.002

Wolter, A., Hager, A.S., Zannini, E., Czerny, M., Arendt, E.K., 2014a. Impact of sourdough fermented with *Lactobacillus plantarum* FST 1.7 on baking and sensory properties of gluten-free breads. European Food Research and Technology 239, 1–12. doi:10.1007/s00217-014-2184-1

Wolter, A., Hager, A.S., Zannini, E., Galle, S., Gänzle, M.G., Waters, D.M., Arendt, E.K., 2014b. Evaluation of exopolysaccharide producing *Weissella cibaria* MG1 strain for the production of sourdough from various flours. Food Microbiology 37, 44–50. doi:10.1016/j.fm.2013.06.009

Zannini, E., Pontonio, E., Waters, D.M., Arendt, E.K., 2012. Applications of microbial fermentations for production of gluten-free products and perspectives. Applied Microbiology and Biotechnology 93, 473–485. doi:10.1007/s00253-011-3707-3

Chapter 6 Antifungal activity of three different *Lactobacillus* species and their production of antifungal carboxylic acid in wheat sourdough.

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6.1 Abstract

This study was undertaken to assess the antifungal performance of three different *Lactobacillus* species. Experiments were conducted *in vitro* and *in situ* to extend the shelf of wheat bread. Standard sourdough analyses were performed characterising acidity and carbohydrate levels. Overall, the strains showed good inhibition *in vitro* against the indicator mould *Fusarium culmorum* TMW4.2043. Sourdough bread fermented with *Lactobacillus amylovorus* DSM19280 performed best in the *in situ* shelf life experiment. An average shelf life extension of six more mould-free days was reached when compared to the non-acidified control bread. A range of antifungal-active acids like 3-phenyllactic acid, 4-hydroxyphenyllactic acid and 2-hydroxyisocaproic acid in quantities between 0.1 ppm and 360 ppm were present in the freeze-dried sourdoughs. Their concentration differed greatly among the species. However, a higher concentration of these compounds could not completely justify the growth inhibition of environmental moulds. In particular, although *Lb. reuteri* R29 produced the highest total concentration of these active compounds in the sourdough, its addition to bread did not result in a longest shelf life. Nevertheless, when the artificial compounds were spiked into a chemically acidified dough, it succeeded in a longer shelf life than achieved only by acidifying the dough. This provides evidence of their synergistic antifungal activity in concentration levels far below their single minimal inhibition concentrations under acidic conditions.

6.2 Introduction

The application of antifungal-active starter cultures in sourdough is a promising strategy to extend the microbial shelf life of bread and lactic acid bacteria (LAB) are well known to produce a range of functional metabolites contributing to the inhibition of mould. Since active compounds responsible for biopreservation are produced *in situ*, the sourdough can replace chemical preservatives like calcium propionate guaranteeing a clean label besides other advantages like improved flavour, texture and nutritional properties along with higher consumer acceptance (Pawlowska et al., 2012). In general, biopreservatives should feature activity against a broad range of microbial contaminants. A comprehensive overview about individual substances, their possible antifungal mechanism and some applications of antifungal acting LAB was recently reviewed by Crowley et al. (2013). Antifungal compounds include organic acid, fatty acids, carboxylic and phenolic acids, proteinaceous compounds and a variety of other low-molecular weight compounds. As a part of antifungal research, great attention has been placed to study the production and activity of 3-phenyllactic acid in growth media and sourdough; for review see Mu et al. (2012). 3-Phenyllactic acid originates from the catabolism of phenylalanine initiated by a transamination reaction to phenylpyruvic acid where the α-amino group is transferred to a keto acid acceptor by an aminotransferase. Phenylpyruvic acid is further reduced to 3-phenyllactic acid by a dehydrogenase. Subsequently, investigations have demonstrated the substrate and strain dependency of the production of this antifungal compound. Due to its relatively high minimal inhibition concentration (MIC), a complex synergistic mechanism between 3-phenyllactic acid and other metabolites has been proposed to play the key role in antifungal action (Cortés-Zavaleta et al., 2014; Miescher Schwenninger et al., 2008; Ndagano et al., 2011; Niku-Paavola et al., 1999). Accordingly, more antifungal carboxylic acids resulting from the LAB amino acid metabolism, like 4-hydroxyphenyllactic acid from tyrosine, benzoic acid and salicylic acid also from phenylalanine or 2-hydroxyisocaproic acid from leucine, have been detected in LAB fermented MRS broth and sourdough and were related with synergistic antifungal activity (Axel et al., 2015; Brosnan et al., 2014; Dallagnol et al., 2013; Ryan et al., 2011).

Some antifungal starter cultures have been already applied successfully *in situ* bread systems (Axel et al., 2015; Black et al., 2013; Coda et al., 2011; Dal Bello et al., 2007; Ryan et al., 2011; Varsha et al., 2014). However, data for the antifungal metabolites profile produced by LAB in food substrates is still scarce.

Our research aimed to investigate the antifungal performance of three different species of lactobacilli in a wheat sourdough system and their contribution to shelf life extension on bread. Additionally, the synergistic antifungal effect among the compounds was tested *in situ*. Characterisation of the sourdough was carried out and the analysis of antifungal compounds was performed using UHPLC-MS/MS. *Lb. amylovorus* DSM19280 has previously been reported to be effective against a range of different food spoilage (Axel et al., 2015; Belz et al., 2012; Lynch et al., 2014; Oliveira et al., 2015; Ryan et al., 2011; Waters et al., 2012). *Lb. reuteri* R29 revealed antifungal activity in malting trials (Oliveira et al., 2015). Therefore, these strains were further investigated in wheat sourdough fermentations along with another strain *Lb. brevis* R2Δ, selected for its antifungal traits.

6.3 Materials and Methods

6.3.1 Materials

The characteristics of the wheat flour (baker's flour, Odlums, Dublin, Ireland) used were the following: moisture, 13.5 %; protein (N x 5.83), 11.5 %; fat, 1.1 %.; ash, 1.9 % and total starch, 67.1 %. Additionally, dry yeast (Puratos, Groot-Bijgaarden, Belgium), sugar (Suicra, Dublin, Ireland) and salt (Glacia British Salt Limited, Cheshire, UK) were used in the bread recipe.

Chemicals and analytical standards were mainly purchased from Sigma Aldrich (Dublin, Ireland). The antifungal compound 3-phenyllactic acid was acquired from BaChem (Weil am Rhein, Germany). All analytical standards had a purity of ≥ 95 %.

6.3.2 Cultures, media and growth conditions

The strains *Lb. amylovorus* DSM19280 patented by Arendt et al. (2009), *Lb. brevis* R2Δ and *Lb. reuteri* R29 were obtained from the culture collection of UCC (School of Food and Nutritional Science, University College Cork, Cork, Ireland). The cultures, stored in 35 % glycerol stock solution at -80 °C, were routinely refreshed on deMan-Rogosa-Sharpe agar (MRS) 5 agar (Meroth et al., 2003) at 30 °C (*Lb. amylovorus* and *Lb. brevis*) and 37 °C (*Lb. reuteri*) for 48 h under anaerobic conditions. For cell counts commercial MRS agar was used (Oxoid, Basingstoke, Hampshire, England) and plates were incubated at same conditions. Both MRS5 and MRS agar were dyed with 0.05 g/L bromocresol green (Sigma-Aldrich, Steinheim, Germany).

Fusarium culmorum TMW4.2043, isolated from barley and kindly provided by the culture collection of TU-München Weihenstephan (TMW), was used as indicator fungus. The mould was cultivated on potato-dextrose agar plates (Fluka Chemie AG, Buchs, Switzerland) at 25 °C until sporulation occurred and then stored at 4 °C until further use. The macroconidia were collected by brushing the plate surface with physiological solution and filtering through a 30 µm pore size filter paper (Filter Paper 113 wet strengthened, Whatman International Ltd, Maidstone, England) removing fungal mycelia. Macroconidia in this suspension were counted using a Thoma chamber haemocytometer (Hawksley, Sussex, UK).

6.3.3 Amylolytic and proteolytic activity test

To test the amylolytic activity of the strains, starch agar was prepared containing 3 g/L meat extract, 10 g/L soluble starch as well as 12 g/L agar. After 48 h incubation time using LAB spots (20 µL of a 16 h culture) in wells (4x4 mm), the plate surface was flooded with 10 mL Gram's iodine solution to make starch hydrolysis visible by a colourless zone surrounding colonies.

The proteolytic activity was tested out on gluten containing agar, pH 4 and 7 according to Wehrle et al. (1999) and on milk agar plates containing 28 g/L skim milk powder, 5 g/L casein peptone, 2.5 g/L yeast extract, 1 g/L glucose, and 15 g/L agar. LAB were spotted and incubated like described for the amylolytic test. After incubation the agar layer was stained for 5 min with 10 mL of a solution of 5 g Coomassie brilliant blue R-250, 500 mL methanol and 92 mL acetic acid in 1 L distilled water. Subsequently the plates were destained overnight with 10 mL using a solution of 250 mL ethanol and 50 mL acetic acid in 1 L of distilled water. Proteolytic activity was indicated as clear zone surrounding LAB spots. The protease and amylase enzymes were not determined.

6.3.4 Preparation of culture cell-free supernatant

Overnight cultures of LAB were inoculated in 500 mL of modified MRS5 broth (1 %), where sodium acetate as well as potassium dihydrogenphosphate were omitted. LAB were grown for 48 h at their optimal temperature mentioned above. Cell-free supernatant (cfs) was prepared by centrifugation (7,500 g for 15 min; 4 °C) and sterile filtration (0.45 µm-pore-size filter; Millipore). Subsequently the cfs was frozen at -80 °C, freeze dried and stored at 4 °C for further antifungal activity investigation. A control was prepared accordingly containing only mMRS5 broth.

6.3.5 Sourdough Type II preparation and analysis

Single colonies from MRS5 agar plates were pre-inoculated in 10 mL MRS5 broth at 30/37 °C for 24 h and subcultured (1 %) in 45 mL of MRS5 broth at 30/37 °C for 16 h. Cells were harvested by centrifugation (5000 rpm, 10 min), washed once and resuspended in 45 mL sterile tap water. Sourdoughs were prepared with an equal

weight of wheat flour and sterile tap water, dough yield (DY) of 200. The starter cultures were added to the sourdough to an initial inoculum of 7 log cfu/g dough. The doughs were fermented at 30 (*Lb. amylovorus* DSM19280 and *Lb. brevis* R2Δ) and 37 °C (*Lb. reuteri* R29) for 48 h (stirring thoroughly after 24 h). LAB cell counts were determined at 0 h and 48 h of fermentation. The identity of starter cultures was confirmed by colony morphology and metabolic patterns (Wolter et al., 2014). Fermentation microbiota were dominated by the starter cultures since uniform colony morphology on agar plates were observed with absence of contaminations. Total titratable acidity (TTA) and pH values of the fermented sourdoughs were measured using a standard procedure (Arbeitsgemeinschaft Getreideforschung e.V., 1994).

6.3.6 Metabolite analysis in sourdough

An Agilent 1260 high performance liquid chromatography system equipped with a refractive index detector (RID) and an ultra violet-diode array detector (UV/DAD) was used to quantify sugars (0.125-2.5 mM) and organic acids (2-32 mM), respectively. Standard calibration curves were prepared with 5 different concentrations in duplicate. Calibration curves showed good linearity with correlation coefficients of ≥ 0.999 for all compounds. For sugar (fructose, glucose, maltose, raffinose and sucrose) and acid analyses (lactic acid and acetic acid), freeze-dried sourdough samples and flour were extracted with distilled water, clarified with 7 % perchloric acid overnight (16 h, 4 °C) and filtered (0.2 µm pore size filter). Sugars were quantified over the RID (35 °C) by elution of the extract from a Hi-Plex H column (300 x 7.7 mm, 8 µm; Agilent, Cork, Ireland), equipped with a guard column (50 x 7.7 mm, 8 µm; Agilent, Cork, Ireland), using water at a flow rate of 0.6 mL/min at 25 °C. Setting the UV/DAD at 210 nm, lactic and acetic acid in the sourdough were determined after elution with 0.004 M sulphuric acid at 65 °C from the same column and a flow of 0.5 mL/min. Injection volumes were 20 µL.

The analysis of 20 known antifungal compounds (azelaic acid, benzoic acid, caffeic acid, catechol, *p*-coumaric acid, ferulic acid, hydrocaffeic acid, hydrocinnamic acid, hydroferulic acid, 4-hydroxybenzoic acid, 2-hydroxydodecanoic acid, β-hydroxylauric acid, 2-hydroxyisocaproic acid, hydroxymyristic acid, 4-hydroxyphenyllactic acid,

methylcinnamic acid, 3-phenyllactic acid, phloretic acid, salicylic acid, vanillic acid) was carried out using a Waters (Milford MA, USA) Acquity UHPLC system employing an Acquity BEH shield RP18 analytical column (Brosnan et al., 2015). Antifungal compounds were detected using a Waters Quattro Premier triple quadrupole instrument operated in negative electrospray ionisation mode (Milford, MA, USA). QuEChERS method (quick, easy, cheap, effective, rugged and safe) was applied to extract *in situ* antifungal compounds from the freeze dried wheat sourdough. Recoveries of the compounds in the wheat matrices were found to range from 60.3 % (hydroxymyristic acid) – 97.2 % (*p*-coumaric acid) (Brosnan et al., 2015).

6.3.7 Evaluation of *in vitro* antifungal activity

Two different assays, the overlay method and the microtiter plate assay, were employed to determine the *in vitro* antifungal activity of the strains.

For the overlay method, overnight mMRS5 broth culture at 30°C (*Lb. amylovorus* DSM19280 and *Lb. brevis* R2Δ) and 37 °C (*Lb. reuteri* R29) at concentration 10^7 cfu/mL were placed as two 5 µL cell spots in the centre of mMRS5 agar plates. These LAB spots were incubated anaerobically for 48 h at 30/37 °C. Subsequently, plates were overlaid with 10 mL cooled malt extract soft agar (0.7 % agar) containing 10^4 cfu/mL *Fusarium* spores. Plates were incubated aerobically at room temperature for another 48 h and the antifungal activity was measured as clear zones of inhibition around the bacterial spots.

The microtiter plate assay was performed according to Mauch et al. (2010). The experiments were performed in duplicate.

6.3.8 Evaluation of *in situ* antifungal activity

A bread shelf life study using a mould environmental challenge method described by Dal Bello et al. (2007) was conducted to assess the *in situ* antifungal activity of the LAB strains.

To test the antifungal synergy effect of the LAB organic acids, four different types of artificially acidified sourdoughs were prepared. One type of this chemically acidified sourdough (CA) was acidified to 1.4 % (w/w) with a mixture of lactic and acetic acid

(4:1) according to Ryan et al. (2008). The remaining three types were chemically acidified in the same manner, but further spiked with a "cocktail" of the antifungal carboxylic acids according to their production [ppm] in the wheat sourdough which was individually fermented by the *Lactobacillus* strains – $CAS_{DSM19280}$, $CAS_{R2\Delta}$ and CAS_{R29}, as measured in Section 6.3.6 (the concentration were divided in half, considering the dilution factor of 2 from freeze dried sourdough to fresh sourdough). Aqueous stock solutions of the compounds (2 mg/mL) were prepared individually and equivalent aliquots were added to the acidified flour water mix. The water level was corrected accordingly. Sourdoughs were produced with a DY of 200 and to exclude acidification by naturally occurring LAB, chloramphenicol and erythromycin (60 and 200 μg/g dough, respectively) were added to the dough, which was then fermented at 30 °C for 24 h.

To conduct the *in situ* shelf life test, 20 % w/w flour-based LAB fermented sourdough and the artificial acidified sourdoughs were incorporated into a simple bread recipe (2 % salt, 2 % sugar and 3 % yeast, 63 % water based on flour weight). Non acidified bread was used as a control. Another control bread contained 0.3 % of calcium propionate (CAP) to compare the inhibitory activity of the sourdoughs to that of a chemical preservative.

After baking, the breads were cooled for 2 h at ambient temperature (Ryan et al., 2008). Bread slices of 20 mm thickness were put on a sterile metal rack, exposed to the bakery environment for 5 min on each side and then packed separately into a sterile plastic bag and heat sealed. Ensuring comparable aerobic conditions in each bag, two filter pipette tips were inserted. The bags were examined for mould growth during a 14-day storage period at an average temperature of 20 ± 2 °C. Mould growth was evaluated based on the percentage of the total surface area of each slice where fungal outgrowth occurred (Axel et al., 2015).

6.3.9 Statistical analysis

Sourdough extractions were carried out from two independent fermentations, extracting each sample twice. Statistical analysis was performed using Minitab 17 software. Data were checked for outliers 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's *post hoc* test to describe the statistical significance between the antifungal, non-antifungal and non-acidified control.

6.4 Results

6.4.1 Enzymatic activity

Figure 6-1 presents the amylase as well as the protease activity tested on starch and milk agar, respectively. All lactobacilli tested were proteolytically active on the casein containing agar; the highest activity were found for *Lb. reuteri* R29 showing the biggest halo, *Lb. brevis* R2Δ demonstrated the lowest activity visible in a weak clear zone around the inoculation spot. In addition to proteolytic activity, only *Lb. amylovorus* DSM19280 exhibited amylolytic activity on starch agar. However, the strains did not show any clear zone on any of the gluten containing plates (data not shown).

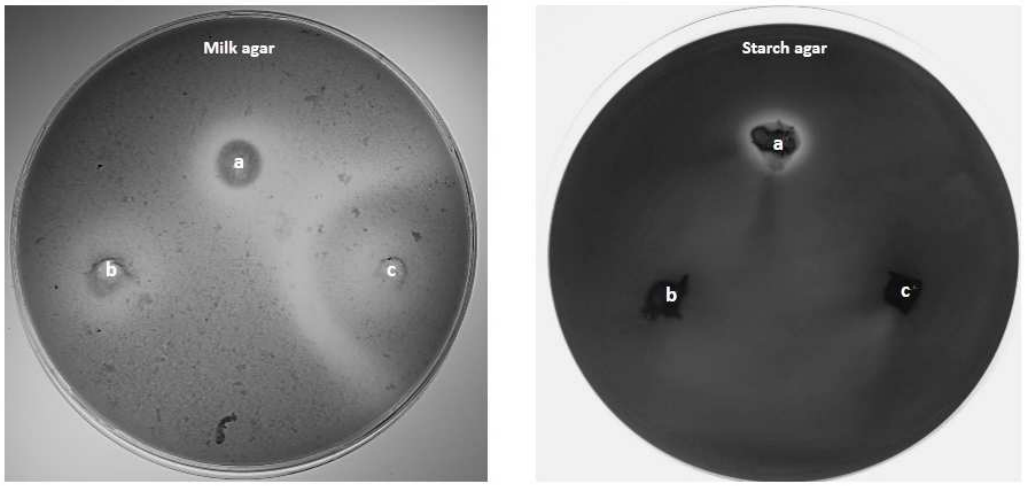


Figure 6-1 Proteolytic activity on milk agar (left) and amylolytic activity on starch agar (right) of *Lb. amylovorus* DSM19280 (a), *Lb. brevis* R2Δ (b) and *Lb. reuteri* R29 (c).

6.4.2 *In vitro* antifungal activity

The *in vitro* antifungal activity of three different LAB strains was investigated using *F. culmorum* TMW4.2043 as target organism.

Using the overlay method, the size of clear zone surrounding the LAB spots varied in degree of antifungal activity between the lactobacilli strains tested against the indicator mould (Figure 6-2). The biggest clear zone was observed for *Lb. brevis* R2Δ. Although the antifungal strain *Lb. amylovorus* DSM19280 showed the smallest inhibition zone, the fungal mycelia surrounding its spots were rather thin collapsed

when compared with the smooth and velvety mycelia surface from the other strains and the control plate.

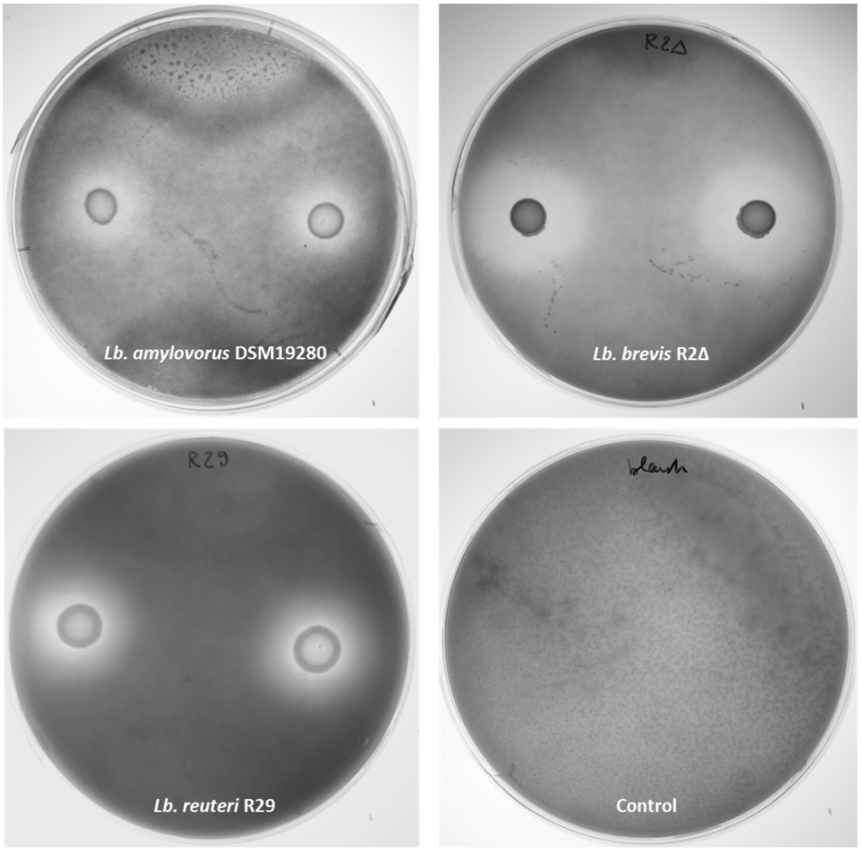


Figure 6-2 Inhibitory zones formed around *Lb. amylovorus* DSM19280, *Lb. brevis* R2Δ and *Lb. reuteri* R29 against *F. culmorum* TMW4.2043 after 2 days of incubation in comparison with substrate control (only mMRS5-agar).

