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UNIVERSITY COLLEGE, CORK

School of Food and Nutritional Sciences



REDUCTION OF SALT IN YEASTED WHEAT BREAD: IMPACT ON BREAD QUALITY AND SOLUTIONS USING SOURDOUGH FERMENTED BY FUNCTIONAL LACTIC ACID BACTERIA STRAINS

Thesis presented by
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To obtain the degree of
Doctor of Philosophy - PhD in Food Science and Technology

Head of School
Prof. Yrjo Roos

April 2016

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.

_____

Markus Belz

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Abbreviations

2D-HRGC-MS	2-dimensional high-resolution gas chromatography mass spectrometer
AIB International	
AESAN	Spanish Food Safety Authority (Spain)
AFSSA	French Food Safety Authority (France)
AGF	Arbeitsgemeinschaft Getreideforschung e.V.
a_w	water activity
CFU	cell forming unit
CP	calcium propionate
DAD	diode array detector
DGE	German Nutrition Society (Germany)
DSM	Deutsche Sammlung von Mikroorganismen
DW	dough weight
EA	ethyl acetate
EPS	exopolysaccharides
FA	formic acid
FAO	Food and Agriculture Organistation (USA)
FDA	Food & Drug Administration (USA)
FFF	field-flow fractionation
FOPH	Federal Office of Public Health (Switzerland)
FSA	Food Safety Authority (UK)
FSAI	Food Safety Authority Ireland
FST	Food Science & Technology
HPLC	high pressure liquid chromatography

IUNA	Irish University Nutrition Alliance
LTH	Lund university, Faculty of Engineering
MRS	de Man, Rogosa, Sharpe
n/a	not applicable
NaCl	sodium chloride
n/d	not detectable
PC	principal component
PCA	principal component analysis
PCR	polymer chain reaction
PTR-MS	proton-transfer-reaction mass spectrometry
RID	refractive index detector
RH	relative humidity
SAFE	solvent-assisted flavour evaporation
SACN	Scientific Advisory Committee on Nutrition (UK)
SIDA	stable isotope dilution assay
TMW	Technische Mikrobiologie Weihenstephan
TTA	titratable acid
TUM	Technische Universität München
UV	ultraviolet
WHO	World Health Organisation

Abstract

The dietary intake of sodium chloride has increased considerably over the last few decades due to changes in the human diet. This higher intake has been linked to a number of diseases including hypertension and other cardiovascular diseases. Numerous international health agencies, as well as the food industry, have now recommended a salt intake level of 5-6 g daily, approximately half of the average current daily intake level. Cereal products, and in particular bread, are a major source of salt in the Western diet. Therefore, any reduction in the level of salt in bread could have a major impact on global health. However, salt is a critical ingredient in bread production, and its reduction can have a deleterious effect on the production process as well as on the final bread quality characteristics such as shelf-life, bread volume and sensory characteristics, all deviating from the bakers' and consumers' expectations. This work addresses the feasibility of NaCl reduction in wheat bread focusing on options to compensate NaCl with the use of functional sourdoughs. Three strains were used for the application of low-salt bread; *L. amylovorus* DSM19280, *W. cibaria* MG1 and *L. reuteri* FF2hh2. The multifunctional strain *L. reuteri* FF2hh2 was tested the first time and its application could be demonstrated successfully. The functionalities were based on the production of exopolysaccharides as well as the production of antifungal compounds. While the exopolysaccharides, mainly high molecular dextrans, positively influenced mainly bread loaf volume, crumb structure and staling rate, the strains producing antifungal compounds prolonged the microbial shelf life significantly and compensated the lack of salt. The impact on the sensory characteristics of bread were evaluated by descriptive sensory evaluation. The increase in surface area as well as the presence of organic acids impacted significantly on the flavour profile of the sourdough bread samples. The flavour attribute "salt" could be enhanced by sourdough addition and increased the salty perception. Furthermore, a trained sensory panel evaluated for the first time the impact of yeast activity, based on different salt and yeast concentrations, on the volatile aroma profile of bread crumb samples. The analytical measurements using high resolution gas chromatography and proton-transfer-reaction mass spectrometry (PTR-MS) resulted in significantly different results based on different yeast activities. Nevertheless, the extent of the result could not be recognised by the sensory panel analysing the odour profile of the bread crumb samples. Hence, the consumer cannot recognised low-salt bread by its odour. The use of sourdough is a natural option to

overcome the broad range of technological issues caused by salt reduction and also a more popular alternative compared to existing chemical salt replacers.