The impact on the growth of *F. culmorum* TMW4.2043 in MEB containing different concentrations of antifungal cfs was determined using a microtiter plate assay (Figure 6-3). In the control, where no cfs was added to the MEB, the fungus had a lag phase of about 4 h reaching an optical density at 620 nm (OD620) of 0.6 after 48 h of growth. When 15 % cfs was used, no change in OD620 (meaning no growth of the fungus) was observed over 48 h for all the three cfs produced by the antifungal LAB strains. 7.5 % cfs of *Lb. reuteri* R29 still highly retarded the growth until 44 h. For half of this concentration, the fungus started to grow after 20 h. When the same cfs concentration produced by *Lb. brevis* R2Δ was added, the growth of *F. culmorum* TMW4.2043 was delayed for 24 h. The antifungal *Lb. amylovorus* DSM19280 only

inhibited its outgrowth for 16 h at 3.75 % cfs concentration. Reducing the concentration of cfs in MEB to less than 2 % caused the loss of antifungal activity for all three LAB strains (data not shown).

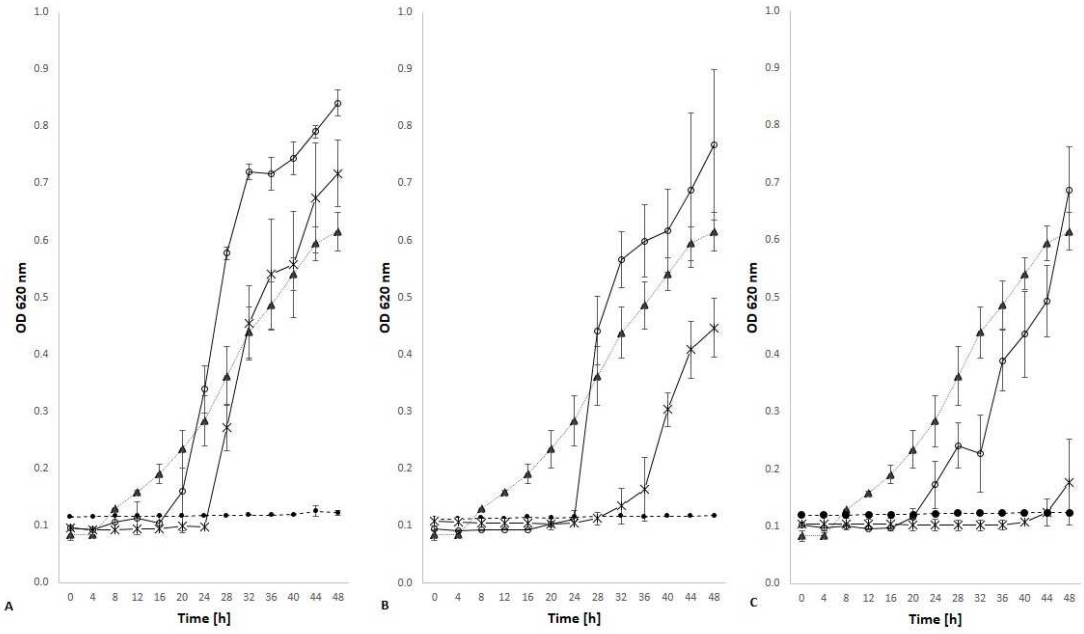


Figure 6-3 Growth of *F. culmorum* TMW4.2043 (10^4 cfu/mL) in malt extract broth (pH 4) containing: 15 % (--●--), 7.5 % (—x—), 3.75 % (—○—) or 0 % (•••▲•••) of cell-free supernatant of (A) *Lb. amylovorus* DSM19280; (B) *Lb. brevis* R2Δ and (C) *Lb. reuteri* R29. Data represents the average of a duplicate with standard deviations.

6.4.3 Sourdough characterisation

Cell counts after 48 h of fermentation were comparable within the different LAB species and reached concentration between 9.6 and 9.9 log cfu/g (data not shown). Regarding the carbohydrate catabolism during sourdough fermentation different uptake and release of sugars were observed between the lactobacilli species (Table 6-1). In the sourdough sample fermented with the obligate homofermentative *Lb. amylovorus* DSM19280 glucose and fructose concentration were at the lowest level and maltose content were highest. Glucose contents were higher and similar in *Lb. brevis* R2Δ and *Lb. reuteri* R29 fermented sourdough. Highest amounts of fructose were found in sourdough inoculated with *Lb. brevis* R2Δ. Remarkably, *Lb. reuteri* R29 metabolized the highest amount of maltose resulting in the lowest level of this

disaccharide found in the sourdoughs analysed. Residual sucrose could not be found in any sourdough sample.

Table 6-1 Sourdough characterisation of the fermentation of wheat flour (DY 200) after 48 h fermentationa different species of LAB[a].

Wheat Metabolites [mmol/kg flour]	**Unfermented flour[b]**	***Lb. amylovorus* DSM19280[b]**	***Lb. brevis* R2Δ**	***Lb. reuteri* R29[c]**
Fructose	ND	27±1c	52±5a	42±4b
Glucose	ND	14±2b	121±5a	145±14a
Sucrose	42±8	ND	ND	ND
Maltose	ND	176±8a	148±2b	47±9c
Raffinose	5±1	ND	ND	ND
Lactate	ND	368±11ab	323±20b	406±27a
Acetate	ND	ND	4±1b	49±5a
TTA [mL]	2.4±0.0d	16.3±1.2b	14.9±0.1c	18.7±0.2a
pH	6.20±0.01c	3.79±0.04a	3.93±0.01b	3.81±0.02a

[a] Shown results are average values and standard deviations of two independent fermentations, each sample extracted twice. Values in one row followed by the same lower case letter are not significantly different ($P < 0.05$); [b] see also Table 4-2; [c] see also Table 5-2, ND means not detected.

In order to compare the sourdough fermentation performance by different LAB starter species in wheat as a substrate, further sourdough analysis were performed (Table 6-1). The final pH values ranged closely from 3.8 to 3.9 and the highest TTA (18.7 mL) was observed in the wheat sourdoughs fermented with *Lb. reuteri* R29. A pH of 3.9 was measured in the CA. The values correlated well with acetate and lactate production. Most lactate and acetate were produced in doughs fermented with *Lb. reuteri* R29, 406 and 49 mmol/kg flour, respectively. Whereas *Lb. brevis* R2Δ showed the lowest formation of acetate and lactate. *Lb. amylovorus* DSM19280 as an obligate homofermentative LAB produced 368 mmol/kg flour lactate.

6.4.4 *In situ* antifungal activity and antifungal compounds of LAB fermented sourdough

Out of the 20 antifungal compounds analysed, extracted wheat sourdough fermented with *Lb. amylovorus* DSM19280 and *Lb. reuteri* R29 detected the greatest number of antifungal compounds (n = 9) at a higher concentration than what was detected from *Lb. brevis* R2Δ fermented sourdough (n = 8). The quantification results given in parts per million (ppm) are presented in Table 6-2.

Table 6-2 Antifungal compounds [ppm] analysed in freeze dried sourdoughs[a] and their single MIC.

LAB isolate/ Antifungal compounds	*Lb. amylovorus* DSM19280	*Lb. brevis* R2Δ	*Lb. reuteri* R29[b]	MIC (reference)
Azelaic acid	19.0±6.2	ND	7.2±3.9	0.1[c]
Benzoic acid	ND	ND	ND	1-100[c]
Caffeic acid	ND	0.4±0.0	2.1±0.1	200[d]
Catechol	ND	ND	ND	1,000[c]
p-Coumaric acid	ND	0.4±0.0	0.2±0.0	25-100[c]
Ferulic acid	ND	21.5±1.3	12.9±0.1	100[c]
Hydrocaffeic acid	1.2±0.1	ND	ND	10,000[c]
Hydrocinnamic acid	ND	ND	ND	100-1,000[c]
Hydroferulic acid	43.5±2.2	ND	ND	1,000[c]
4-Hydroxybenzoic acid	ND	ND	ND	1,000[c]
2-Hydroxydodecanoic acid	ND	ND	ND	5-25[e]
β-Hydroxylauric acid	ND	ND	ND	10-50[e]
2-Hydroxyisocaproic acid	44.8±2.5	130.6±4.1	360.0±0.6	72,000[f]
Hydroxymyristic acid	ND	0.1±0.0	ND	10-100[e]
4-Hydroxyphenyllactic acid	22.2±2.2	ND	ND	5,000[g]
Methylcinnamic acid	ND	ND	ND	670[g]
3-Phenyllactic acid	67.6±1.6	7.78±0.19	194.1±3.5	2,500-10,000[c,g]
Phloretic acid	1.3±0.1	ND	1.9±0.0	100-10,000[c]
Salicylic acid	0.6±0.1	1.2±0.2	3.3±0.0	0.1-1[c]
Vanillic acid	18.5±1.8	8.9±0.3	6.9±0.2	100[c]
Total concentration	218.6	171.0	588.7	-

[a] Shown results are average values ± standard deviations of two independent fermentations, each sample extracted three times, [b] see also Table 5-4, [c] Broberg et al. 2007, [d] Aziz et al. 1998, [e] Sjögren et al. 2003, [f] Sakko et al. 2014, [g] Ryan et al. 2011, ND means not detected.

The composition of the antifungal compounds produced during sourdough fermentation differed enormously among the LAB species. Overall, *Lb. reuteri* R29

was the best producer reaching a total concentration of 589 ppm. 2-Hydroxyisocaproic acid and 3-phenyllactic acid were the main compounds formed by this strain reaching a concentration of 360 and 194 ppm, respectively. Hydrocaffeic acid (1 ppm) and hydroferulic acid (44 ppm) and 4-hydroxyphenyllactic acid (22 ppm) resulted only from the metabolism of *Lb. amylovorus* DSM19280. For this strain the sum of antifungal carboxylic acids yielded a concentration of about 219 ppm. *Lb. brevis* R2Δ produced with 171 ppm a slightly lower total concentration of antifungal acids whereas 2-hydroxyisocaproic acid (131 ppm) and ferulic acid (22 ppm) were the main compounds. Furthermore, its 3-phenyllactic acid production yielded only 8 ppm. Chromatograms and mass spectra of 3-phenyllactic acid and 2-hydroxyisocaproic acid are depicted in Figure 6-4. The variance in peak size points out the differences in concentration of 3-phenyllactic acid and 2-hydroxyisocaproic acid between the three *Lactobacillus* strains.

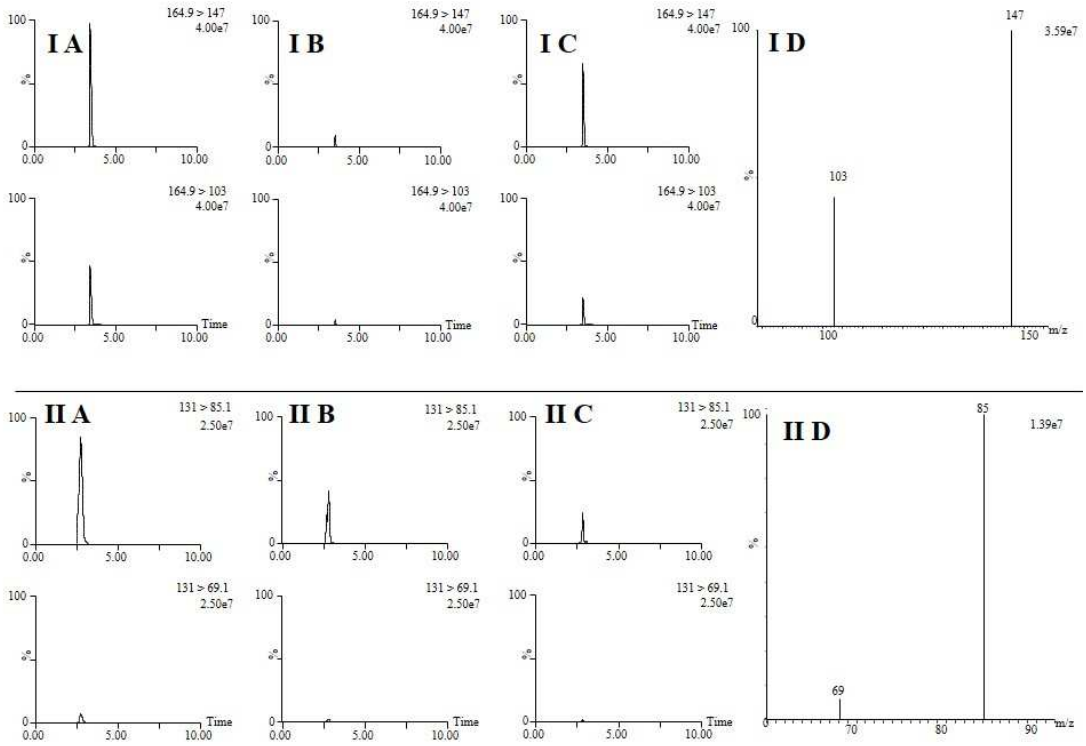


Figure 6-4 Chromatograms of 3-phenyllactic acid (I) and 2-hydroxyisocaproic acid (II) derived from extracts of wheat sourdough fermented with *Lb. reuteri* 29 (A), *Lb. brevis* R2Δ (B) and *Lb. amylovorus* DSM19280 (C). Mass spectra of 3-phenyllactic acid (ID) and 2-hydroxyisocaproic acid (IID).

Figure 6-5 represents an overview of the shelf life for the LAB sourdough breads as well as for the control breads and artificial spiked breads. The higher spoilage rate of the non-acidified control bread in comparison to the biologically acidified breads revealed that LAB fermented sourdough breads retarded naturally, with different extent, the outgrowth of environmental fungi. The maximum average shelf life of 10 mouldy-free days was obtained for the sourdough bread fermented with the antifungal strain *Lb. amylovorus* DSM19280. The non-acidified control showed first fungal spoilage after 4 days. Furthermore, fungi grew more rapidly on this control, whereas further fungal outgrowth was prevented for the sourdough breads. At the end of the storage period, about 30 % of the control bread slices were completely spoiled. *Lb. brevis* R2Δ and *Lb. reuteri* R29 fermented sourdough breads, in the same experimental conditions, extended the bread shelf life for two and three days,

respectively. Compared to the non-acidified control breads, the addition of CA increased the shelf-life by one more day. The CAS were spiked additionally with antifungal acids to test synergy among the compounds in different concentration levels. Remarkably, due to the inclusion of this antifungal "cocktail", the shelf life increased for another day for $CAS_{DSM19280}$ and $CAS_{R2\Delta}$ and 2 days for CAS_{R29} in comparison to the CA bread. Like that the shelf life of CAS_{R29} and *Lb. reuteri* R29 fermented sourdough bread was comparable. The bread with added CAP achieved a mould-free shelf life of 6 days.

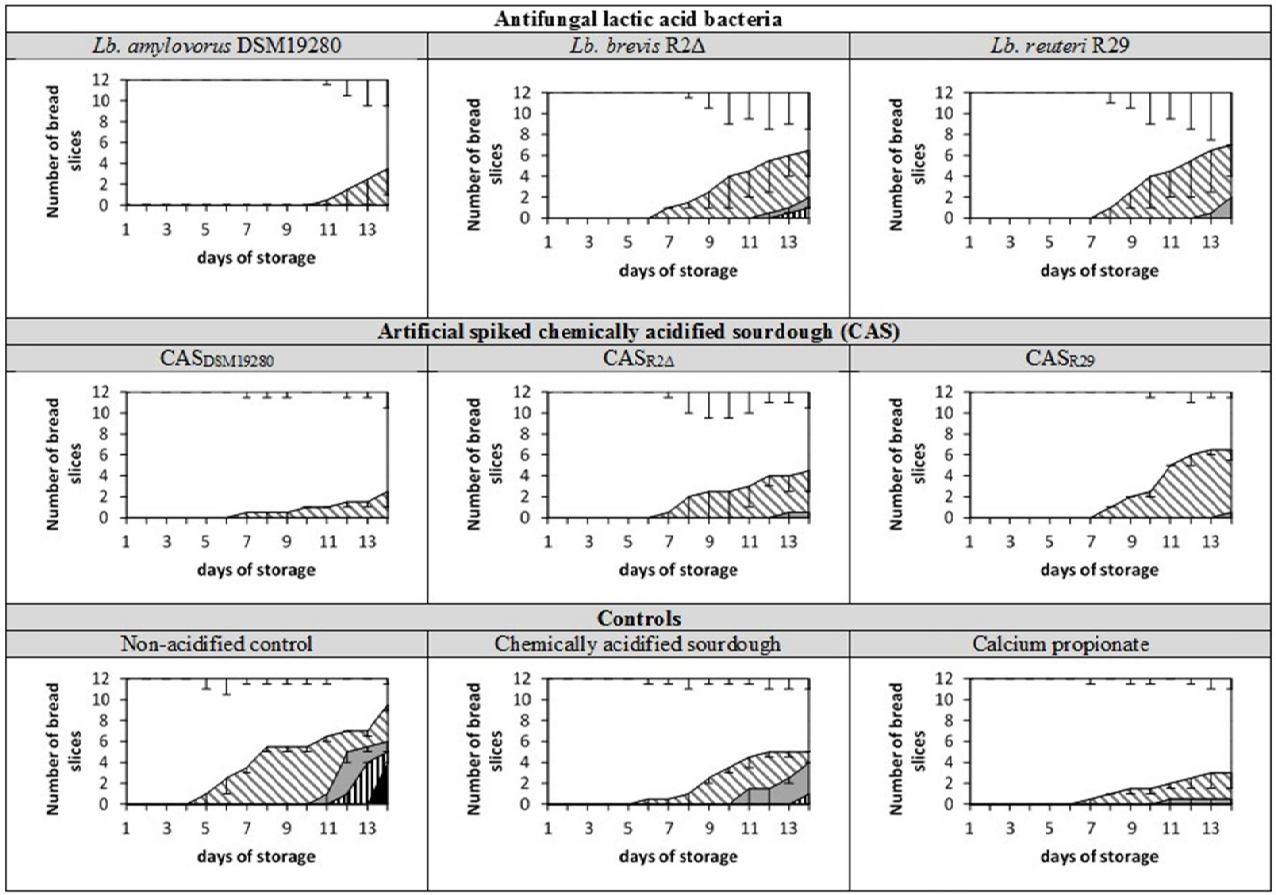


Figure 6-5 Shelf life of wheat bread against environmental moulds during a 14-day storage period. Bread spoilage is indicated as percentage of the total surface area of each of the 12 slices where fungal outgrowth occurred: Mould-free slices (white area), <10 % mouldy (grey diagonally striped area), 10–24 % mouldy (grey area), 25-49 % mouldy (black horizontally striped area) and >50 % mouldy (black area). Mean values are shown (n = 2); error bars indicate standard deviations.

6.5 Discussion

As expected, due to the different metabolism of the lactobacilli species, the strains showed different carbohydrate catabolism and acid production (Table 6-1). The preference of glucose fermentation by *Lb. amylovorus* DSM19280 was evident showing the lowest concentration left in the sourdough sample. The high amylolytic activity of this strain (Figure 6-1) further contributed to glucose provision and depletion. Hexose metabolism in homofermentative LAB occurs via the Emden-Meyerhoff pathway wherein glucose is generally metabolised before fructose and maltose (Gänzle et al., 2007). In heterofermentative LAB like *Lb. brevis* and *Lb. reuteri* maltose is metabolized by choice via the maltose phosphorylase pathway and fructose is utilized as an electron acceptor to mannitol (Gänzle et al., 2007).

All the three lactobacilli strains showed good inhibition *in vitro* against the indicator mould *F. culmorum* TMW4.2043 (Figure 6-2 and Figure 6-3). Nevertheless, technological application of biopreservatives requires appropriate *in situ* tests against a broad range of contaminants. Thus, a bakery environmental challenge test was applied on the different produced bread samples. The antifungal abilities of *Lb. amylovorus* DSM19280 showed the best performance in the *in situ* shelf life experiment, although the *in vitro* activity against the indicator mould showed higher inhibition with the other species. This result reinforces the suitability of *Lb. amylovorus* DSM19280 as an antifungal adjunct for food products.

The higher glucose concentration in the sourdoughs fermented by the heterofermentative *Lb. brevis* R2Δ and *Lb. reuteri* R29 and accordingly in the bread recipe could have also contributed to the higher mould growth on their bread slices. However, it is likely that most of this glucose was depleted during the bread dough fermentation by the Baker's yeast. Furthermore, due to the relatively low binding specificity of the fungi's transport protein for glucose other sugars will be transported if glucose is absent (Deacon, 2005).

Overall, a specific different profile of antifungal compounds was found in the sourdough samples. These variations are partially explained by species specific metabolic differences; however, as only one strain was chosen for each of the species, strain specific variations may additionally influence the profile of antifungal

compounds in wheat sourdough (Ryan et al., 2009). Some of the antifungal compounds analysed in the sourdough samples are derivates resulted from amino acid metabolism during LAB fermentation. Thus, the higher the proteolysis in sourdough fermentation takes place, the more potential substrates for antifungal compounds formation like 3-phenyllactic acid and 2-hydroxyisocaproic acid are formed. Previous studies have reported the 3-phenyllactic production by *Lb. amylovorus*, *Lb. reuteri* and for some strains of *Lb. brevis* (Gerez et al., 2009; Ryan et al., 2011; Valerio et al., 2004). All the lactobacilli tested in our study showed proteolytic activity on milk agar. *Lb. reuteri* R29 was the highest proteolytic active strain (Figure 6-1). Likewise, this strain also produced the highest amounts of 3-phenyllactic acid and 2-hydroxyisocaproic acid (Table 6-2). Phenolic acids like caffeic, ferulic or *p*-coumaric acid occur naturally in wheat, predominantly in bound form (Li et al., 2008). During sourdough fermentation free phenolics are released by cereal enzymes or chemical reactions which can be catalysed by LAB (Gänzle, 2014). Further metabolism of these free phenolics is possible due to the activity of strain-specific phenolic acid decarboxylases and cinnamic acid reductases (Svensson et al., 2010). As a result of cinnamic acid reductase activity hydrocaffeic acid, hydroferulic acid and phloretic acid can be formed. Caffeic acid, *p*-coumaric acid, and ferulic acid were not metabolized by *Lb. reuteri* (Svensson et al., 2010). In our study, the sourdough samples of *Lb. brevis* R2Δ and *Lb. reuteri* R29 still contained these unreduced forms (Table 6-2) indicating low or absent reductase activity. It seems that *Lb. amylovorus* DSM19280 metabolism harbours cinnamic acid reductases activity. In the sourdough sample fermented with this strain, remarkable amounts of hydroferulic acid and also hydrocaffeic and phloretic acid were present indicating that free ferulic acid, caffeic acid and *p*-coumaric acid were further metabolised (Table 6-2). More research is required to confirm which enzymes are involved in the ability of *Lb. amylovorus* DSM19280 to degrade phenolic acids in foods. Decarboxylation of phenolic acids and reduced phenolic acids can result into vinyl and ethyl phenols, respectively (Curiel et al., 2010). Low ferulic acid decarboxylation rate was observed for strains *Lb. brevis*, whereas *p*-coumaric acid and caffeic acid were completely decarboxylated (Curiel et al., 2010). The extracted sourdough sample derived from the *Lb. brevis* R2Δ fermentation resulted in the

highest ferulic acid content and very low contents of *p*-coumaric acid and caffeic acid were detected. However, the method of Brosnan et al. (2015) does not include the detection of vinyl and ethyl phenols. This leads to the need of further studies to clarify the metabolism of phenolic compounds on LAB.

In relation to the shelf life extension of the wheat sourdough breads, the analysis of antifungal carboxylic acids revealed that a higher concentration of these compounds cannot justify completely the growth inhibition of environmental moulds on the bread slices. In particular, although *Lb. reuteri* R29 produced the highest total concentration of these active compounds in the sourdough (Table 6-2), the bread did not show the longest shelf life (Figure 6-5). Cortés-Zavaleta et al. (2014) tested *in vitro* the antifungal activity of 7 lactobacilli. The maximal antifungal activity was found in both the highest 3-phenyllactic acid producer *Lb. casei* 21/1 (46 ppm) and the non-PLA producer *Lb. acidophilus* ATCC-4495. In our study, the longest shelf life was achieved with the *Lb. amylovorus* DSM19280 fermented sourdough which yielded in less than half of the active antifungal acids (Table 6-2). In an additional experiment, antifungal carboxylic acids were spiked as a "cocktail" into a chemically acidified sourdough. A mould-free shelf life of 7 days was reached with the highest concentration CAS_{R29} which was similar to the shelf life of the *Lb. reuteri* R29 fermented sourdough bread (Figure 6-5). The use of 20 % sourdough in the bread recipe correlates to a 1:5 dilution (in bread). Taking bake loss into account (12 %), the average concentration of the antifungal carboxylic acids in the bread would amount about 30 ppm and for lactic and acetic acid maximal 4500 and 360 ppm, respectively. An overview about the single MIC for the compounds is also presented in Table 6-2. For example, the MIC ranging from 2,500 to 10,000 ppm for 3-phenyllactic acid and 5,000 ppm for 4-hydroxyphenyllactic acid (Broberg et al., 2007; Ryan et al., 2011). 2-Hydroxyisocaproic acid has a broad antibacterial activity, but little is known of the efficacy against pathogenic fungi. Sakko et al. (2014) determined a very high single MIC (72,000 ppm) for 2-hydroxyisocaproic acid against *Aspergillus fumigatus* and *A. terreus*. At pH 5 the MIC values for acetic acid ranged 1200–7200 ppm and above 45,000 ppm for lactic acid (Lind et al., 2005). Our results confirm that under acidic conditions synergistic activity of these compounds in concentration levels far below their single MIC exist for mould inhibition.

3-Phenyllactic acid and the other compounds are not allowed by legislation to be used as food additives. Researching the metabolism pathway, health and possible toxicity effects of those antifungal compounds should be conducted to ascertain their food safety regardless the authorised use of many lactobacilli within the European Union (EFSA, 2012).

Ryan et al. (2011), Lynch et al. (2014) and Oliveira et al. (2015) investigated antifungal metabolites produced by antifungal *Lb. amylovoru*s DSM19280 and *Lb. reuteri* R29 in MRS broth and wort. Due to the higher production of the antifungal carboxylic acids in this study, wheat flour seems to be a better substrate. This is not surprisingly, flour has a higher protein content than wort and MRS and since during sourdough fermentation flour enzymes are activated more cofactors are provided for LAB metabolism. In a previous study of our group, UV-active antifungal compounds extracted from quinoa and wheat sourdough were analysed using a HPLC-DAD method (Axel et al., 2015). Low recovery rates and high matrix effects complicated data evaluation. In the current study, matrix effects could be minimalized by matrix calibration and peak purity was confirmed with mass spectrometry, Figure 6-4 (Brosnan et al., 2015). However, the diversity of antifungal compounds and different methodical approaches to analyse them in the literature, leads the authors to speculate that other unknown antifungal compounds not analysed in this study could also be present and further contribute to mould inhibition. This could one reason, why *Lb. amylovorus* DSM19280 fermented wheat sourdough bread reached the longest shelf life. Further investigations could explore peptide-based antifungal compounds, which might act additionally for the distinct shelf life extension.

In conclusion, *Lb. amylovorus* DSM19280, *Lb. brevis* R2Δ and *Lb. reuteri* R29 produced a range of known antifungal active carboxylic acids in different quantities during wheat flour fermentation. Interestingly, the maximal antifungal activity *in situ* could not be related to *Lb. reuteri* R29 being the highest producer of these compounds. However, the addition of CAS to the bread recipe succeeded in a longer shelf life than achieved only by acidifying the dough evidencing their contribution to the antifungal activity and their synergy.

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6.7 References

Arbeitsgemeinschaft Getreideforschung e.V., 1994. Standard-Methoden für Getreide, Mehl und Brot. Verlag Moritz Schäfer: Detmold, Germany, vol. 7.

Arendt, E.K., Dal Bello, F., Ryan, L.A.M., 2009. Increasing the shelf life of bakery and patisserie products by using the antifungal *Lactobacillus amylovorus* DSM 19280. Patent: WO/2009/14

Axel, C., Roecker, B., Brosnan, B., Zannini, E., Furey, A., Coffey, A., Arendt, E.K., 2015. Application of *Lactobacillus amylovorus* DSM19280 in gluten-free sourdough bread to improve the microbial shelf life. Food Microbiology 47, 36–44. doi:10.1016/j.fm.2014.10.005

Aziz, N.H., Farag, S.E., Mousa, L.A.A., Abo-Zaid, M.A., 1998. Comparative antibacterial and antifungal effects of some phenolic compounds. Microbios 93, 43–54.

Belz, M.C.E., Mairinger, R., Zannini, E., Ryan, L.A.M., Cashman, K.D., Arendt, E.K., 2012. The effect of sourdough and calcium propionate on the microbial shelf-life of salt reduced bread. Applied Microbiology and Biotechnology 96, 493–501. doi:10.1007/s00253-012-4052-x

Black, B.A., Zannini, E., Curtis, J.M., Gänzle, M.G., 2013. Antifungal hydroxy fatty acids produced during sourdough fermentation: microbial and enzymatic pathways, and antifungal activity in bread. Applied and Environmental Microbiology 79, 1866–73. doi:10.1128/AEM.03784-12

Broberg, A., Jacobsson, K., Ström, K., Schnürer, J., 2007. Metabolite profiles of lactic acid bacteria in grass silage. Applied and Environmental Microbiology 73, 5547–5552. doi:10.1128/aem.02939-06

Brosnan, B., Axel, C., Coffey, A., Arendt, E.K., Furey, A., 2015. Development of an UHPLC-MS/MS method for the detection of antifungal compounds in lactic acid bacteria fermented sourdough. Food Chemistry (Manuscript for submission)

Brosnan, B., Coffey, A., Arendt, E.K., Furey, A., 2014. The QuEChERS approach in a novel application for the identification of antifungal compounds produced by lactic acid bacteria cultures. Talanta 129, 364–373. doi:10.1016/j.talanta.2014.05.006

Coda, R., Cassone, A., Rizzello, C.G., Nionelli, L., Cardinali, G., Gobbetti, M., 2011. Antifungal activity of *Wickerhamomyces anomalus* and *Lactobacillus plantarum* during sourdough fermentation: identification of novel compounds and long-term effect during storage of wheat bread. Applied and Environmental Microbiology 77, 3484–92. doi:10.1128/AEM.02669-10

Cortés-Zavaleta, O., López-Malo, a, Hernández-Mendoza, a, García, H.S., 2014. Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. International Journal of Food Microbiology 173, 30–5. doi:10.1016/j.ijfoodmicro.2013.12.016

Crowley, S., Mahony, J., van Sinderen, D., 2013. Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. Trends in Food Science & Technology 33, 93–109. doi:10.1016/j.tifs.2013.07.004

Curiel, J.A., Rodríguez, H., Landete, J.M., de las Rivas, B., Muñoz, R., 2010. Ability of *Lactobacillus brevis* strains to degrade food phenolic acids. Food Chemistry 120, 225–229. doi:10.1016/j.foodchem.2009.10.012

Dal Bello, F., Clarke, C.I., Ryan, L.A.M., Ulmer, H., Schober, T.J., Ström, K., Sjögren, J., van Sinderen, D., Schnürer, J., Arendt, E.K., Strom, K., Sjogren, J., Schnurer, J., 2007. Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. Journal of Cereal Science 45, 309–318. doi:10.1016/j.jcs.2006.09.004

Dallagnol, A.M., Pescuma, M., De Valdez, G.F., Rollán, G., 2013. Fermentation of quinoa and wheat slurries by *Lactobacillus plantarum* CRL 778: proteolytic activity. Applied Microbiology and Biotechnology 97, 3129–40. doi:10.1007/s00253-012-4520-3

Deacon, J. W., 2005. Chapter 6 Fungal nutrition: The carbon and energy sources of fungi, in Fungal Biology (4th ed), Wiley-Blackwell, pp. 111-113. ISBN 978-1-4051-3066-0

EFSA, 2012. Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update). EFSA Journal;10(12):3020. 1–84. doi:10.2903/j.efsa.2012.3020.

Gänzle, M.G., 2014. Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiology 37, 2–10. doi:10.1016/j.fm.2013.04.007

Gänzle, M.G., Vermeulen, N., Vogel, R.F., 2007. Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiology 24, 128–38. doi:10.1016/j.fm.2006.07.006

Gerez, C.L., Torino, M.I., Rollán, G., Font de Valdez, G., 2009. Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. Food Control 20, 144–148. doi:10.1016/j.foodcont.2008.03.005

Li, L., Shewry, P.R., Ward, J.L., 2008. Phenolic acids in wheat varieties in the HEALTHGRAIN Diversity Screen. Journal of Agricultural and Food Chemistry 56, 9732–9. doi:10.1021/jf801069s

Lind, H., Jonsson, H., Schnürer, J., 2005. Antifungal effect of dairy propionibacteria - Contribution of organic acids. International Journal of Food Microbiology 98, 157–165. doi:10.1016/j.ijfoodmicro.2004.05.020

Lynch, K.M., Pawlowska, A.M., Brosnan, B., Coffey, A., Zannini, E., Furey, A., McSweeney, P.L.H., Waters, D.M., Arendt, E.K., 2014. Application of *Lactobacillus amylovorus* as an antifungal adjunct to extend the shelf-life of Cheddar cheese. International Dairy Journal 34, 167–173. doi:10.1016/j.idairyj.2013.07.017

Mauch, A., Dal Bello, F., Coffey, A., Arendt, E.K., 2010. The use of *Lactobacillus brevis* PS1 to *in vitro* inhibit the outgrowth of *Fusarium culmorum* and other common *Fusarium* species found on barley. International Journal of Food Microbiology 141, 116–21. doi:10.1016/j.ijfoodmicro.2010.05.002

Meroth, C.B., Walter, J., Hertel, C., Brandt, J., Hammes, W.P., Brandt, M.J., 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69, 475–482. doi:10.1128/aem.69.1.475-482.2003

Miescher Schwenninger, S., Lacroix, C., Truttmann, S., Jans, C., Spörndli, C., Bigler, L., Meile, L., 2008. Characterization of low-molecular-weight antiyeast metabolites produced by a food-protective *Lactobacillus-Propionibacterium* coculture. Journal of Food Protection 71, 2481–2487.

Mu, W., Yu, S., Zhu, L., Zhang, T., Jiang, B., 2012. Recent research on 3-phenyllactic acid, a broad-spectrum antimicrobial compound. Applied Microbiology and Biotechnology 95, 1155–63. doi:10.1007/s00253-012-4269-8

Ndagano, D., Lamoureux, T., Dortu, C., Vandermoten, S., Thonart, P., 2011. Antifungal activity of 2 lactic acid bacteria of the *Weissella* genus isolated from food. Journal of Food Science 76, M305–311. doi:10.1111/j.1750-3841.2011.02257.x

Niku-Paavola, M.L., Laitila, A., Mattila-Sandholm, T., Haikara, A., 1999. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. Journal of Applied Microbiology 86, 29–35.

Oliveira, P.M., Brosnan, B., Furey, A., Coffey, A., Zannini, E., Arendt, E.K., 2015. Lactic acid bacteria bioprotection applied to the malting process. Part I: Strain characterization and identification of antifungal compounds. Food Control 51, 433–443. doi:10.1016/j.foodcont.2014.07.004

Pawlowska, A.M., Zannini, E., Coffey, A., Arendt, E.K., 2012. "Green preservatives": combating fungi in the food and feed industry by applying antifungal lactic acid bacteria. Advances in Food and Nutrition Research 66, 217–238. doi:10.1016/b978-0-12-394597-6.00005-7

Ryan, L.A.M., Dal Bello, F., Arendt, E.K., 2008. The use of sourdough fermented by antifungal LAB to reduce the amount of calcium propionate in bread. International Journal of Food Microbiology 125, 274–278. doi:10.1016/j.ijfoodmicro.2008.04.013

Ryan, L.A.M., Dal Bello, F., Czerny, M., Koehler, P., Arendt, E.K., 2009. Quantification of phenyllactic acid in wheat sourdough using high resolution gas chromatography-mass spectrometry. Journal of Agricultural and Food Chemistry 57, 1060–4. doi:10.1021/jf802578e

Ryan, L.A.M., Zannini, E., Dal Bello, F., Pawlowska, A., Koehler, P., Arendt, E.K., 2011. *Lactobacillus amylovorus* DSM 19280 as a novel food-grade antifungal agent for bakery products. International Journal of Food Microbiology 146, 276–283. doi:10.1016/j.ijfoodmicro.2011.02.036

Sakko, M., Moore, C., Novak-Frazer, L., Rautemaa, V., Sorsa, T., Hietala, P., Järvinen, a, Bowyer, P., Tjäderhane, L., Rautemaa, R., 2014. 2-Hydroxyisocaproic acid is fungicidal for *Candida* and *Aspergillus* species. Mycoses 57, 214–21. doi:10.1111/myc.12145

Sjögren, J., Magnusson, J., Broberg, A., Schnürer, J., Kenne, L., 2003. Antifungal 3-Hydroxy Fatty Acids from Lactobacillus plantarum MiLAB 14. Applied and Environmental Microbiology 69, 7554–7557. doi:10.1128/AEM.69.12.7554

Svensson, L., Sekwati-Monang, B., Lutz, D.L., Schieber, A., Gänzle, M.G., 2010. Phenolic acids and flavonoids in nonfermented and fermented red sorghum (*Sorghum bicolor (L.) Moench*). Journal of Agricultural and Food Chemistry 58, 9214–20. doi:10.1021/jf101504v

Valerio, F., Lavermicocca, P., Pascale, M., Visconti, A., 2004. Production of phenyllactic acid by lactic acid bacteria: an approach to the selection of strains contributing to food quality and preservation. FEMS Microbiology Letters 233, 289–95. doi:10.1016/j.femsle.2004.02.020

Varsha, K.K., Priya, S., Devendra, L., Nampoothiri, K.M., 2014. Control of spoilage fungi by protective lactic acid bacteria displaying probiotic properties. Applied Biochemistry and Biotechnology 172, 3402–13. doi:10.1007/s12010-014-0779-4

Waters, D.M., Parlet, C., Moroni, A., Arendt, E.K., 2012. Identification of the fungal microflora of coffee beans from different origins and evaluation of different decontamination concepts, in: 24th International Conference on Coffee Science, ASIC Costa Rica 24th. pp. 145–152.

Wehrle, K., Crowe, N., van Boeijen, I., Arendt, E.K., 1999. Screening methods for the proteolytic breakdown of gluten by lactic acid bacteria and enzyme preparations. European Food Research and Technology 209, 428–433. doi:10.1007/s002170050521

Wolter, A., Hager, A.S., Zannini, E., Czerny, M., Arendt, E.K., 2014. Impact of sourdough fermented with *Lactobacillus plantarum* FST 1.7 on baking and sensory properties of gluten-free breads. European Food Research and Technology 239, 1–12. doi:10.1007/s00217-014-2184-1

Chapter 7 General Discussion

7.1 General Discussion

In recent years, interest in food biopreservation has dramatically increased. Evidence for this movement is shown in the bakery market since the advertisement of the absence of additives/preservatives represented 21 % of new bread products launched in Europe in 2013/2014 (Mintel, 2014). Fermentation, a process based on the growth of microorganisms in foods, is considered to be one of the most established practices in food biopreservation. The microorganism most widely used as starter cultures are LAB. A literature review was conducted as part of this thesis (Chapter 2). This study revealed that sourdough fermented with antifungal strains of LAB is the most studied biopreservation method for bread products and serves as a high-potential biological ingredient to produce gluten-containing and gluten-free breads with an improved nutritional value, bread quality and safety due to an extended shelf life meeting present consumer needs (Arendt et al., 2007; Moroni et al., 2009; Pawlowska et al., 2012; Zannini et al., 2012). Nevertheless, only a few applications of selected LAB starters are currently documented both at artisan and industrial levels (Corsetti, 2013). This makes the investigation of more suitable LAB strains important. Moreover, increasing the knowledge about the origin of the antifungal effect is fundamental for further enhancement of biopreservation. The production of antifungal compounds depends on the microbial growth and metabolic activity of each strain which are further influenced by endogenous factors such as substrate composition, enzymes and microbial interaction (Meroth et al., 2003; Van der Meulen et al., 2007; Vermeulen et al., 2006).

The LAB strains *Lb. amylovorus* DSM19280, *Lb. brevis* R2Δ and *Lb. reuteri* R29 were investigated for *in situ* cereal fermentations based on preliminary *in vitro* trials and previous antifungal studies.

A HRGC/MS method for the identification and quantification of the antifungal active compounds cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Met-Pro) and cyclo(Phe-Pro) was successfully developed by using stable isotope dilution assays with the deuterated counterparts (Chapter 3). This study represents the first report of quantification of

cyclic dipeptides using SIDAs in LAB fermentates. Overall, the concentrations of cyclo(Leu-Pro), cyclo(Pro-Pro), and cyclo(Phe-Pro) increased only moderately by the fermentation activity of the selected microorganism (Table A-I). Statistical analysis of the quantitative results showed, that in the *Lb. brevis* R2Δ fermented MRS-broth, the concentrations of cyclo(Leu-Pro), cyclo(Pro-Pro) and cyclo(Phe-Pro) were significantly higher ($P < 0.05$), as in wort for cyclo (Leu-Pro) when compared to their controls. A connection was linked between the formation of these three cyclic dipeptides and *Lb. brevis* R2Δ fermentation. However, the determined increases induced by *Lb. brevis* R2Δ amounted only to about 20 % in MRS broth and 10 % in wort. This was associated most likely as a sight effect resulted by acid induced cyclization of more precursor formed linear dipeptides. In *Lb. reuteri* R29 fermented MRS-broth the concentration of only two cyclic dipeptides, cyclo(Leu-Pro) and cyclo(Phe-Pro) increased significantly ($P < 0.05$). Whereas *Lb. amylovorus* DSM19280 fermentation induced the least increase in cyclic dipeptides (Table A-I). Concentrations of cyclic dipeptides investigated in the LAB fermented supernatants in this study are much below the MIC necessary to inhibit many of the fungi reported, as an individual antifungal compound (Ryan et al., 2011; Ström et al., 2002; Yan et al., 2004). Thus, the antifungal activity of the cyclic dipeptides plays here a minor role. Synergistic effects along with other antifungal compounds are possible, but require further studies. The novel application of deuterated cyclic dipeptides for SIDA reported here can potentially be used for analyses of cyclic dipeptides in other liquid matrices, including beverages. However, the synthesis of the ^{2}H-labelled cyclic dipeptides should be improved since it resulted in low yields and multicomponent mixtures of labelled isotopomers. A better H–D exchange might overcome the encountered problems by using heterogeneous catalyser systems (Pd/C-, Pt/C-, or/and Rh/C) in the presence of deuterium oxide and hydrogen gas (Modutlwa et al., 2010).

The potential and suitability of the antifungal strains to improve the microbial shelf life of gluten-containing and gluten-free bread was evaluated in the second part of this thesis (Chapter 4, Chapter 5 and Chapter 6). Therefore, three different flours

(quinoa, rice and wheat) were selected as fermentation substrates and fully characterised (Table 5-1). The shelf life of the breads was monitored using the mould environmental challenge method as developed by Dal Bello et al. (2007).