Chapter 1

Introduction

1.1 Introduction

Mean daily salt intakes of the population in developed countries are well in excess of their dietary needs. While the human body requires 3-4 g sodium chloride (NaCl) per day, the average daily salt intake ranges between 7-18 g NaCl (European Commission, 2008, European Food Saftey Authority, 2005). It has been widely reported that high daily salt intake results in hypertension and numerous other health problems (du Cailar et al., 2002, Elliott et al., 1996). In particular, cardio-vascular diseases were determined as the most concerning impact on human health (Farquhar et al., 2015, O'Donnell et al., 2015, Brown et al., 2009, Angus, 2007, Stamler, 1997, Reddy and Marth, 1991, Freis, 1976). This results in a serious strain on health systems and has a negative impact on society. The importance of sodium chloride reduction in the Western diet has been highlighted by global organisations (WHO and FAO, 2003, Codex Alimentarius, 1997) and has been put on the agenda of national authorities in Europe by the European Union (European Parliament and the Council of the EU, 2006, European Commission, 2008), Ireland (FSAI, 2005, FSAI, 2015), Germany (German Nutrition Society (DGE), 2009), the United Kingdom (SACN, 2003), France (French Food Safety Agency (AFSSA), 2009), Spain (Spanish Food Safety Agency (AESAN), 2010), Switzerland (Federal Office of Public Health (FOPH), 2009), Norway (Norwegian Ministry of Health and Care, 2007), Iceland (Public Health Institute of Iceland, 2007), Turkey (Turkish Society of Hypertension and Renal Diseases, 2008), Canada (Canadian Food Inspection Agency, 2004), the United States of America (FDA, 2005), South Africa (Charlton et al., 2008), Korea (Korean Food and Drug Administration, 2007) and Australia (Neal, 2008).

Different studies pointed out that the vast majority of processed foods have significant levels of sodium added during the manufacturing process, leading to excessive sodium intake (He et al., 2001). In addition, increasing consumption of processed food was reported by several authors (Reddy and Marth, 1991, Gibson et al., 2000, SACN, 2003). Depending on the country and the respective culinary tradition, up to 40% of the daily NaCl intake is attributed to cereal products (Thomson, 2009, Cauvain, 2007, Angus, 2007). Bread is classed as a staple food worldwide and has been found to be a major source of dietary sodium (James et al., 1987, Greenfield et al., 1984), being responsible for an average of 30% of the daily salt intake (Girgis et al., 2003). Hence, one of the most efficient way to decrease the daily NaCl intake is the reduction of NaCl in bread (Girgis et al., 2003).

About 20 years ago, Irish bread contained an average of 1.2 % NaCl (Lynch et al., 2009, Gormley and Morrissey, 1993). Due to the fact that bread is a leading source of sodium, the Food Safety Authority of Ireland (FSAI) required a reduction in the salt level in bread. Hence, the NaCl reduction programme was started by the FSAI in 2003. The periodic monitoring over the last 12 years presented Ireland as a successful example showing that the concentration of salt in processed foods were reduced towards the set targets (FSAI, 2015). The bread category showed NaCl reduction of 18-34% depending on the type of bread resulting in an average reduction of NaCl from 1.2% to 1.0%.

However, reduction of salt in foods influences many quality characteristics which are important for consumer acceptance and industrial suitability. These include the direct salty taste in foods as well as the enhancing effect salt has on other flavour constituents. Furthermore, salt serves as a preservative agent against microbial growth by reduction of the water activity. The technological process of bread baking as well as some of the final quality characteristics of bread such as shelf life (Samapundo et al., 2010, Pateras, 2007, Filtenborg et al., 1996), aroma and flavour profile, (Lynch et al., 2009) and crumb structure (Beck et al., 2012) are affected by the reduction of NaCl.