The suitability of *Lb. amylovorus* DSM19280 to ferment gluten-free quinoa flour, where the sourdough was then incorporated into a gluten-free bread recipe was investigated (Chapter 4). Evaluation of bread characteristics such as specific volume or crumb hardness, revealed that the addition of *Lb. amylovorus* fermented sourdough improved gluten-free bread quality like higher specific volumes, softer crumb structure and slower staling rates. Sourdough addition to gluten-free bread is reported to better keep freshness over storage (Moore et al., 2007; Wolter et al., 2014). High starch contents trigger staling in gluten-free breads (Hager et al., 2012). The low starch content of quinoa flour seemed to have a significant influence on its low rate of staling, which can be even improved by the addition of sourdough. Nevertheless, since a basic recipe was used without the inclusion of other ingredients/additives like fibres, hydrocolloids or egg protein usually added to gluten-free bread formulation (Zannini et al., 2012), the quinoa bread quality in this study was inferior in comparison to available commercial products. However, the antifungal quinoa sourdough serves as a high potential biopreservative ingredient for such commercial products.

The mould free shelf life of the quinoa breads increased for 4 days (+ 200 %) in comparison to the non-acidified control when the sourdough fermented with *Lb. amylovorus* DSM19280 was incorporated in the bread recipe (Figure 4-3). A prolongation of only 2 days (+ 100 %) was achieved with addition of *Lb. amylovorus* DSM20531^T fermented and CA sourdough. *Lb. amylovorus* DSM20531^T was chosen as a non-antifungal negative control strain. The better biopreservative efficacy of *Lb. amylovorus* DSM19280 fermented quinoa sourdough was related to the higher concentration of antifungal compounds analysed in this sourdough. The concentration of 4-hydroxyphenyllactic acid, phloretic acid, 3-phenyllactic acid and hydroferulic acid were significantly higher ($P<0.01$) in the quinoa sourdough fermented with the antifungal *Lb. amylovorus* DSM19280 when compared to the control strain, thus indicating their contribution to the antifungal activity. These

results confirm previous studies conducted, that the antifungal activity is strain specific (Oliveira et al., 2015; Ryan et al., 2011, 2009). 15 UV-active antifungal compounds extracted from quinoa and wheat sourdough were analysed using a HPLC-DAD method (Figure 4-4); the extraction method was adapted from Brosnan et al. (2014). Low recovery rates and high matrix effects complicated data evaluation. Since the method of Brosnan et al. (2014) describes the extraction of antifungal compounds from MRS-broth matrices, the difficulties encountered with the analysis of the antifungal compounds in Chapter 4 were probably matrix related applying the extraction method to the freeze dried sourdough samples. These matrix effects were minimalized with an improved extraction procedure and matrix calibration (Brosnan et al., 2015). Moreover, peak purity of the antifungal compounds was confirmed with mass spectrometry which further implemented the detection of 5 more non-UV-active compounds (Brosnan et al., 2015).

Using this improved method, the production of the antifungal compounds was studied as part of the sourdough investigation using the antifungal strain *Lb. reuteri* R29 for the fermentation of quinoa, rice and wheat flour (Chapter 5). The flour characterisation and known metabolic pathways generally described for *Lb. reuteri* enabled correlations between the sourdough fermentation, *Lb. reuteri* R29 metabolism and substrate features. *Lb. reuteri* R29 sourdough fermentation of the different flour substrates generated a complex and significantly different profile of antifungal compounds (Table 5-4). The protein content of quinoa flour was found to be superior for the production of the antifungal carboxylic acids, 3-phenyllactic acid and 2-hydroxyisocaproic acid originated from the metabolism of the amino acids phenylalanine and leucine, respectively. As found for rice and wheat sourdough, a lower protein content in the substrate resulted in a lower production of these antifungal compounds. In contrast, a higher protease activity during fermentation (rice) might have compensated its lack of protein which could give rise to more amino acid or peptide availability and concomitantly higher antifungal compounds. This could explain the higher 2-hydroxyisocaproic acid content in the rice sourdough when compared to the wheat sourdough. Another group of antifungal compounds belong to phenolic acids which include derivatives of either cinnamic acid (*p*-coumaric,

caffeic, ferulic acids) or benzoic acid (4-hydroxybenzoic, vanillic acids). Repeatedly, the composition of the quinoa flour favoured the formation of antifungal compounds, here *p*-coumaric, caffeic, ferulic acid and vanillic acid.

Regarding the shelf life, the addition of *Lb. reuteri* R29 fermented sourdough could prolong the mould-free days (Figure 5-3). However, a better preservative effect in quinoa sourdough bread was achieved when *Lb. amylovorus* DSM19280 fermented quinoa sourdough was used (Chapter 4), although the concentration of the antifungal compounds found in that biopreservative were much lower.

The same effect was observed in the last study conducted for this thesis (Chapter 6). *Lb. amylovorus* DSM19280, *Lb. brevis* R2Δ and *Lb. reuter*i R29 were used for wheat flour fermentations and their *in situ* antifungal performance in the breads were tested. As expected, due to the different metabolism of the *Lactobacillus* species, the strains showed different carbohydrate catabolism and acid production (Table 6-1). Overall a specific different profile of antifungal compounds was found in the sourdough samples (Table 6-2). *Lb. reuteri* R29 was the best producer reaching a total concentration of the antifungal compounds of 589 ppm. 2-Hydroxyisocaproic acid and 3-phenyllactic acid were the main compounds formed by this strain reaching a concentration of 360 and 194 ppm, respectively. *Lb. brevis* R2Δ produced with 171 ppm the lowest total concentration of antifungal acids with 131 ppm 2-hydroxyisocaproic acid and 22 ppm ferulic acid being the main compounds. Furthermore, its 3-phenyllactic acid production yielded only 8 ppm. The variations in concentration of the antifungal compounds were partially explained by species-specific metabolic differences; however, as only one strain was chosen for each of the species, strain specific variations may additionally influence the profile of antifungal compounds in wheat sourdough (Ryan et al., 2009). This was also found in the study conducted in Chapter 4. The highest proteolytic activity of the antifungal *Lb. reuteri* R29 might have contributed to the higher level of 2-hydroxyisocaproic acid and 3-phenyllactic acid. However, this would need to be confirmed in further studies. In relation to the shelf life extension of the wheat sourdough breads, the analysis of antifungal carboxylic acids revealed, that a higher concentration of these compounds cannot justify completely the growth inhibition of environmental moulds on the bread

slices. In particular, although *Lb. reuteri* R29 produced the highest total concentration of these active compounds in the sourdough (Table 6-2), the bread did not show the longest shelf life (Figure 6-5) as this was assigned to *Lb. amylovorus* DSM19280. In an additional experiment, antifungal carboxylic acids, based on the analysis of the biologically acidified samples, were spiked into a chemically acidified sourdough. A mould-free shelf life of 7 days (+ 75 %) was reached with the highest concentration CAS_{R29} which was similar to the shelf life of the *Lb. reuteri* R29 fermented sourdough bread (Figure 6-5). The shelf life for $CAS_{DSM19280}$ and $CAS_{R2\Delta}$ increased only by + 50 %. Conclusively, the chemically application of the antifungal compounds succeeded in a longer shelf life than achieved only by acidifying the dough (+ 25 %) evidencing their contribution to the antifungal activity and their synergy. Considering the addition of sourdough at 20 % flour based to the bread recipe and taking bake loss into account, the average concentration of the antifungal compounds in the bread would amount about 30 ppm and for lactic and acetic acid maximal 4500 and 360 ppm, respectively. The results further confirm, that under acidic conditions synergistic activity of these compounds in concentration levels far below their single MIC exist for mould inhibition as proposed by other studies (Cortés-Zavaleta et al., 2014; Miescher Schwenninger et al., 2008; Ndagano et al., 2011; Niku-Paavola et al., 1999). Bread is baked at high oven temperatures (200-220 °C). Therefore, bread preservatives should be heat stable and losses due to evaporation should also be avoided. The antifungal carboxylic acids analysed in this study have boiling points generally higher than 250 °C. Heat stability of 3-phenyllactic acid was further proven (Cortés-Zavaleta et al., 2014; Gerez et al., 2013). Moreover, the bread crust which is formed during baking can act as a barrier to prevent compounds from being transferred from the crumb to external space (Onishi et al., 2011).

For the outstanding antifungal performance of *Lb. amylovorus* DSM19280, it leads to speculate, that other unknown antifungal compounds not analysed in this study are also produced by this strain and further contribute to mould inhibition. However, this requires further studies. Furthermore, it would be interesting to study, how the production of the antifungal compounds could be improved for examples by the

additional usage of protein/peptides and proteolytic enzymes or by the fermentation of phenolic rich flour bran fractions.

Overall, the findings of this study showed that *Lb. amylovorus* DSM19280, *Lb. brevis* R2Δ and *Lb. reuteri* R29 are competitive bio-protective starters for the successful production of antifungal active sourdough using wheat and gluten-free flours as substrate. Furthermore, this study contributed to further knowledge about the origin of the antifungal effect helping to further enhance biopreservation.

7.2 References

Arendt, E.K., Ryan, L.A.M., Dal Bello, F., 2007. Impact of sourdough on the texture of bread. Food Microbiology 24, 165–174. doi:10.1016/j.fm.2006.07.011

Brosnan, B., Axel, C., Coffey, A., Arendt, E.K., Furey, A., 2015. Development of an UHPLC-MS/MS method for the detection of antifungal compounds in lactic acid bacteria fermented sourdough. Food Chemistry (Manuscript for submission)

Brosnan, B., Coffey, A., Arendt, E.K., Furey, A., 2014. The QuEChERS approach in a novel application for the identification of antifungal compounds produced by lactic acid bacteria cultures. Talanta 129, 364–373. doi:10.1016/j.talanta.2014.05.006

Corsetti, A., 2013. Chapter 4 Technology of Sourdough Fermentation and Sourdough Applications, in: Gobetti, M., Gänzle, M.G. (Eds.), Handbook on Sourdough Biotechnology. Springer, pp. 85–103. doi:10.1007/978-1-4614-5425-0

Cortés-Zavaleta, O., López-Malo, a, Hernández-Mendoza, a, García, H.S., 2014. Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. International Journal of Food Microbiology 173, 30–5. doi:10.1016/j.ijfoodmicro.2013.12.016

Dal Bello, F., Clarke, C.I., Ryan, L.A.M., Ulmer, H., Schober, T.J., Ström, K., Sjögren, J., van Sinderen, D., Schnürer, J., Arendt, E.K., Strom, K., Sjogren, J., Schnurer, J., 2007. Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. Journal of Cereal Science 45, 309–318. doi:10.1016/j.jcs.2006.09.004

Gerez, C.L., Torres, M.J., Font de Valdez, G., Rollan, G., 2013. Control of spoilage fungi by lactic acid bacteria. Biological Control 64, 231–237. doi:10.1016/j.biocontrol.2012.10.009

Hager, A.S., Wolter, A., Czerny, M., Bez, J., Zannini, E., Arendt, E.K., 2012. Investigation of product quality, sensory profile and ultrastructure of breads made from a range of commercial gluten-free flours compared to their wheat counterparts. European Food Research and Technology 235, 333–344. doi:10.1007/s00217-012-1763-2

Meroth, C.B., Walter, J., Hertel, C., Brandt, J., Hammes, W.P., Brandt, M.J., 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69, 475–482. doi:10.1128/aem.69.1.475-482.2003

Miescher Schwenninger, S., Lacroix, C., Truttmann, S., Jans, C., Spörndli, C., Bigler, L., Meile, L., 2008. Characterization of low-molecular-weight antiyeast metabolites produced by a food-protective *Lactobacillus-Propionibacterium* coculture. Journal of Food Protection 71, 2481–2487.

Mintel, 2014. Category Insight: Bread & Bread Products. mintel.com 1–6.

Modutlwa, N., Maegawa, T., Monguchi, Y., Sajiki, H., 2010. Synthesis of deuterium-labelled drugs by hydrogen-deuterium (H-D) exchange using heterogeneous catalysis. Journal of Labelled Compounds and Radiopharmaceuticals 53, 686–692. doi:10.1002/jlcr.1848

Moore, M.M., Juga, B., Schober, T.J., Arendt, E.K., 2007. Effect of lactic acid bacteria on properties of gluten-free sourdoughs, batters, and quality and ultrastructure of gluten-free bread. Cereal Chemistry 84, 357–364. doi:10.1094/cchem-84-4-0357

Moroni, A. V, Dal Bello, F., Arendt, E.K., 2009. Sourdough in gluten-free bread-making: An ancient technology to solve a novel issue? Food Microbiology 26, 676–684. doi:10.1016/j.fm.2009.07.001

Ndagano, D., Lamoureux, T., Dortu, C., Vandermoten, S., Thonart, P., 2011. Antifungal activity of 2 lactic acid bacteria of the *Weissella* genus isolated from food. Journal of Food Science 76, M305–311. doi:10.1111/j.1750-3841.2011.02257.x

Niku-Paavola, M.L., Laitila, A., Mattila-Sandholm, T., Haikara, A., 1999. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. Journal of Applied Microbiology 86, 29–35.

Oliveira, P.M., Brosnan, B., Furey, A., Coffey, A., Zannini, E., Arendt, E.K., 2015. Lactic acid bacteria bioprotection applied to the malting process. Part I: Strain characterization and identification of antifungal compounds. Food Control 51, 433–443. doi:10.1016/j.foodcont.2014.07.004

Onishi, M., Inoue, M., Araki, T., Iwabuchi, H., Sagara, Y., 2011. Odorant transfer characteristics of white bread during baking. Bioscience, Biotechnology, and Biochemistry 75, 261–267. doi:10.1271/bbb.100572

Pawlowska, A.M., Zannini, E., Coffey, A., Arendt, E.K., 2012. "Green preservatives": combating fungi in the food and feed industry by applying antifungal lactic acid bacteria. Advances in Food and Nutrition Research 66, 217–238. doi:10.1016/b978-0-12-394597-6.00005-7

Ryan, L.A.M., Dal Bello, F., Arendt, E.K., Koehler, P., 2009. Detection and quantitation of 2,5-diketopiperazines in wheat sourdough and bread. Journal of Agricultural and Food Chemistry 57, 9563–8. doi:10.1021/jf902033v

Ryan, L.A.M., Zannini, E., Dal Bello, F., Pawlowska, A., Koehler, P., Arendt, E.K., 2011. *Lactobacillus amylovorus* DSM 19280 as a novel food-grade antifungal agent for bakery products. International Journal of Food Microbiology 146, 276–283. doi:10.1016/j.ijfoodmicro.2011.02.036

Ström, K., Sjögren, J., Broberg, A., Schnürer, J., 2002. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Applied and Environmental Microbiology 68, 4322–4327. doi:10.1128/aem.68.9.4322

Van der Meulen, R., Scheirlinck, I., Van Schoor, A., Huys, G., Vancanneyt, M., Vandamme, P., De Vuyst, L., 2007. Population dynamics and metabolite target analysis of lactic acid bacteria during laboratory fermentations of wheat and spelt sourdoughs. Applied and Environmental Microbiology 73, 4741–50. doi:10.1128/AEM.00315-07

Vermeulen, N., Gänzle, M.G., Vogel, R.F., 2006. Influence of Peptide Supply and Cosubstrates on Phenylalanine Metabolism of *Lactobacillus sanfranciscensis* DSM20451T and *Lactobacillus plantarum* TMW1.468. Journal of Agricultural and Food Chemistry 54, 3832–3839.

Wolter, A., Hager, A.S., Zannini, E., Czerny, M., Arendt, E.K., 2014. Influence of dextran-producing *Weissella cibaria* on baking properties and sensory profile of gluten-free and wheat breads. International Journal of Food Microbiology 172, 83–91. doi:10.1016/j.ijfoodmicro.2013.11.015

Yan, P., Song, Y., Sakuno, E., Nakajima, H., Nakagawa, H., Yabe, K., 2004. Cyclo (L -Leucyl- L -Prolyl) produced by *Achromobacter xylosoxidans* inhibits aflatoxin production by *Aspergillus parasiticus* 70, 7466–7473. doi:10.1128/AEM.70.12.7466

Zannini, E., Pontonio, E., Waters, D.M., Arendt, E.K., 2012. Applications of microbial fermentations for production of gluten-free products and perspectives. Applied Microbiology and Biotechnology 93, 473–485. doi:10.1007/s00253-011-3707-3

Appendix

Appendix A Additional table

Table A-1 Concentration of cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Met-Pro) and cyclo(Phe-Pro) in cultures of *Lb. amylovorus* DSM19280, *Lb. brevis* R2Δ, *Lb. reuteri* R29, acid treated broth and in unfermented controls.

Sample	Cyclo (Leu-Pro) [mg/L]	Cyclo (Pro-Pro) [mg/L]	Cyclo (Met-Pro) [mg/L]	Cyclo (Phe-Pro) [mg/L]
Wort				
Lb. amylovorus DSM19280	0.55 ± 0.01^{B}	< 0.06*	< 0.1*	0.68 ± 0.01^{A}
Lb. brevis R2Δ	0.60 ± 0.01^{A}	< 0.06*	< 0.1*	0.64 ± 0.03^{A}
Lb. reuteri R29	0.61 ± 0.01^{A}	< 0.06*	< 0.1*	0.67 ± 0.03^{A}
unfermented	0.55 ± 0.01^{B}	< 0.06*	< 0.1*	0.64 ± 0.03^{A}
MRS-Broth				
Lb. amylovorus DSM19280	7.57 ± 0.12^{b}	0.53 ± 0.04^{b}	14.32 ± 0.16^{a}	9.09 ± 0.45^{b}
Lb. brevis R2Δ	8.13 ± 0.07^{a}	0.63 ± 0.04^{a}	14.20 ± 0.28^{a}	11.85 ± 0.24^{a}
Lb. reuteri R29	8.12 ± 0.05^{a}	0.58 ± 0.04^{ab}	14.29 ± 0.17^{a}	11.71 ± 0.21^{a}
acidified	7.77 ± 0.11^{b}	0.61 ± 0.03^{a}	13.60 ± 0.09^{b}	11.21 ± 0.50^{a}
unfermented	6.81 ± 0.13^{c}	0.52 ± 0.03^{b}	14.28 ± 0.14^{a}	9.81 ± 0.16^{b}

Mean values ± SD (n = 3); analysis performed by one-way ANOVA and pairwise comparisons using Fisher's LSD procedure; mean values within columns labelled with a common upper case letter from the wort samples are not significantly different ($P < 0.05$); mean values within columns labelled with a common lower case letter from the broth samples are not significantly different ($P < 0.05$);* concentration lower than limit of detection.

Appendix B Publications and presentations

First author publications

Claudia Axel, Emanuele Zannini, Elke K. Arendt, Deborah M. Waters and Michael Czerny

Quantification of cyclic dipeptides from cultures of *Lactobacillus brevis* R2Δ by HRGC/MS using Stable Isotope Dilution Assay

Published: *Analytical and Bioanalytical Chemistry (2014) 406 (9-10): 2433-2444,* doi: 10.1007/s00216-014-7620-3

Claudia Axel, Bettina Röcker, Brid Brosnan, Emanuele Zannini, Ambrose Furey, Aidan Coffey and Elke K. Arendt

Application of *Lactobacillus amylovorus* DSM19280 in gluten-free sourdough bread to improve the microbial shelf

Published: *Food Microbiology (2015) 47: 36-44,* doi: 10.1016/j.fm.2014.10.005

Claudia Axel, Emanuele Zannini and Elke K. Arendt

Mould spoilage of bread and its biopreservation: A review of current strategies for bread shelf life extension

Submitted: *Critical reviews in food science and nutrition*

Claudia Axel, Brid Brosnan, Emanuele Zannini, Ambrose Furey, Aidan Coffey and Elke K. Arendt

Antifungal carboxylic acids produced by *Lactobacillus reuteri* R29 during sourdough fermentation: Substrate dependency and *in situ* antifungal activity in bread

Submitted: *International Journal of Food Microbiology*

Claudia Axel, Brid Brosnan, Emanuele Zannini, Lorenzo C. Peyer, Ambrose Furey, Aidan Coffey and Elke K. Arendt

Antifungal activity of three different *Lactobacillus* species and their production of antifungal carboxylic acids in wheat sourdough

Submitted: *Applied Microbiology and Biotechnology*

Second author publication

Brid Brosnan, Claudia Axel, Aidan Coffey, Elke K. Arendt and Ambrose Furey

Development of an UHPLC-MS/MS method for the detection of antifungal compounds in lactic acid bacteria fermented sourdough

Manuscript for submission: *Food Chemistry*

Oral presentations

Claudia Axel and Elke K. Arendt

Lactic acid bacteria producing anti-fungal compounds: biopreservation in cereal products

International Commission on Food Mycology Workshop, Freising, Germany, 3rd to 5th June 2013 (oral presentation)

Claudia Axel and Elke K. Arendt

Lactic acid bacteria with antifungal activity and their application in gluten-free bread

GDL-Forum (Society of German Food Technologists) "Sourdough V", Münster, Germany, 20th to 21st May 2014 (oral presentation in German)

Claudia Axel, Brid Brosnan, Emanuele Zannini, Ambrose Furey, Aidan Coffey and Elke K. Arendt

Antifungal compounds from lactic acid bacteria – biopreservation in bread

VIth Sourdough Symposium, Nantes, France, 30th September to 2nd October 2015 (oral presentation)

Food Chemistry

Manuscript for submission

Development of an UHPLC-MS/MS method for the detection of antifungal compounds in lactic acid bacteria fermented sourdough

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Keywords: UHPLC–MS/MS, QuEChERS, Lactic acid bacteria, antifungal, sourdough

Abstract

Issues with fungal contamination are of major concern to consumers who demand safe food products with reduced chemical preservatives. The use of lactic acid bacteria (LAB) starter cultures with antifungal activity have been employed to combat such issues by causing a delay/reduction in fungal contamination improving food quality, safety and extending product shelf-life. Most studies to date have been completed under laboratory conditions. The number of *in situ* applications to food is increasing but knowledge of the compounds produced by these antifungal LAB *in situ* is still very limited. Characterisation of these compounds *in situ* is vital to enhance the understanding of how antifungal activity is occurring. A QuEChERS method to detect and quantify antifungal compounds from LAB in MRS broth has previously been developed by this group. The method described herein demonstrates the application of this QuEChERS method to extract *in situ* antifungal compounds from sourdough (rice, wheat and quinoa). These *in situ* compounds (n = 20) were detected by the most comprehensively validated UHPLC-MS/MS chromatography developed to date that allows for a wide range of antifungal compounds to be identified in a single method with a short analysis time (7 minutes).

1. Introduction

Food safety is a common worldwide concern; therefore regulators and food scientists are constantly looking for safe methods to combat such issues. Fungal contamination of food is a major problem; as it causes enormous economic losses (i.e., yield and production losses, increased waste and food shortages) and may cause human intoxications following the consumption of foods contaminated with fungal mycotoxins, which are associated with several acute and chronic diseases in humans [1-3]. Consumers desire minimally processed foods, without the use of chemical preservatives that provide little or no compromise to the nutritional status [4]. This is a challenge for the food industry, which has led to the revival of food fermentations and biopreservation techniques. The shelf life of food can be enhanced by directly adding lactic acid bacteria (LAB) as starter cultures to food as they compete with other microflora (both bacterial and fungal) for nutrients and by their production of

antimicrobial metabolites [4]. LAB are an important group of industrial starter cultures that can be applied to the production of fermented foods such as yogurt, cheese, dry sausage, sauerkraut and sourdough [5]. The suitability of using LAB for this is mainly due to their GRAS (Generally Recognised As Safe) and QPS (Qualified Presumption of Safety) status. When used in foods they provide improved organoleptic attributes and help to extend the shelf-life of the food material [6].

The addition of sourdough to bread has been shown to be a promising procedure for reducing / delaying the occurrence of fungal spoilage [7-12]. Sourdough is a fermented flour and water mixture that can be inoculated with LAB starter cultures. As a technology, sourdough has been long established to improve and diversify the sensory quality of bread [13]. This is advantageous as it complements consumers' demands for natural and additive-free food products [14]. LAB are the predominant microorganisms used as starter cultures for sourdough [15]. Recently some of these starter cultures have been shown to provide extended shelf life due to their antifungal activity [16, 17]. The antifungal activity observed is attributed to the lowering of the pH by lactic and acetic acids as well as from the production of low molecular weight compounds formed by antifungal LAB during fermentation [5]. Hence, use of the appropriate LAB starter cultures in sourdough fermentation can reduce the risks of the growth and survival of food borne pathogens and food spoilage organisms [18]. Comprehensive studies have shown the *in vitro* activity of LAB against various fungi [19-21]. However, their role in preventing food spoilage *in situ* needs more scientific investigations including the analysis of antifungal analytes present *in situ*.

Several studies have been completed to investigate the *in situ* use of antifungal LAB strains as starter cultures in sourdough [7-9, 11, 22-26]. There use has been shown to provide increased shelf-life and improve the quality for wheat bread [7, 22, 26] and gluten free bread [9]. Studies also showed a reduction in the amount of chemical preservatives required due to use of the antifungal LAB strain [8, 11, 24]. Some of these studies identified the antifungal compounds produced by the LAB strain in cell free supernatant by employing SPE, semi-preparative HPLC, NMR, MS and GC analysis [25]. From these studies very few studies have been completed where compounds were extracted directly from the food matrices [23]. The methods detailed

long multistep procedure for the isolation of antifungal compounds from bread. While these methods detect antifungal compounds use of multiple extraction steps are time consuming, require large amounts of solvents and material and the methods are more prone to losses. For these reasons, a high throughput and sensitive analytical method for the confirmation of antifungal compounds in several food matrices is required. Recently, an extraction procedure defined as quick, easy, cheap, effective, rugged and safe (QuEChERS) has been developed for the extraction of antifungal compounds from MRS broth [27].

QuEChERS has several advantages as a method. It is simple, uses small amounts of solvent, has minimal waste steps and is very effective for cleaning up complex samples [28-30].

This paper reports an ultra-high performance liquid chromatography (UHPLC)-ESI-MS/MS method for the confirmation of twenty previously identified antifungal compounds from LAB in sourdough (rice, wheat and quinoa as flour substrates). The method consisted of a newly developed QuEChERS clean-up step which examined the extraction methods efficiency over several different sourdough matrices. These sourdoughs have been inoculated with an antifungal strain that has shown good antifungal activity *in vitro* and *in situ* [25]. The final extracts were separated by UHPLC and analysed by ESI-MS/MS in 11 minutes. This was accomplished with matrix matched calibration curves to assess matrix effects.

2. Materials and Methods

2.1 Chemicals, Reagents and Standards

Ultra-pure water (18.2MΩ/cm) was generated in-house using a Millipore (Cork, Ireland) water purification system. LC-MS grade acetonitrile (ACN), dimethyl sulphoxide puriss P.A. (DMSO), magnesium sulphate puriss p,a, anhydrous (MgSO4), ethyl acetate (EA), de Man, Rogosa and Sharpe (MRS) broth was sourced from Sigma Aldrich (Dublin, Ireland). Acetic acid (AA) (eluent additive for LC-MS) was purchased from Fluka (Ireland). Formic acid (FA) (~99 %) was bought from Fluka (Germany). Sodium chloride p.a. (NaCl) came from AppliChem GmbH (Darmstadt,

Germany). The dispersive SPE Kit Cat #: 5982-4956 (15 mL; 150 mg C18, 900 mg $MgSO_4$ were received from Agilent (Dublin, Ireland).

1,2-dihydroxybenzene (A), DL-4-hydroxyphenyllactic acid (B), 4-hydroxybenzoic acid (C), 3,4-dihydroxyhydrocinnamic acid (D), (S)-(−)-2-hydroxyisocapric acid (E), Vanillic acid (F), caffeic acid (G), 3-(4-hydroxyphenyl)propionic acid (I), 3-(4-hydroxy-3-methoxyphenyl)propionic acid (J), benzoic acid (K), *p*-coumaric acid (L), ferulic acid (M), salicylic acid (N), azelaic acid (O), hydrocinnamic acid (P), methylcinnamic acid (Q), 2-hydroxydodecanoic acid (R), DL- β -hydroxylauric acid (S) and DL- β -hydroxymyristic acid (T) were received from Sigma Aldrich (Dublin, Ireland). Phenyllactic acid (H) was obtained from Bachem (Weil am Rhein, Germany). Hydrocinnamic acid D9 and salicylic acid D6 deuterated internal standards were purchased from Sigma Aldrich (Dublin, Ireland). All analytes had a purity of ≥ 95%. Molecular formula and weight of individual compounds are included in Table 1. Polypropylene tubes (50 and 15 mL) with screw caps were obtained from Starstedt Ltd., (Wexford, Ireland). A VWR International (Dublin, Ireland) multi-tube vortexer, a mistral 3000i centrifuge from Davidson and Hardy (Dublin, Ireland), Turbovap LV evaporator from Caliper Life Sciences (Runcorn, UK), a DeLonghi coffee grinder model KG49 EX:A and Transsonic 780 LH ultrasonic bath from Mason Technology (Dublin, Ireland) were used during sample preparation.

2.2 Preparation of Standard Solutions

Twenty 2 mg/mL stock solutions were prepared by dissolving 4 mg of each of the compounds individually in H_2O or ACN (2 mL) as appropriate. 100 µL of each of the individual stock solution (2 mg/mL) was combined (2 mL; 20 x 100 µL) in a vial to make a 100 µg/mL (100 ppm) standard mix solution. The deuterated internal standards were prepared as follows 50 µL of both deuterated stock solutions (2 mg/mL) were combined in a vial and 1900 µL of DMSO was added to make a 50 µg/mL (50 ppm) deuterated internal standard mix solution.

2.3 Preparation of extracted matrix calibrants and controls

Sourdough (Rice, Wheat and Quinoa)

Extracted sourdough matrix calibrants were prepared by spiking chemically acidified (1.4 % with a mixture of lactic and acetic acid (4:1, v/v)) control sourdough that was not inoculated with a LAB strain with 100 µL of the deuterated internal standard mix (50 ppm) and the appropriate amount of the standard mix (100 ppm) to make the calibration curve points and controls. A calibration range of 6 points for all standards was employed (1-25 µg/mL) and a 7 point calibration range (1-50 µg/mL) for (S)-(−)-2-hydroxyisocapric acid, DL-4- hydroxyphenyllactic acid and phenyllactic acid. 10, 50, 100, 150, 200, 250, 500 µL of the 100 ppm standard mix was added to make calibrant concentrations of 1, 5, 10, 15, 20, 25 and 50 ppm. Standard controls were prepared in the same manner at concentrations of 2.5, 12.5, 17.5 and 30 ppm by adding 25, 125, 175 and 300 µL of the 100 ppm standard mix. 3 of each of these standard controls were prepared for each calibration run.

2.4 Sample Preparation

Sourdough samples were freeze dried and grinded to a fine powder. These freeze dried sourdough samples (2.0 g ± 0.01 g) were weighed into individually labelled polypropylene tubes (50 mL) and H_2O (10 mL) was added and vortexed for 30 seconds. Samples were then fortified with deuterated internal standard (100 µL) and left to stand for 15 minutes. EA (10 mL) with 0.1% FA was dispensed into the samples which were then vortexed for 30 seconds. NaCl (1 g) and $MgSO_4$ (4 g) were added and shaken immediately upon addition for 1 minute. The samples were then centrifuged for 10 minutes at 3500 rpm (2842 x g). The organic supernatant containing the targeted compounds was transferred to a 15 mL Agilent dSPE tube, vortexed for 30 seconds and centrifuged for 10 minutes at 3500 rpm (2842 x g). A 5 mL aliquot of the supernatant (equivalent to 1/2 of the original samples; 1.0 g) was transferred to a 15 mL polypropylene tube with 500 µL of DMSO and evaporated under nitrogen at 50 °C on a Turbovap LV system. Extracts were filtered through 0.2 µm PTFE 13 mm millex syringe filters (Millipore) and 5 µL was injected onto the UHPLC-MS/MS system.

2.5 UHPLC-MS/MS conditions

Separations were performed using a Waters (Milford MA, USA) Acquity UPLC system employing an Aquity BEH shield RP18 analytical column (2.1 x 100 mm, particle size 1.8 μm) maintained at a temperature of 50 °C and the pump was operated at a flow rate of 0.6 mL/min. A binary gradient system was used to separate analytes comprising of mobile phase A, 0.1 % acetic acid in water and mobile phase B, 0.1 % acetic acid in ACN. The gradient profile was as follows: (1) 0-2 min, 95 % A, (2) 2-5 min, 70 % A, (3) 5-7 min, 0 % A, (4) 7-7.5 min, 0 % A, (5) 7.51-11 min, 95 % A. The UHPLC autosampler was sequentially rinsed using strong and weak washes that consisted of methanol/isopropanol/water (80/10/10), and water/methanol (80/20) respectively. These washes were required to clean the needle and reduce carryover between injections.

Antifungal compounds were detected using a Waters Quattro Premier triple quadrupole instrument operated in negative electrospray ionisation mode (Milford, MA, USA). The UHPLC-MS/MS system was controlled by MassLynxTM software and data was processed using TargetLynxTM software (both from Waters). The electrospray voltage was set at 2.5 kV in negative mode. The desolvation and source temperatures were set at 400 and 150 °C, respectively. Nitrogen was employed as the desolvation and cone gases and was set at 1000 and 50 L/h, respectively. Argon was employed as the collision gas, at a flow rate of 0.21 μg/mL, which typically gave pressures of 3.52 x 10-3 mBar. The MS conditions were optimised by teed infusion of 10 μg/mL standard solutions into 50 % mobile phase A and B at a flow rate of 20 μL/min and 0.2 mL/min, respectively.

2.6 Validation

A validation of the method was performed in compliance with the EC [31] and ICH [32] guidelines taking into account specificity, linearity, limits of detection and quantitation, trueness and precision. Validation was completed by analysing standard concentrations (1 ppm, 5 ppm, 10 ppm, 15 ppm, 20 ppm, 25 ppm, 50 ppm) in triplicate on 1 day and over three consecutive days. Controls (2.5 ppm, 12.5 ppm 17.5 ppm and 30 ppm) were run three times each on 1 day and over three consecutive

days. Signal to Noise (S/N) values of S/N = 3 was selected to determine the limit of detection (LOD) and S/N = 10 used to calculate the limit of quantitation (LOQ).

3. Results and discussion

UHPLC-MS/MS optimisation

A UHPLC-MS/MS method was established to determine the presence of 20 antifungal compounds in sourdough fermented with LAB. The MS conditions were optimised initially by infusing standards at a 10 µg/mL concentration and tuning the capillary voltage, cone voltage, desolvation gas flow, cone gas flow and collision energies using mobile phase A containing water with 0.1 % AA and mobile phase B containing acetonitrile with 0.1 % AA. Previous methods have employed water and acetonitrile with formic acid [25, 27, 33-36] or acetic acid [37-39] as the mobile phase. It was decided to compare using 0.1 % formic acid (FA) to 0.1 % acetic acid (AA) as the mobile phase additive to determine if any difference would be encountered when assessing and optimising the ESI ionising efficiencies for all twenty compounds. From the results (Figure 1) it can be seen that DL-4-hydroxyphenyllactic acid (B) , 3,4-dihydroxyhydrocinnamic acid (D) and caffeic acid (G) have higher intensities (i.e., greater ionisation) with 0.1 % FA than with 0.1 % AA but a reduction in intensity is observed for 0.1 % FA for all other compounds with major ionisation losses (i.e., significant ionisation inefficiencies) for vanillic acid (F), 3-(4-hydroxyphenyl)propionic acid (I), ferulic acid (M) and hydrocinnamic acid (P). These results indicate that if 0.1% FA was employed samples containing vanillic acid (F), 3-(4-hydroxyphenyl) propionic acid (I), ferulic acid (M) and hydrocinnamic acid (P) could be reported as negative due to insufficient ionisation using electrospray. Due to this study 0.1 % AA provides better overall ionisation results for each of the twenty compounds allowing for both the qualitative and quantitative detection of all the target compounds. It also gives confidence that unknown compounds within LAB samples will have a better probability of being detected using 0.1 % AA (i.e., the optimised ionisation buffer) in the mobile phase than if 0.1 % FA was used.

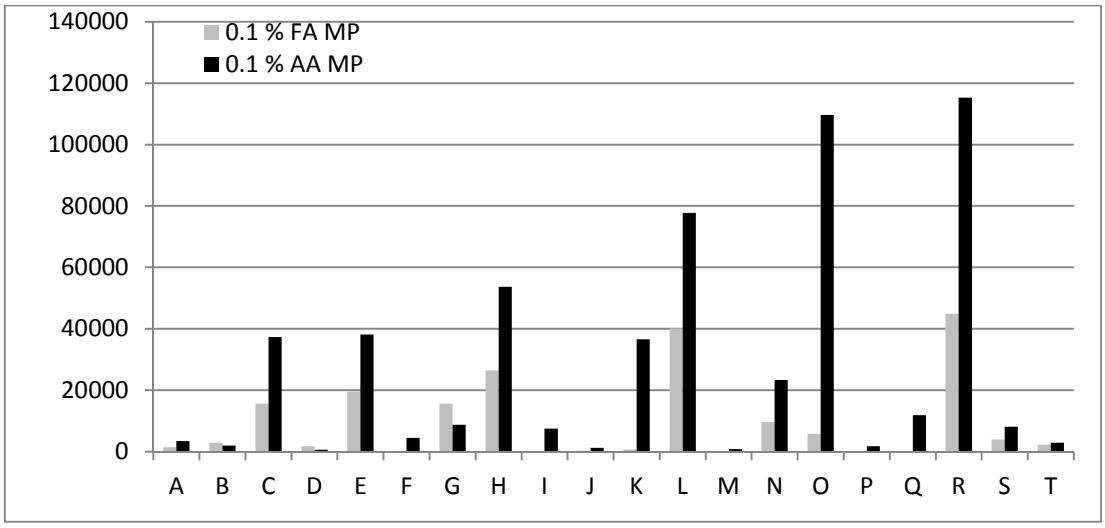


Figure 1 Chromatographic peak area comparison of twenty antifungal compounds injected into mobile phases made of water and acetonitrile with (i) 0.1 % formic acid (FA) and (ii) 0.1 % acetic acid (AA). Compounds are as follows: A) 1,2-dihydroxybenzene, B) DL-4-hydroxyphenyllactic acid, C) 4-hydroxybenzoic acid, D) 3,4-dihydroxyhydrocinnamic acid, E) (S)-(−)-2-hydroxyisocapric acid, F) vanillic acid, G) caffeic acid, H) phenyllactic acid, I) 3-(4-hydroxyphenyl)propionic acid, J) 3-(4-hydroxy-3- methoxyphenyl)propionic acid, K) benzoic acid, L) *p*-coumaric acid, M) ferulic acid, N) salicylic acid, O) azelaic acid, P) hydrocinnamic acid, Q) methylcinnamic acid, R) 2-hydroxydodecanoic acid, S) DL- β -hydroxylauric acid and T) DL- β -hydroxymyristic acid.

The MS tune file was generated with the optimised conditions from the tuning by infusion for each precursor ion and the two most abundant product fragment ions (if available) produced were selected. The SRM (Selected reaction monitoring) windows were time-sectored, and dwell time and inter-scan delays were set to get maximum response from the instrument. These conditions are outlined in Table 1. Inter-scan delay was set to 5 ms between successive SRM windows and dwell times ranged from 1 ms to 10 ms.

Table 1 Name, chemical formula, molecular weight and MS conditions determined with the Waters Quattro Premier XE mass spectrometer for 20 LAB standards

Analyte	Empirical formula	MW[1] (g/mol)	Tr[2] (min)	Transition (m/z)	Dwell time (s)	Cone (V)	CE[3] (V)	SRM[4]
A) 1,2 dihydroxy-benzene	$C_6H_6O_2$	110.11	1.58	109 → 81 109 → 90.9	0.1	40	16	1
B) DL-4-hydroxyphenyllactic acid	$C_9H_{10}O_4$	182.17	1.52	181.5 → 135 181.5 → 163	0.05	25	20	1
C) 4-hydroxybenzoic acid	$C_7H_6O_3$	138.12	2.43	137.4 → 93	0.05	25	15	2
D) 3,4-dihydroxyhydro-cinnamic acid	$C_9H_{10}O_4$	182.17	2.29	181.5 → 109 181.5 → 137	0.05	25	12	2
E) (S)-(−)-2-hydroxyisocapric acid	$C_6H_{12}O_3$	132.16	2.77	131 → 69.1 131 → 85.1	0.05	30	14	3
F) vanillic acid	$C_8H_8O_4$	168.15	3.05	167.4 → 108 167.4 → 123	0.01	25	20	4
G) caffeic acid	$C_9H_8O_4$	180.16	3.32	179.4 → 135	0.05	30	18	4
H) phenyllactic acid	$C_{10}H_{12}O_3$	166.17	3.55	164.9 → 103 164.9 → 147	0.05	25	17	5
I) 3-(4-hydroxyphenyl) propionic acid	$C_9H_{10}O_3$	166.17	3.51	165.4 → 93 165.4 → 121	0.01	25	14 11	5
J) 3-(4-hydroxy-3-meth-oxyphenyl)-propionic acid	$C_{10}H_{12}O_4$	196.20	3.85	195.7 → 121 195.7 → 136	0.1	30	30 15	5
K) benzoic acid	$C_7H_6O_2$	122.12	4.21	121 → 77.1	0.1	25	13	6
L) *p*-coumaric acid	$C_9H_8O_3$	164.16	4.23	163.3 → 118.9	0.01	25	15	6
M) ferulic acid	$C_{10}H_{10}O_4$	194.18	4.39	193.7 → 134 193.7 → 177.9	0.1	25	20 10	6
N) salicylic acid	$C_7H_6O_3$	138.12	4.59	137.5 → 93	0.05	25	15	7
salicylic acid D6	$C_7D_6O_3$	144.16	4.58	141.4 → 97	0.05	25	15	7
O) azelaic acid	$C_9H_{16}O_4$	188.22	4.74	187.3 → 97 187.3 → 125	0.01	25	20 14	7
P) hydrocinnamic acid	$C_9H_{10}O_2$	150.17	5.20	149.7 → 105.9	0.1	30	10	8
hydrocinnamic acid D9	$C_9HO_2D_9$	159.23	5.20	158.5 → 114.5	0.1	30	10	8
Q) methylcinnamic acid	$C_{10}H_{10}O_2$	162.19	6.02	161.5 → 117	0.01	25	10	9
R) 2-hydroxydodecanoic acid	$C_{12}H_{24}O_3$	216.32	6.72	215.4 → 169.1 215.4 → 197.1	0.01	30	20	10
S) DL-β-hydroxylauric acid	$C_{12}H_{24}O_3$	216.32	6.52	215.8 → 59.2	0.05	30	15	10
T) DL-β-hydroxymyristic acid	$C_{14}H_{28}O_3$	244.37	6.83	243.9 → 59.2	0.01	30	15	10

[1]MW= molecular weight, [2]tr = retention time, [3]CE = collision energy, [4]SRM 1 (1.25 – 2.0 min), SRM 2 (2.0 - 2.80 min), SRM 3 (2.5 – 3.2 min), SRM 4 (2.9 – 3.6 min), SRM 5 (3.3 – 4.1 min), SRM 6 (4.0 – 4.6 min), SRM 7 (4.2 – 5.1 min), SRM 8 (5.0 – 5.4 min), SRM 9 (5.8 – 6.2 min), SRM 10 (6.4 – 7.0 min)

Use of internal standards can provide improved quantitative accuracy and repeatability to help to counteract the effects of matrix ion suppression and/or enhancement. Unfortunately only two internal standards were available: hydrocinnamic acid-D9 and salicylic acid-D6. These were spiked into each standard, blank, control and samples at a known concentration (100 µL of 50 ppm IS mix) to provide continuous monitoring of the extraction method and mass spectrometer's performance during analysis [40, 41]. Rice, wheat and quinoa sourdough samples were spiked after extraction and compared to standards in mobile phase (water/acetonitrile (90/10)). Figure 2 shows the simple calibration curves for four of the compounds from the method DL-4-hydroxyphenyllactic acid (B), phenyllactic acid (H), 2-hydroxydodecanoic acid (R) and hydrocinnamic acid (P). As can be seen from the figure differences between the slope of the lines for each of the calibration curves prepared in mobile phase or spiked into rice, wheat, and quinoa was observed. If the individual simple calibration curve for each standard prepared in mobile phase overlaid the corresponding standard calibration curve spiked into a samples matrix (i.e., rice, wheat and/or quinoa) then no ion suppression/enhancement effects is occurring. When simple calibration curve overlay does not occur (i.e., slope of the line is different) then either ion suppression/enhancement effects are occurring [42]. DL-4-hydroxyphenyllactic acid (B) (Figure 2 I) showed enhancement when spiked into rice, wheat and quinoa sourdough compared to the simple calibration curve generated in mobile phase. This is an issue as if the mobile phase calibration curve was used to quantify the presence of this compound; a larger concentration would be indicated than the concentration present. While rice and quinoa produce a similar calibration curve, the wheat sourdough curve is not comparable. Differences are again observed for phenyllactic acid (H) (Figure 2 II) and 2-hydroxydodecanoic acid (R) (Figure 2 III) calibration curves but the pattern is not the same for all 3 compounds as both enhancement and suppression are occurring. An internal standard is available for hydrocinnamic acid (P) and as can be seen from the calibration curves: simple calibration curve (Figure 2 IV) and with internal standard employed (Figure 2 V) the improvement in the curves.

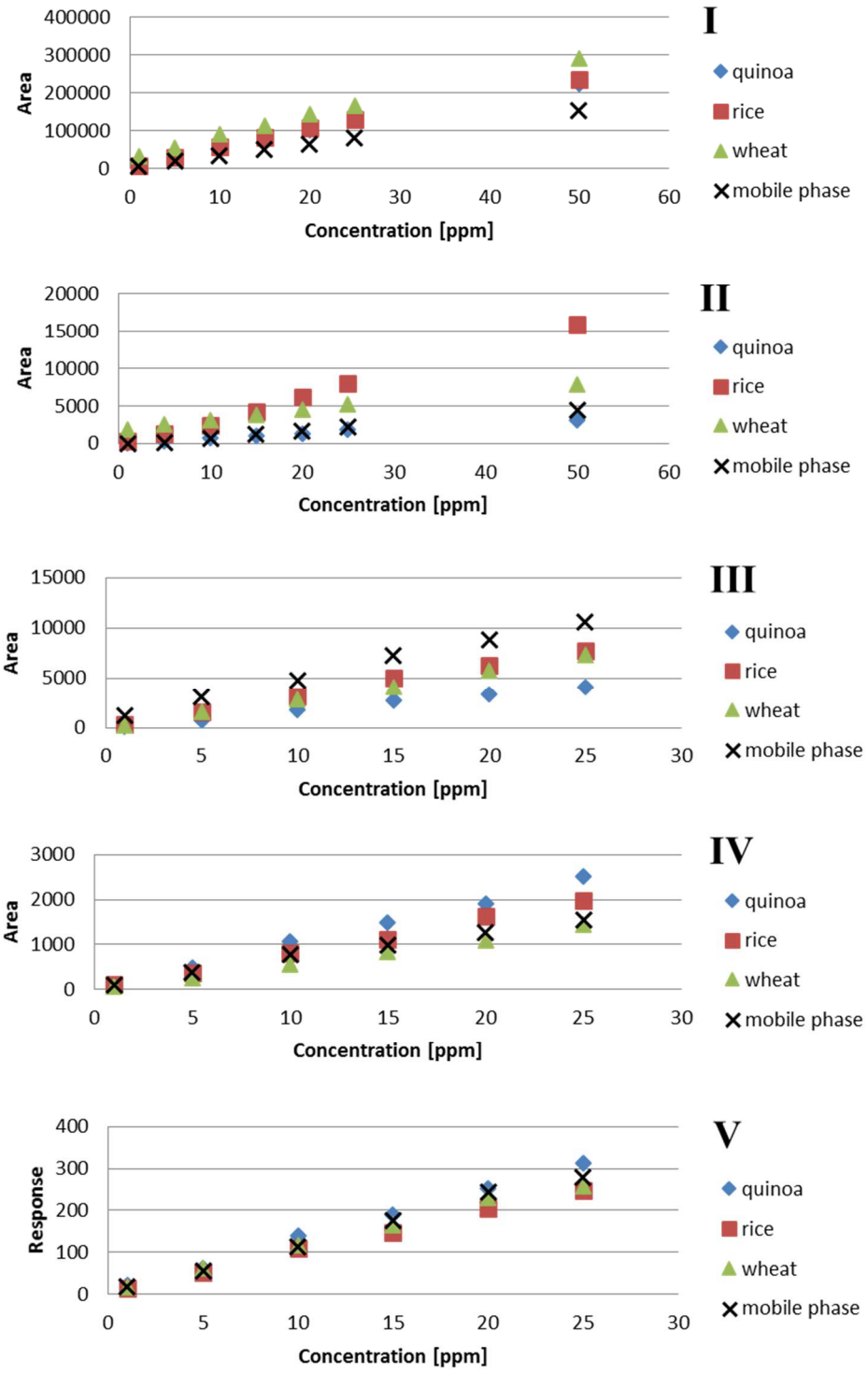


Figure 2 Matrix assessment through the comparison of calibration curves of standards spiked into extracted rice, wheat and quinoa sourdough matrices. Compounds shown A) DL-4-hydroxyphenyllactic acid, B) phenyllactic acid, C) 2-hydroxydodecanoic acid, D) hydrocinnamic acid and E) hydrocinnamic acid with internal standard hydrocinnamic acid D9.

Employment of the internal standard helps minimise matrix effects occurring between the different matrices providing identical/similar slopes of the line for all matrices compared to the standard in mobile phase. As internal standards are not available for the majority of compounds (n = 18) the application of matrix matched calibration curves was undertaken to assess the extent of samples (i.e., rice, wheat and quinoa) matrix effects.

Matrix effect is an issue that can cause signal enhancement or suppression during LC-MS/MS analysis, mainly when employing electrospray ionisation (ESI) as the ionisation technique. Matrix effect was investigated as part of the method validation. The matrix effect appears to be compound dependant potentially due to target analytes co-eluting with matrix components from the sourdough samples. As compounds were subject to matrix effect, matrix matched calibration curves were used to accurately quantify the antifungal compounds. The topic of ion suppression/enhancement is discussed in more detail by the following articles; Trufelli et al. [43], Ciric et al. [44], Chambers et al. [45], Van Eeckhaut et al. [46], Fong et al. [47], Oldekop et al. [48], Furey et al. [42].

The majority of previous methods that identify antifungal compounds from LAB employ HPLC [11, 23, 33, 36-38, 49-56]. This is the second application of UHPLC for the identification of antifungal compounds. The previous method by Wang et al. [35] identified two compounds 3-phenyllactic acid and benzeneacetic acid, 2-propenyl ester with only 3-phenyllactic acid being confirmed by the UPLC system with an ESQUIRE-LC quadrupole ion trap mass spectrometer (Bruker Daltonic, CA, USA) with electrospray ionization. A BEH C18 column (50 x 2.1 mm, 1.7 mm; Thermo Fisher Scientific) at 25 °C was employed. The mobile phases used were: (A) acetonitrile with 0.1 % formic acid; and (B) water with 0.1 % formic acid. A linear gradient from 20 % - 80 % A was employed at a flow rate of 0.3 mL/min which was split to allow a flow rate of 0.2 mL/min to enter the MS. The second compound identified benzeneacetic acid, 2- propenyl ester was completed by GC-MS. The method developed here provides a sensitive and reliable quantitative multi-antifungal compound method for the simultaneous determination of 20 previously identified antifungal compounds (1,2-dihydroxybenzene (A), DL-4-hydroxyphenyllactic acid

(B), 4-hydroxybenzoic acid (C), 3,4-dihydroxyhydrocinnamic acid (D), (S)-(−)-2-hydroxyisocapric acid (E), Vanillic acid (F), caffeic acid (G), 3-(4-hydroxyphenyl)propionic acid (I), Phenyllactic acid (H), 3-(4-hydroxy-3-methoxyphenyl)propionic acid (J), benzoic acid (K), *p*-coumaric acid (L), ferulic acid (M), salicylic acid (N), azelaic acid (O), hydrocinnamic acid (P), methylcinnamic acid (Q), 2-hydroxydodecanoic acid (R), DL-β-hydroxylauric acid (S) and DL-β-hydroxymyristic acid (T)) within a single method. All analytes are eluted in less than 7 min with a run time of 11 min. Following the theory that antifungal activity is occurring due to the synergist interactions caused by the presence of multiple compounds, detection of more than 1 – 2 compounds is required to accurately profile LAB samples. [34, 49, 53, 57, 58].

Method validation

Linearity was evaluated by constructing matrix matched simple calibration curves over the concentration range of 1, 5, 10, 15, 20, 25 for all compounds in blank (un-inoculated) chemically acidified sourdough with an extra point 50* µg/mL employed for DL-4-hydroxyphenyllactic acid (B), (S)-(−)-2-hydroxyisocapric acid and phenyllactic acid (H) due to the higher concentrations generally present of these compounds. The majority of analytes gave linear regression values greater than 0.99. Table 2 shows the linear range, the equation of the line and the R^2 value determined for each compound from i) quinoa, ii) wheat and iii) rice. Method accuracy and precision was evaluated using controls samples at low (2.5 µg/mL), medium (12.5 µg/mL), and high (17.5 or 30* µg/mL) concentrations. The intraday variability was evaluated from three replicate controls determined at three concentrations in a single run (n = 9). The recoveries and the repeatability of the method were established in order to evaluate the methods' trueness and precision, respectively (Table 2 and 3). Table 2 details the intraday results achieved for rice, wheat and quinoa with relative percentage error values ranging from -0.27 – 9.3 % and RSD values of 2.0 – 12.7 achieved for rice. For wheat relative percentage error values ranging from -0.06 – 8.9

% with RSD values of 0.95 – 13.4 were achieved and quinoa obtained relative percentage error values ranging from -0.06 – 9.8 % with RSD values of 1.1 – 14.0.
The interday variability was obtained from nine replicate controls at the same three concentrations analysed over three sequential days (n = 27). Table 3 details the interday results with relative percentage error values ranging from 0.06 – 9.8 % and RSD values of 0.15 – 14.7 achieved for rice, the relative percentage error values for wheat ranged from -0.03 – 10.9 % with relative RSD values of 1.3 – 14.6 and relative percentage error values ranging from 0.06 – 9.3 % with RSD values of 1.3 – 14.3 were found for quinoa. Limit of quantification (LOQ) and limit of detection (LOD) were selected by employing a signal to noise (S/N) values = 3 for the LOD and S/N = 10 used to calculate the LOQ. Values are shown in Table 2. To confirm the presence of a compound, its retention time was compared with its known standard retention time, within ± 0.3 min and the ion ratio conforms to within
± 20 %.

Table 2 Results for linear range, LOD, LOQ, trueness, and precision for known antifungal standards. These 20 standards were assessed in three sourdough matrix solutions: i) quinoa, ii) wheat and iii) rice.

Analyte	equation of the line	Linear range µg mL^{-1}	R^2	LOD µg mL^{-1}	LOQ µg mL^{-1}	2.5 µg mL^{-1}		12.5 µg mL^{-1}		17.5 or 30 * µg mL^{-1}	
						RE % (n= 9)	RSD % (n=9)	RE % (n= 9)	RSD % (n=9)	RE % (n= 9)	RSD % (n=9)
A) 1,2-dihydroxybenzene	i) 117.4 x - 97.5	1 - 25	0.9930	0.05	0.1	5.78	10.38	1.33	3.03	-0.57	2.80
	ii) 160.2 x - 22.7		0.9920			-1.78	8.17	-0.18	2.07	-0.06	5.47
	ii) 211.8 - 32.9		0.9916			-3.11	5.37	1.24	2.13	-0.89	4.77
B) DL-4-hydroxyphenyllactic acid *	i) 66.5 x + 26323	1 - 50	0.9954	0.05	0.1	7.11	6.67	-0.27	2.41	-3.19	5.15
	ii) 122.9 x + 1951		0.9919			0.44	13.42	-1.24	1.90	-3.15	1.86
	iii) 312.2 x - 107.0		0.9922			-0.89	12.73	1.24	2.56	6.89	7.27
C) 4-hydroxybenzoic acid	i) 238.2 x + 6071	1 - 25	0.9958	0.05	0.1	4.00	6.66	1.42	3.02	2.35	1.44
	ii) 454.3 x + 5873		0.9916			8.00	7.86	-1.69	11.15	2.29	4.65
	iii) 1741 x + 1471		0.9902			9.33	9.51	4.27	3.34	3.81	2.00
D) 3,4-dihydroxyhydrocinnamic acid	i) 26.1 x - 89.3	1 - 25	0.9942	0.5	0.75	3.56	6.24	0.62	2.17	0.83	3.16
	ii) 131.9 x - 5.9		0.9907			-2.67	4.59	-2.58	2.84	-3.87	8.46
	iii) 361.8 x + 16.5		0.9907			3.56	5.61	-0.71	2.40	6.22	6.55
E) (S)-(−)-2-hydroxyisocapric acid *	i) 4115 x + 42818	1 - 50	0.9981	0.05	0.1	-0.89	5.63	1.16	1.86	1.33	2.41
	ii) 6756 x + 12339		0.9948			-1.78	6.15	0.98	0.95	-2.63	1.24
	iii) 2127 x + 786.5		0.9971			5.33	3.80	-6.49	9.48	-3.33	9.93
F) Vanillic acid	i) 13.3 + 1158	1 - 25	0.9959	0.05	0.1	-2.22	6.50	-0.09	1.72	2.41	1.36
	ii) 18.1 x + 19.5		0.9911			3.11	13.41	2.67	4.82	4.00	3.95
	iii) 46.9 x - 20.5		0.9982			1.78	12.45	0.27	2.15	3.94	7.06

(continued on next page)

Analyte	equation of the line	Linear range µg mL^{-1}	R^2	LOD µg mL^{-1}	LOQ µg mL^{-1}	2.5 µg mL^{-1}		12.5 µg mL^{-1}		17.5 or 30 * µg mL^{-1}	
						RE % (n= 9)	RSD % (n=9)	RE % (n= 9)	RSD % (n=9)	RE % (n= 9)	RSD % (n=9)
G) caffeic acid	i) 208.2 x - 188.7	1 - 25	0.9919	0.05	0.1	9.78	4.50	4.98	14.04	1.33	1.81
	ii) 957.7 x - 95.3		0.9951			8.44	7.25	-0.53	1.89	-6.73	9.10
	iii) 2556 x + 189.0		0.9918			-4.44	7.38	3.82	5.87	5.78	7.15
H) Phenyllactic acid *	i) 4616 x + 4168	1 - 50	0.9925	0.05	0.1	5.78	5.38	0.71	2.04	-0.48	8.02
	ii) 5458 x + 31232		0.9928			-0.44	3.14	-0.98	5.40	4.78	9.51
	iii) 5406 x + 2328		0.9967			8.44	8.34	0.71	2.12	0.85	9.86
I) 3-(4-hydroxyphenyl)propionic acid	i) 27.5 x + 271.3	1 - 25	0.9921	0.05	0.1	1.78	11.64	-0.27	2.69	2.22	1.76
	ii) 96.8 x + 24.5		0.9910			-3.56	7.02	0.80	4.47	0.51	1.41
	iii) 127.7 x + 332.3		0.9902			8.00	2.62	1.24	1.94	1.78	6.65
J) 3-(4-hydroxy-3-methoxyphenyl)-propionic acid	i) 29.5 x - 6.8	1 - 25	0.9966	0.05	0.1	1.33	10.44	0.71	2.46	-0.06	1.58
	ii) 53.1 x + 414.4		0.9970			5.33	4.65	-1.96	6.08	-5.65	10.23
	iii) 73.2 x + 30.9		0.9912			-3.56	6.70	0.44	2.49	4.06	3.05
K) benzoic acid	i) 2340 x + 12911	1 - 25	0.9961	0.05	0.1	2.67	12.47	0.71	2.33	5.97	8.67
	ii) 2132 x + 24046		0.9935			2.67	8.93	6.67	3.82	2.10	1.75
	iii) 2066 x + 7386		0.9929			0.44	10.43	0.98	2.50	-0.38	6.17
L) *p*-coumaric acid	i) 2512 x + 60371	1 - 25	0.9903	0.05	0.1	8.44	5.03	0.71	2.33	2.92	2.88
	ii) 4008 x + 2601		0.9956			8.89	5.74	4.71	6.67	4.25	3.21
	iii) 3006 x +8338		0.9937			7.11	9.66	1.96	2.99	7.68	10.73
M) ferulic acid	i) 20.6 x + 788.7	1 - 25	0.9948	0.05	0.1	0.89	6.20	-0.27	2.23	-0.44	1.57
	ii) 48.7 x + 163.9		0.9907			-2.22	11.59	-1.60	6.73	-4.89	8.04
	iii) 30.4 x + 455.8		0.9925			-2.67	8.72	-1.51	3.43	2.10	6.28

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Analyte	equation of the line	Linear range µg mL^{-1}	R^2	LOD µg mL^{-1}	LOQ µg mL^{-1}	2.5 µg mL^{-1}		12.5 µg mL^{-1}		17.5 or 30 * µg mL^{-1}	
						RE % (n= 9)	RSD % (n=9)	RE % (n= 9)	RSD % (n=9)	RE % (n= 9)	RSD % (n=9)
N) salicylic acid	i) 0.23 x - 0.67	1 - 25	0.9926	0.05	0.1	1.78	7.12	0.62	1.24	0.70	1.57
	ii) 0.08 x -0.05		0.9952			4.44	3.55	2.49	2.83	-1.97	3.03
	iii) 0.11 x - 0.02		0.9946			-3.11	9.18	0.27	2.19	1.46	1.44
O) azelaic acid	i) 6378 x + 167010	1 - 25	0.9977	0.05	0.1	8.00	6.68	0.71	2.79	0.38	1.78
	ii) 8216 x + 202946		0.9967			4.00	3.85	2.67	3.21	-0.44	3.95
	iii) 5616 x + 115637		0.9929			-2.67	8.22	-1.07	1.85	0.19	1.85
P) hydrocinnamic acid	i) 21.6 x - 0.78	1 - 25	0.9904	0.05	0.1	-0.44	12.78	0.71	2.59	-0.76	1.12
	ii) 10.6 x 2.37		0.9916			0.89	5.87	2.40	3.45	3.49	5.37
	iii) 1.66 x + 2.99		0.9942			3.11	10.58	-0.27	2.27	1.33	2.01
Q) methylcinnamic acid	i) 17.1 x + 5.90	1 - 25	0.9930	0.05	0.1	7.56	4.34	-0.09	2.17	0.63	1.69
	ii) 12.0 x 1.23		0.9926			-0.44	6.80	0.18	2.45	5.02	2.17
	iii) 49.1 x + 8.55		0.9938			4.89	6.82	0.09	1.85	2.41	2.76
R) 2-hydroxydodecanoic acid	i) 339.4 x + 389.7	1 - 25	0.9904	0.05	0.1	-0.89	8.27	0.18	2.79	0.70	1.32
	ii) 360.7 x + 416.4		0.9927			1.78	7.12	-0.27	2.50	-3.81	8.88
	iii) 397.0 x + 823.9		0.9930			3.56	8.52	0.44	2.29	2.10	4.77
S) DL-β-hydroxylauric acid	i) 189.4 x - 88.7	1 - 25	0.9924	0.05	0.1	-4.89	7.52	0.27	2.26	-0.06	1.82
	ii) 287.0 x - 50.2		0.9991			4.44	9.07	2.49	7.43	-6.41	10.84
	iii) 290.0 x -55.0		0.9938			3.11	5.75	-1.07	2.43	1.84	1.58
T) DL-β-hydroxymyristic acid	i) 189.8 x + 42.9	1 - 25	0.9957	0.05	0.1	-2.22	6.50	2.13	0.68	1.65	1.07
	ii) 462.6 x + 171.9		0.9990			8.89	4.78	3.91	4.61	4.00	2.98
	iii) 640.0 x + 1088		0.9915			4.00	9.62	0.53	1.49	-1.40	5.79

* Indicates where a different calibration range was employed with an extra calibration point of 50 µg/mL.

Table 3 Interday relative percentage error and RSD values. These 20 standards were assessed in three sourdough matrix solutions: i) quinoa, ii) wheat and iii) rice.

compound		2.5		12.5		17.5 or 30*	
		RE % (n= 27)	RSD % (n=27)	RE % (n= 27)	RSD % (n=27)	RE % (n= 27)	RSD % (n=27)
A) 1,2-dihydroxybenzene	i)	4.44	10.48	0.89	2.64	0.70	2.71
	ii)	-1.04	9.15	-0.09	1.64	2.05	6.88
	iii)	-0.74	14.66	2.34	18.70	0.55	4.51
B) DL-4-hydroxyphenyllactic acid *	i)	9.33	7.59	0.06	2.07	-1.88	10.25
	ii)	3.56	11.65	-0.39	1.87	-7.77	5.99
	iii)	6.96	10.66	1.01	2.39	5.99	12.83
C) 4-hydroxybenzoic acid	i)	3.70	5.65	0.89	2.45	2.29	1.71
	ii)	8.15	9.26	0.36	7.70	1.93	5.06
	iii)	9.78	12.80	2.19	6.34	-1.48	10.15
D) 3,4-dihydroxyhydrocinnamic acid	i)	1.63	10.08	-0.09	2.54	1.86	2.47
	ii)	-3.85	7.52	-3.50	3.02	-1.42	5.57
	iii)	2.96	8.92	-7.97	11.34	2.03	9.56
E) (S)-(−)-2-hydroxyisocapric acid *	i)	1.48	9.29	1.36	1.66	2.28	1.41
	ii)	-4.15	12.33	1.75	3.09	-6.85	5.41
	iii)	2.52	12.81	-2.25	6.22	0.11	8.09
F) vanillic acid	i)	4.15	8.49	0.80	2.04	2.01	1.96
	ii)	5.04	10.59	3.23	3.94	5.40	5.81
	iii)	1.93	10.80	2.52	3.26	3.22	10.60
G) caffeic acid	i)	9.33	10.59	1.99	8.55	2.07	2.06
	ii)	-3.70	13.03	-0.03	2.52	-5.93	8.42
	iii)	-7.85	9.82	-0.92	7.78	4.97	10.74
H) Phenyllactic acid *	i)	8.30	10.89	0.44	2.18	-4.52	10.09
	ii)	-6.81	21.65	-0.65	9.08	5.83	11.77
	iii)	2.81	12.44	-1.78	7.67	-3.02	18.46
I) 3-(4-hydroxyphenyl)propionic acid	i)	3.56	11.10	0.50	2.82	2.60	1.82
	ii)	-2.96	10.20	5.45	14.55	1.67	3.03
	iii)	8.74	10.10	0.74	6.63	3.03	9.73
J) 3-(4-hydroxy-3-methoxyphenyl)propionic acid	i)	3.41	9.93	1.39	2.40	1.25	2.07
	ii)	5.33	9.00	-1.51	6.73	-3.11	7.43
	iii)	7.70	12.49	3.76	5.35	0.49	8.12
K) benzoic acid	i)	4.44	10.90	0.18	2.60	5.84	14.26
	ii)	3.70	8.55	4.62	3.77	2.50	3.15
	iii)	2.37	12.35	3.02	8.28	-3.81	10.17
L) *p*-coumaric acid	i)	5.93	8.27	1.51	2.53	2.65	2.16
	ii)	9.33	8.37	4.56	5.80	5.14	2.82
	iii)	3.85	42.70	-2.81	8.20	1.12	38.25
M) ferulic acid	i)	-0.59	8.62	0.36	2.64	0.23	1.57
	ii)	3.26	11.82	-1.51	8.04	-3.07	6.54
	iii)	1.33	9.91	-7.08	11.58	-6.71	13.72

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compound		2.5		12.5		17.5 or 30*	
		RE % (n= 27)	**RSD % (n=27)**	**RE % (n= 27)**	**RSD % (n=27)**	**RE % (n= 27)**	**RSD % (n=27)**
N) salicylic acid	i)	3.41	8.50	1.39	1.60	1.80	1.79
	ii)	7.56	7.10	1.60	3.04	-0.13	4.18
	iii)	4.15	11.20	-1.78	5.72	2.71	2.07
O) azelaic acid	i)	4.15	10.02	1.10	4.72	0.91	1.93
	ii)	5.93	7.86	1.27	9.45	1.38	3.39
	iii)	-1.93	8.93	-0.86	3.86	-8.80	13.66
P) hydrocinnamic acid	i)	4.15	10.46	1.42	2.14	0.87	1.95
	ii)	4.59	10.75	1.63	3.85	2.52	9.74
	iii)	0.17	9.38	-0.77	2.81	1.93	1.80
Q) methylcinnamic acid	i)	5.93	7.72	2.19	8.49	0.93	1.83
	ii)	4.89	8.61	1.78	3.83	8.30	7.10
	iii)	3.70	8.88	0.27	2.32	2.48	2.34
R) 2-hydroxydodecanoic acid	i)	1.93	8.32	0.74	2.46	1.42	2.09
	ii)	5.33	8.29	0.41	4.89	-2.07	6.41
	iii)	-0.30	10.37	2.76	3.49	4.25	8.58
S) DL-β-hydroxylauric acid	i)	2.96	12.41	0.50	2.12	0.23	2.24
	ii)	6.81	10.32	2.19	7.52	-4.25	8.51
	iii)	1.93	7.80	0.15	2.96	3.83	2.87
T) DL-β-hydroxymyristic acid	i)	3.70	7.25	1.88	1.55	2.62	1.31
	ii)	10.81	4.57	4.15	3.75	4.04	3.01
	iii)	8.00	9.25	0.89	2.10	0.06	6.66

* Indicates where a different control point was employed of 30 µg/mL due to an extra calibration point of 50 µg/mL being employed by the calibration curve.

Sample preparation

A previous method employed by Ryan et al. [23] to identify antifungal compounds from sourdough involved numerous steps to extract the compounds of interest – 1) samples frozen using liquid nitrogen, 2) freeze dried, 3) crushed using a mortar and pestle, 4) samples separated into crust and crumb, 5) blitzed in a food processor, 6) n-pentane wash, 7) centrifugation, 8) acetone/water (70/30) LLE, 9) centrifugation and supernatant dried under N_2, 10) redissolved in acetonitrile/water (50/50) and anhydrous sodium sulphate, 11) filter, 12) removal of acetonitrile under reduced pressure, 13) aqueous solution LLE with dichloromethane, 14) dry the organic layer, 15) redissolve in aqueous hydrochloric acid (0.001 mol/L), 16) add solution to dowex slurry, 17) collect eluent fractions and freeze dry, 18) dissolve in 50% acetonitrile, 19) inject on LC-MS system. The development of this QuEChERS method to analysis these compounds from an *in situ* matrix (sourdough) greatly reduces the number of steps, the solvent quantities, waste generated in comparison to such previous methods employed.

A QuEChERS-based approach that was previously developed by this group for the extraction of antifungal compounds from MRS broth [27] and wort substrate [59] was examined to isolate 20 antifungal analytes from rice, wheat and quinoa sourdough. QuEChERS methods have been successfully adapted and validated in multiple labs to extract hundreds of pesticides [60],[28],[30], drugs [61], veterinary drug residues [29],[30], pharmaceuticals [62], natural metabolites [63], mycotoxins [30],[64] and plant toxins [30]. It's a quick and simple method which is advantageous if numerous samples are required to be analysed in a short time frame. However, this method reports the application of this sample preparation approach in relation to analysing antifungal compounds from an *in situ* matrix (sourdough).

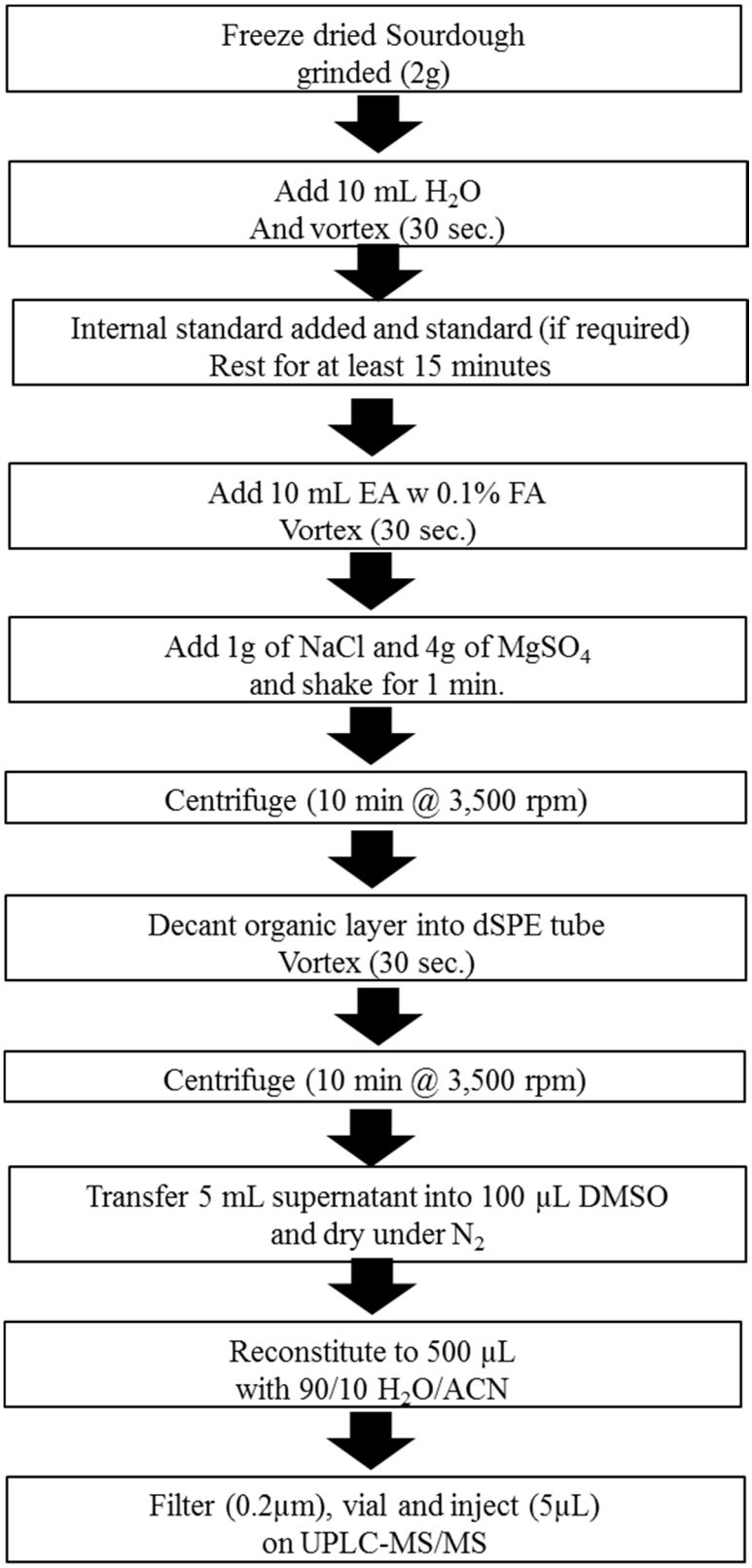


Figure 3 Flow diagram outlining the steps involved in the extraction of sourdough samples (rice, wheat and quinoa).

Figure 3 describes the steps involved in the extraction procedure. An extraction study was performed to evaluate the performance of the procedure for the extraction of compounds from these complex matrices. Compounds were spiked into chemically acidified (CA) sourdough matrix (rice, wheat, quinoa as appropriate) prior to extraction and extracted following the procedure outlined in Figure 3. Compounds were also spiked into the extracted CA sourdough (rice, wheat, quinoa) to compare the results. Figure 4 illustrates the results of this study.

Good recoveries were obtained for all compounds with values ranging from 60.7 % (DL- β -hydroxymyristic acid (T)) -104.3 % (benzoic acid (K)) in rice, 60.3 % (hydroxymyristic acid (T)) – 97.2 % (*p*-coumaric acid (L)) in wheat, and 76.2 % (DL- β -hydroxylauric acid (S)) – 102.1 % (1,2-dihydroxybenzene (A)) in quinoa. RSD values of 0.9 – 8 % were obtained for rice, 0.2 – 8.6 % for wheat and 0.2 - 9.3 % for quinoa. These results indicate the method can adequately extract and identify the 20 known antifungal compounds from these sourdough matrices.

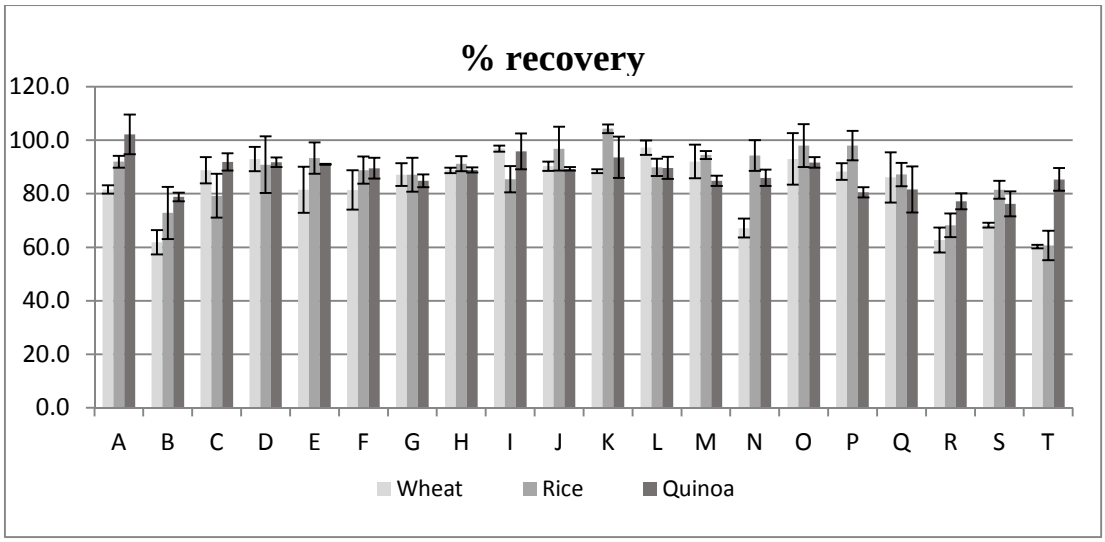


Figure 4 Percentage recovery data from twenty antifungal compounds extracted from wheat, rice and quinoa sourdough. A) 1,2-dihydroxybenzene, B) DL-4-hydroxyphenyllactic acid, C) 4-hydroxybenzoic acid, D) 3,4-dihydroxyhydrocinnamic acid, E) (S)-(−)-2-hydroxyisocapric acid, F) Vanillic acid, G) caffeic acid, H) phenyllactic acid, I) 3-(4-hydroxyphenyl)propionic acid, J) 3-(4-hydroxy-3- methoxyphenyl)propionic acid, K) benzoic acid, L) *p*-coumaric acid, M) ferulic acid, N) salicylic acid, O) azelaic acid, P) hydrocinnamic acid, Q) methylcinnamic acid, R) 2-hydroxydodecanoic acid, S) DL- β -hydroxylauric acid and T) DL- β -hydroxymyristic acid.

Analysis of samples

The achievement of positive *in vitro* results obtained by laboratory experiments does not guarantee for the efficient transfer of antifungal activity to *in vivo* food matrices [65]. Hence, the need for *in situ* testing is required. The same rational can be applied to identifying and quantification of the antifungal compounds produced by LAB. Their production and concentration depend on the conditions available for growth [59, 66]. Samples were extracted as per described in Figure 3. The antifungal strain *Lactobacillus amylovorus* DSM19280 was inoculated into the three different sourdough matrices to examine if similar compounds are produced and if they are produced at what concentration levels within the different matrices by the same strain.

Table 4 Concentrations determined for compounds detected from rice, wheat and quinoa sourdough sample prepared using antifungal strain *Lb. amylovorus* DSM19280.

	quinoa (n=3)		**rice (n=3)**		**wheat (n=3)**	
	µg/mL	**RSD**	**µg/mL**	**RSD**	**µg/mL**	**RSD**
B) DL-4-hydroxyphenyllactic acid	71.9	7.3	39.2	1.8	24.4	6.5
C) 4-hydroxybenzoic acid	7.5	10.5	2.7	1.1		
E) (S)-(−)-2-hydroxyisocapric acid	146	4	100.9	1.1	42.3	1.9
F) vanillic acid	193	6.7	14	6.1	16.7	3.9
H) phenyllactic acid	221.1	1.6	33.1	1.1	69.2	1.8
I) 3-(4-hydroxyphenyl)propionic acid	22.3	12.7			1.4	11.8
J) 3-(4-hydroxy-3-methoxyphenyl)-propionic acid	32.7	5.6	9.1	3.6	41.3	1.1
K) benzoic acid	3.3	2.3				
N) salicylic acid	3.5	10.7	3	2.6		
O) azelaic acid	108.6	2.1	7.9	2.5	25.1	2

As can be seen from the results (Table 4) *Lb. amylovorus* DSM19280 produced varying concentrations of antifungal compounds when used to ferment rice, wheat and quinoa sourdough. Ten antifungal compounds DL-4-hydroxyphenyllactic acid (B), 4-hydroxybenzoic acid (C), (S)-(−)-2-hydroxyisocapric acid (E), vanillic acid (F), phenyllactic acid (H), 3-(4-hydroxyphenyl)propionic acid (I), 3-(4-hydroxy-3-methoxyphenyl)propionic acid (J), benzoic acid (K), salicylic acid (N) and azelaic acid (O) in total were detected from the inoculated rice, wheat and quinoa sourdough matrices. Extracted quinoa sourdough detected all of the ten antifungal compounds

at a much higher concentration than what was detected from rice and wheat sourdough. This confirms previous research, that the production of antifungal compounds is substrate specific. Figure 5 shows the chromatogram, mass spectrum and structures for the antifungal compounds produced from the antifungal strain *Lb. amylovorus* DSM19280 used to prepare quinoa sourdough.

The main active compounds analysed in the sourdough (B, E, H) originate from the LAB amino acid metabolism. As such, phenylalanine is converted by a transamination reaction to phenylpyruvic acid. Phenylpyruvic acid is further reduced to phenyllactic acid by a dehydrogenase. 4-hydroxyphenyllactic acid originates from tyrosine, benzoic acid and salicylic acid also from phenylalanine and 2-hydroxyisocaproic acid is a by-product of the leucine pathway. Quinoa (*Chenopodium quinoa Willd.*) is a so-called pseudo-cereal, which is dicotyledonous that is different to the monocotyledonous true cereal grains like wheat and rice. Quinoa flour has superior protein content which is almost double as high as in rice flour; in contrast, starch content is lowest in quinoa flour [67]. Furthermore, its high mineral content allows the quinoa sourdough to have a higher buffering capacity enabling the production of higher acids contents [68]. Moreover, protein degradation during fermentation is expected to be also higher in quinoa due to the highest protease activity of the flour providing more free amino acids to metabolise for LAB [data not shown]. These differences in the chemical composition of the quinoa flour may provide an explanation for why there are more antifungal compounds (n = 10) in higher concentrations detected by the quinoa sourdough in comparison to rice (n = 8) and wheat (n = 7). Although rice flour has a lower protein content (6.4 %) than wheat flour (11.5 %), its protease activity is higher (data not shown) which could explain similar concentration of antifungal compounds in the corresponding sourdoughs.

However, the preservation effect of the sourdough in the final product when incorporated in the bread is much more complex. Raw materials differ, and in a practical context, the optimal water level has to be adjusted for each bread recipe. In general, gluten free breads have higher water activities due to the higher water level in the recipe, thus they are more susceptible for microbial spoilage. Axel et al. [69] has demonstrated that use of *Lb. amylovorus* DSM19280 fermented quinoa sourdough in

bread production has extended the shelf life of the bread by 4 days compared to the non-acidified control which had only a mould free shelf life of 2 days. This indicates that the antifungal compounds produced contribute towards the preservation effect observed.

Wheat bread has a lower water activity than rice and quinoa bread [70]. Thus, although there are less antifungal compounds present in the wheat sourdough, Ryan et al. [25] showed the potential of *Lb. amylovorus* DSM19280 to extend the shelf life of wheat sourdough bread by up to fourteen days. This paper identified fifteen antifungal compounds from the MRS broth as contributing to the antifungal activity. Six of these identified compounds as well as fourteen extra antifungal compounds were examined in this method. The presence and concentration in the actual sourdough fermented with *Lb. amylovorus* DSM 19280 was determined as shown in Table 4. Ryan et al. [25] paper did not quantify the concentration of these compounds present in the MRS broth but it is likely that all these compounds are produced within the sourdough but are present at levels below the quantification capabilities of this method.

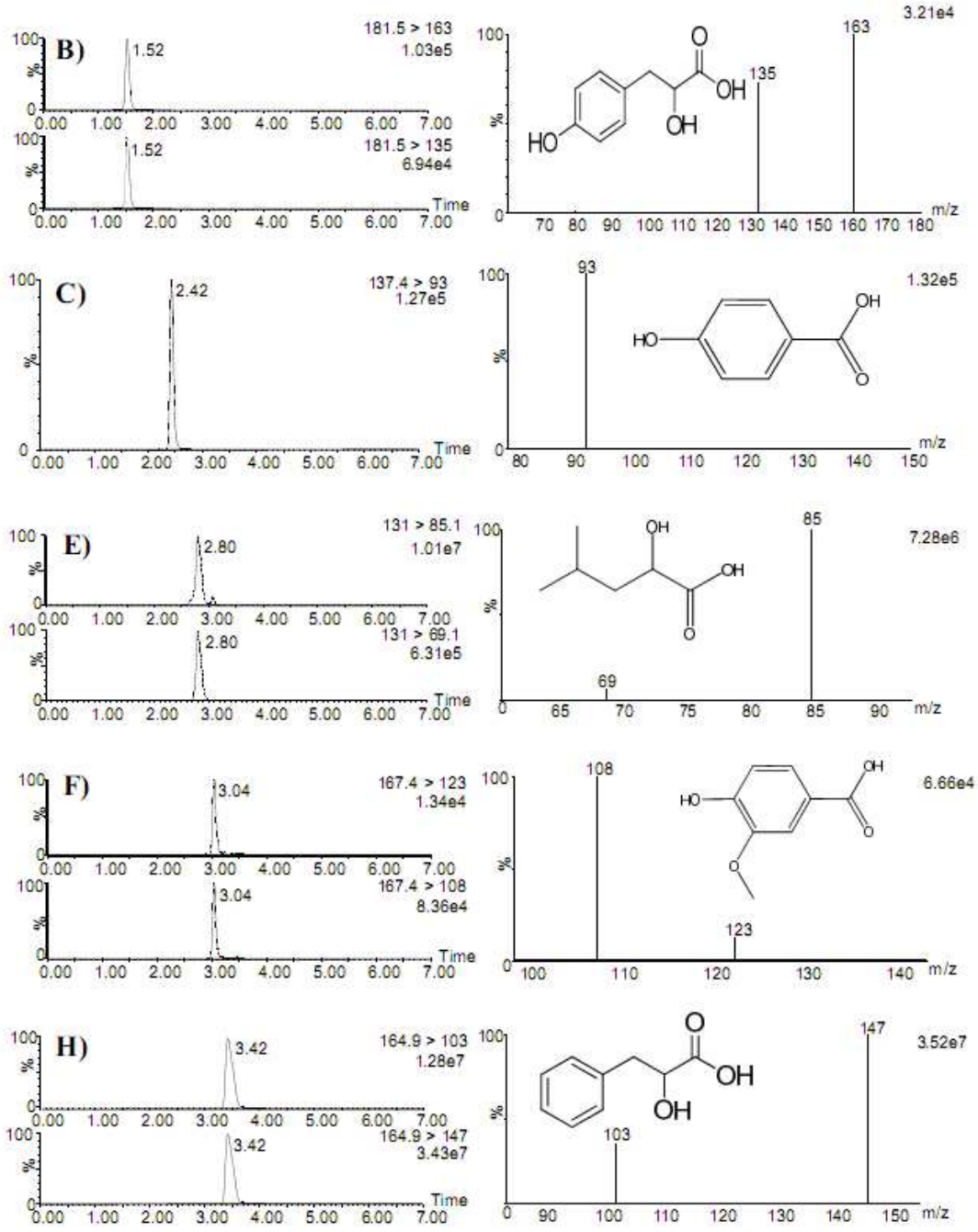


Figure 5 Chromatogram, mass spectrum and structure of the identified antifungal compounds from quinoa sourdough prepared with the antifungal strain *Lb. amylovorus* DSM19280. B) DL-4-hydroxyphenyllactic acid, C) 4-hydroxybenzoic acid, E) (S)-(−)-2-hydroxyisocapric acid, F) vanillic acid, H) phenyllactic acid, I) 3-(4-hydroxyphenyl)propionic acid, J) 3-(4-hydroxy-3- methoxyphenyl)propionic acid, K) benzoic acid, N) salicylic acid and O) azelaic acid.

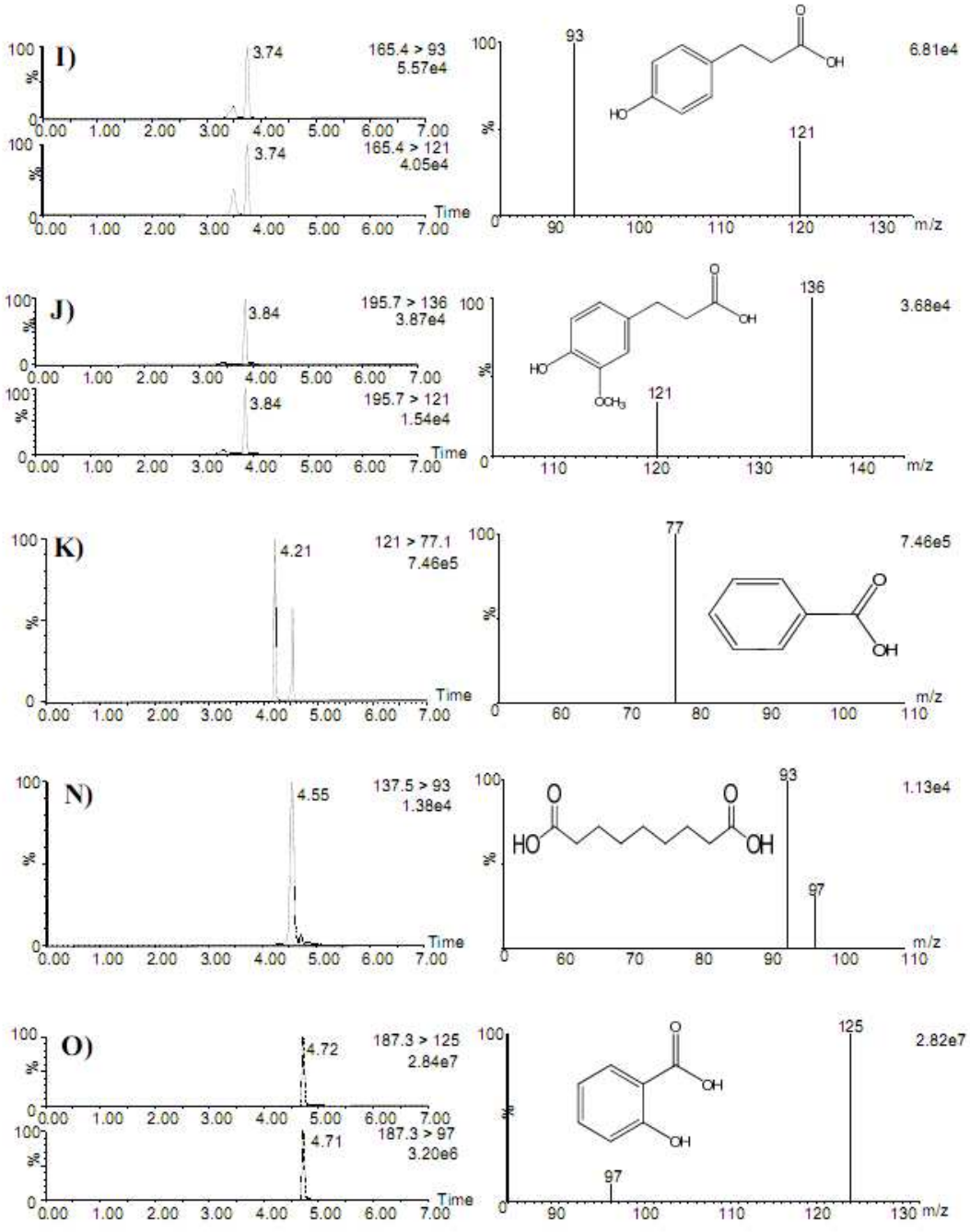


Figure 5 continued

4. Conclusions

Foods fermented with LAB have shown improved textures, flavours and tastes with longer shelf lives due to naturally occurring antimicrobial compounds which prevent food spoilage and maintain food safety. For this reason LABs have seen wide use in food fermentation. Comparison of LAB to previously employed methods *in situ* and their ability to retain the same or improve the nutritive value of the food has been determined [71]. In this study we developed a multi-compound (20) UHPLC-MS/MS method for the determination of *in situ* antifungal compounds from sourdough using QuEChERS.

This method provides separation of twenty antifungal compounds on an UHPLC system a vast improvement from the one compound previously detected by this technique. Use of QuEChERS to extract the sourdough samples also provides a much quicker and more efficient method compared to those techniques previously employed that required numerous long steps to extract antifungal compounds from the same matrices. Assessment of the matrix effect between the different matrices (rice, wheat and quinoa) was also undertaken. The use of two deuterated internal standards (Hydrocinnamic acid D9 and Salicylic acid D6) and matrix matched calibration curves were employed to highlight quantitation issues that can arise and combat these issues that may occur due to differences in the matrices.

Determination of the concentration of antifungal compounds produced by the same antifungal strain *Lb. amylovorus* DSM19280 inoculated at the same concentration into the three different matrices (rice, wheat and quinoa) produced varying amounts of the antifungal compounds detected. These results show the importance of undertaking *in situ* determination of antifungal compounds produced. Development of this method has provided an efficient method for the accurate achievement of this from sourdough with the potential applicability to various other food matrices. Due to LABs wide application possibilities, there is a need for a rapid universal method for the extraction of these compounds from several matrices. The analysis of antifungal compounds from LAB in food can be an extremely challenging task due to the minute amounts that can be present in samples and the potential for co-extraction of similar food flavour compounds present that are not involved in the antifungal activity

observed. This new methodology will allow for the identification and quantification of the key substances causing antifungal activity. Development and application of this rapid multi-compound extraction procedure utilising UHPLC-MS/MS detection to monitor 20 known antifungal compounds from LAB in the fermentation of various sourdough materials will help provide insight into the topic and demonstrates the capabilities of the method to be further employed for other food matrices fermented with antifungal LAB.

References

1. Köppen, R., et al., Determination of mycotoxins in foods: current state of analytical methods and limitations. Applied Microbiology and Biotechnology, 2010. **86**(6): p. 1595-1612.
2. Oliveira, P.M., E. Zannini, and E.K. Arendt, Cereal fungal infection, mycotoxins, and lactic acid bacteria mediated bioprotection: From crop farming to cereal products. Food Microbiology, 2014. 37(0): p. 78-95.
3. James, K.J., J. Frahill, and A. Furey, Chapter 11: Analysis of Trichothecenes using Liquid Chromatography-Mass Spectrometry In: Food Contaminants:Mycotoxins and Food Allergens. 2008: Oxford University Press
4. Garcha, S. and N.K. Natt, *In situ* control of food spoilage fungus using *Lactobacillus acidophilus* NCDC 291. Journal of Food Science and Technology-Mysore, 2012. 49(5): p. 643-648.
5. Messens, W. and L. De Vuyst, Inhibitory substances produced by Lactobacilli isolated from sourdoughs—a review. International Journal of Food Microbiology, 2002. 72(1–2): p. 31-43.
6. Caplice, E. and G.F. Fitzgerald, Food fermentations: role of microorganisms in food production and preservation. International Journal of Food Microbiology, 1999. 50(1–2): p. 131-149.
7. Lavermicocca, P., et al., Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. Applied and Environmental Microbiology, 2000. 66(9): p. 4084-4090.
8. Ryan, L.A.M., F. Dal Bello, and E.K. Arendt, The use of sourdough fermented by antifungal LAB to reduce the amount of calcium propionate in bread. International Journal of Food Microbiology, 2008. 125(3): p. 274-278.
9. Moore, M., F. Dal Bello, and E. Arendt, Sourdough fermented by *Lactobacillus plantarum* FST 1.7 improves the quality and shelf life of gluten-free bread. European Food Research and Technology, 2008. 226(6): p. 1309-1316.
10. Clarke, C.I. and E.K. Arendt, A review of the application of sourdough technology to wheat breads. Advances in Food and Nutrition Research, 2005. 49(1): p. 137-161.

11. Gerez, C.L., et al., Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. Food Control, 2009. 20(2): p. 144-148.
12. Pawlowska, A.M., et al., Chapter 5 - "Green Preservatives": Combating Fungi in the Food and Feed Industry by Applying Antifungal Lactic Acid Bacteria, in Advances in Food and Nutrition Research, H. Jeyakumar, Editor. 2012, Academic Press. p. 217-238.
13. Hassan, Y.I. and L.B. Bullerman, Antifungal activity of *Lactobacillus paracasei* ssp. tolerans isolated from a sourdough bread culture. International Journal of Food Microbiology, 2008. 121(1): p. 112-115.
14. Rosenquist, H. and Å. Hansen, The antimicrobial effect of organic acids, sour dough and nisin against *Bacillus subtilis* and *B. licheniformis* isolated from wheat bread. Journal of Applied Microbiology, 1998. 85(3): p. 621-631.
15. Vogel, R.F., et al., Non-dairy lactic fermentations: the cereal world, in Lactic Acid Bacteria: Genetics, Metabolism and Applications. 1999, Springer. p. 403-411.
16. Corsetti, A., et al., Antimould activity of sourdough lactic acid bacteria: identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco* CB1. Applied Microbiology and Biotechnology, 1998. 50(2): p. 253-256.
17. Magnusson, J. and J. Schnürer, *Lactobacillus coryniformis subsp. Coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. Applied and Environmental Microbiology, 2001. 67(1): p. 1-5.
18. Holzapfel, W., R. Geisen, and U. Schillinger, Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. International Journal of Food Microbiology, 1995. 24(3): p. 343-362.
19. Schnürer, J. and J. Magnusson, Antifungal lactic acid bacteria as biopreservatives. Trends in Food Science & Technology, 2005. 16(1-3): p. 70-78.
20. Lacroix, C., Protective Cultures, Antimicrobial Metabolites and Bacteriophages for Food and Beverage Biopreservation. 2010: Elsevier Science.
21. Crowley, S., J. Mahony, and D. van Sinderen, Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. Trends in Food Science & Technology, 2013. 33(2): p. 93-109.

22. Dal Bello, F., et al., Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. Journal of Cereal Science, 2007. 45(3): p. 309-318.
23. Ryan, L.A.M., et al., Detection and quantitation of 2,5-diketopiperazines in wheat sourdough and bread. Journal of Agricultural and Food Chemistry, 2009. 57(20): p. 9563-9568.
24. Gerez, C.L., et al., A Ready-to-use antifungal starter culture improves the shelf life of packaged bread. Journal of Food Protection, 2010. 73(4): p. 758-762.
25. Ryan, L.A.M., et al., *Lactobacillus amylovorus* DSM 19280 as a novel food-grade antifungal agent for bakery products. International Journal of Food Microbiology, 2011. 146(3): p. 276-283.
26. Cizeikiene, D., et al., Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. Food Control, 2013. 31(2): p. 539-545.
27. Brosnan, B., et al., The QuEChERS approach in a novel application for the identification of antifungal compounds produced by lactic acid bacteria cultures. Talanta, 2014. 129(0): p. 364-373.
28. Lehotay, S.J., et al., Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. Journal of Chromatography A, 2010. 1217(16): p. 2548-2560.
29. Kinsella, B., et al., Determination of the new anthelmintic monepantel and its sulfone metabolite in milk and muscle using a UHPLC-MS/MS and QuEChERS method. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences, 2011. 879(31): p. 3707-3713.
30. Mol, H.G., et al., Toward a generic extraction method for simultaneous determination of pesticides, mycotoxins, plant toxins, and veterinary drugs in feed and food matrixes. Analytical Chemistry, 2008. 80(24): p. 9450-9.
31. EU, Commission decision of 12 August 2002 implementing council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (notified under document number C(2002) 3044) (Text

with EEA relevance) (2002/657/EC). . L 221/8-36 EN Official Journal of the European Communities 1782002., 2002.

32. ICH, International Conference on Harmonisation of technical requirements for registration of pharmaceutical for human use: Validation of analytical procedures: Text and Methodology Q2(R1) Current Step 4 version, Parent Guideline dated 27 October 1994 (Complementary Guidelines on Methodology dated 6 November 1996 incorporated in November 2005). 2005.
33. Broberg, A., et al., Metabolite profiles of lactic acid bacteria in grass silage. Applied and Environmental Microbiology, 2007. 73(17): p. 5547-5552.
34. Ndagano, D., et al., Antifungal Activity of 2 Lactic Acid Bacteria of the *Weissella* Genus Isolated from Food. Journal of Food Science, 2011. 76(6): p. M305-M311.
35. Wang, H.K., et al., Production and characterization of antifungal compounds produced by *Lactobacillus plantarum* IMAU10014. Plos One, 2012. 7(1): p. e29452.
36. Brosnan, B., et al., A comprehensive investigation into sample extraction and method validation for the identification of antifungal compounds produced by lactic acid bacteria using HPLC-UV/DAD. Analytical Methods, 2014. 6(14): p. 5331-5344.
37. Brosnan, B., et al., Rapid identification, by use of the LTQ Orbitrap hybrid FT mass spectrometer, of antifungal compounds produced by lactic acid bacteria. Analytical and Bioanalytical Chemistry, 2012. 403(10): p. 2983-2995.
38. Guo, J., et al., Antifungal activity of *Lactobacillus* against *Microsporum canis*, *Microsporum gypseum* and *Epidermophyton floccosum*. Bioeng Bugs, 2012. 3(2): p. 104-13.
39. Lynch, K.M., et al., Application of *Lactobacillus amylovorus* as an antifungal adjunct to extend the shelf-life of Cheddar cheese. International Dairy Journal, 2014. 34(1): p. 167-173.
40. McIlhenny, E.H., et al., Direct analysis of psychoactive tryptamine and harmala alkaloids in the Amazonian botanical medicine ayahuasca by liquid chromatography–electrospray ionization-tandem mass spectrometry. Journal of Chromatography A, 2009. 1216(51): p. 8960-8968.

41. Tarcomnicu, I., et al., High-throughput HPLC-MS/MS method to determine ibandronate in human plasma for pharmacokinetic applications. J Chromatogr B Analyt Technol Biomed Life Sci, 2009. 877(27): p. 3159-68.
42. Furey, A., et al., Ion suppression; A critical review on causes, evaluation, prevention and applications. Talanta, 2013. 115: p. 104-22.
43. Trufelli, H., et al., An overview of matrix effects in liquid chromatography–mass spectrometry. Mass Spectrometry Reviews, 2011. 30(3): p. 491-509.
44. Ćirić, A., et al., Evaluation of matrix effect in determination of some bioflavonoids in food samples by LC–MS/MS method. Talanta, 2012. 99(0): p. 780-790.
45. Chambers, E., et al., Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. Journal of Chromatography B, 2007. 852(1–2): p. 22-34.
46. Van Eeckhaut, A., et al., Validation of bioanalytical LC–MS/MS assays: Evaluation of matrix effects. Journal of Chromatography B, 2009. 877(23): p. 2198-2207.
47. Fong, B.M.-W., et al., Development and validation of a high-throughput double solid phase extraction–liquid chromatography–tandem mass spectrometry method for the determination of tetrodotoxin in human urine and plasma. Talanta, 2011. 83(3): p. 1030-1036.
48. Oldekop, M.-L., K. Herodes, and R. Rebane, Study of the matrix effects and sample dilution influence on the LC–ESI–MS/MS analysis using four derivatization reagents. Journal of Chromatography B, 2014. 967(0): p. 147-155.
49. Niku-Paavola, M.L., et al., New types of antimicrobial compounds produced by *Lactobacillus plantarum*. Journal of Applied Microbiology, 1999. 86(1): p. 29-35.
50. Valerio, F., et al., Production of phenyllactic acid by lactic acid bacteria: an approach to the selection of strains contributing to food quality and preservation. Fems Microbiology Letters, 2004. 233(2): p. 289-295.
51. Lind, H., H. Jonsson, and J. Schnürer, Antifungal effect of dairy propionibacteria - contribution of organic acids. International Journal of Food Microbiology, 2005. 98(2): p. 157-165.

52. Armaforte, E., et al., High-performance liquid chromatography determination of phenyllactic acid in MRS broth. Journal of Chromatography A, 2006. 1131(1-2): p. 281-284.
53. Schwenninger, S.M., et al., Characterization of low-molecular-weight antiyeast metabolites produced by a food-protective *Lactobacillus-Propionibacterium* coculture. Journal of Food Protection, 2008. 71(12): p. 2481-7.
54. Mu, W.M., et al., 3-Phenyllactic acid production by substrate feeding and pH-control in fed-batch fermentation of *Lactobacillus* sp SK007. Bioresource Technology, 2009. 100(21): p. 5226-5229.
55. Mu, W.M., et al., Production of 4-hydroxyphenyllactic acid by *Lactobacillus sp* SK007 fermentation. Journal of Bioscience and Bioengineering, 2010. 109(4): p. 369-371.
56. Cortés-Zavaleta, O., et al., Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. International Journal of Food Microbiology, 2014. 173(0): p. 30-35.
57. Ström, K., et al., *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Applied and Environmental Microbiology, 2002. 68(9): p. 4322-4327.
58. Sjögren, J., et al., Antifungal 3-hydroxy fatty acids from *Lactobacillus plantarum* MiLAB 14. Applied and Environmental Microbiology, 2003. 69(12): p. 7554-7557.
59. Oliveira, P.M., et al., Lactic acid bacteria bioprotection applied to the malting process. Part I: Strain characterization and identification of antifungal compounds. Food Control, (0).
60. Anastassiades, M., et al., Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. Journal of Aoac International, 2003. 86(2): p. 412-431.
61. Westland, J.L. and F.L. Dorman, QuEChERS extraction of benzodiazepines in biological matrices. Journal of Pharmaceutical Analysis, 2013. 3(6): p. 509-517.

62. Bragança, I., et al., QuEChERS: A new sample preparation approach for the determination of ibuprofen and its metabolites in soils. Science of the Total Environment, 2012. 433(0): p. 281-289.
63. Delgado-Zamarreño, M.M., et al., A modified QuEChERS method as sample treatment before the determination of isoflavones in foods by ultra-performance liquid chromatography–triple quadrupole mass spectrometry. Talanta, 2012. 100(0): p. 320-328.
64. Yogendrarajah, P., et al., Development and validation of a QuEChERS based liquid chromatography tandem mass spectrometry method for the determination of multiple mycotoxins in spices. Journal of Chromatography A, 2013. 1297(0): p. 1-11.
65. Devlieghere, F., L. Vermeiren, and J. Debevere, New preservation technologies: possibilities and limitations. International Dairy Journal, 2004. 14(4): p. 273-285.
66. Vermeulen, N., M.G. Ganzle, and R.F. Vogel, Influence of peptide supply and cosubstrates on phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM20451(T) and *Lactobacillus plantarum* TMW1.468. Journal of Agriculture Food Chemestry, 2006. 54(11): p. 3832-9.
67. Hager, A.-S., et al., Nutritional properties and ultra-structure of commercial gluten free flours from different botanical sources compared to wheat flours. Journal of Cereal Science, 2012. 56(2): p. 239-247.
68. Wolter, A., et al., Evaluation of exopolysaccharide producing *Weissella cibaria* MG1 strain for the production of sourdough from various flours. Food Microbiology, 2014. 37: p. 44-50.
69. Axel, C., et al., Application of *Lactobacillus amylovorus* DSM19280 in gluten-free sourdough bread to improve the microbial shelf life. Food Microbiology, (2015) 47: p. 36-44.
70. Hager, A.-S., et al., Investigation of product quality, sensory profile and ultrastructure of breads made from a range of commercial gluten-free flours compared to their wheat counterparts. European Food Research and Technology, 2012. 235(2): p. 333-344.

71. Sangmanee, P. and T. Hongpattarakere, Inhibitory of multiple antifungal components produced by *Lactobacillus plantarum* K35 on growth, aflatoxin production and ultrastructure alterations of *Aspergillus flavus* and *Aspergillus parasiticus*. Food Control, 2014. 40(0): p. 224-233.

Other publications/presentations

Claudia Axel, Emanuele Zannini, Aidan Coffey, Jiahui Guo, Deborah M. Waters, Elke K. Arendt

Eco-friendly control of potato late blight causative agent and the potential role of lactic acid bacteria: a review

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Jiahui Guo, Brid Brosnan, Ambrose Furey, Elke K. Arendt, Claudia Axel, Aidan Coffey

Anti-oomycete potential of *Lactobacillus amylovorus* JG2 against the potato blight pathogen *Phytophthora infestans*

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Anti-oomycete potential of *Lactobacillus amylovorus* JG2 against the potato blight pathogen *Phytophthora infestans*

Society of General Microbiology Spring Conference, Manchester, United Kingdom, 25th to 28th March 2013 (oral presentation)

Claudia Axel, Jiahui Guo, Brid Brosnan, Ambrose Furey, Elke K. Arendt, Aidan Coffey

Anti-oomycete potential of *Lactobacillus amylovorus* JG2 against the potato blight pathogen *Phytophthora infestans*

11th International Symposium on Lactic Acid Bacteria, Egmond aan Zee, Netherlands, August 31st to September 4th 2014 (poster presentation)