Bread is known as a high moisture product with water activity values between 0.96 - 0.98 (Smith et al., 2004). As demonstrated by Doerry (1990), microbial spoilage is the main cause for shelf life issues in intermediate and high moisture food products. Nowadays, mould growth is still a cause of high losses to the bread producing industry (Pateras, 2007, Smith et al., 2004, Corsetti et al., 1998, Legan, 1993). Many references show that fungal spoilage is not a current problem in the baking industry but causing trouble and losses since decades (Samapundo et al., 2010; Pateras, 2007; Filtenborg et al., 1996; Legan, 1993; Jarvis, 1972; Knight and Menlove, 1961). Knight and Menlove (1961) could show that mould spores are killed during the baking process and the issue limiting the long-term shelf life of bread is a post-baking fungal contamination. Sodium chloride, commonly called salt, acts as a preservative agent in bread, due to its ability to reduce the water activity. Increased osmotic pressure causes cells to loose water to the environment, thus, inhibiting cell growth.

The impact of salt reduction on taste profiles has been demonstrated for numerous foods, amongst them white yeasted bread. An investigation by Tuorila-Ollikainen and co-workers (1986) on white yeasted, rye and rye-sourdough breads indicated a low consumer

preference for the reduced-salt breads. In contrast, however, Wyatt (1983) observed no significant difference in consumer preference for white bread with a 50 % reduction in NaCl compared with the reference bread. The current challenge for food producers is to develop products with a reduced salt content but an unimpaired and consistent taste. This has been investigated with the use of salt replacers such as potassium chloride, magnesium chloride, ammonium chloride, calcium chloride and calcium carbonate (Bassett et al., 2014, Charlton et al., 2007), the use of sourdough (Rizzello et al., 2010) or yeast extract (Spina et al., 2015), the inclusion of flavour-enhancing acids and other aroma-intensive compounds (Ghawi et al., 2014, Jimenez-Maroto et al., 2013, Breslin, 1996, Hellemann, 1992, Reddy and Marth, 1991) or by changes to the bread crumb texture influencing the saltiness perception (Kuo and Lee, 2014, Pflaum et al., 2013a, Pflaum et al., 2013b, Noort et al., 2012, Noort et al., 2010).

In contrast to taste, little is known about the influence of salt reduction on the volatile aroma profile of food and there are no reports of studies investigating the relationship of these properties in bread crumb. Volatile aroma compounds impart flavour to food, and the volatile fraction of bread is highly complex with about 600 volatile compounds reported to be present in bread crumb (Schieberle and Grosch, 1991). In particular, the yeast metabolism plays a key role in the development of a bread's aroma profile and salt, primarily its sodium ions, has a direct impact on yeast activity (Matz, 1992). In addition to ethanol and carbon dioxide, many low molecular weight flavour compounds such as further alcohols, aldehydes, acids, esters, sulphides and carbonyl compounds are produced by the yeast metabolism. These volatile compounds are essential contributors to the flavour of fermented foods and beverages (McKinnon et al., 1996, Suomalainen and Lehtonen, 1979, Whiting, 1976). The Ehrlich pathway is one of several routes responsible for the generation of aroma compounds by yeast in bread. In particular, it leads to the formation of potent compounds such as fusel alcohols and acids. The efficacy of the Ehrlich pathway in converting amino acids into alcoholic odorants was investigated, amongst others, by Czerny and Schieberle (2006), who used stable isotope dilution assays (SIDA) to demonstrate the conversion of C^{13}(6)-leucine to the metabolite 3-methylbutanol. Transamination and decarboxylation were described as the key reaction sequence of the Ehrlich pathway followed by either an oxidation to the corresponding acid or a reduction to the corresponding alcohol (Sentheshanmuganathan, 1960). The cultivation conditions

dictate which of the two reactions are favoured. During bread dough fermentation, the reduction towards alcohol dominates over the oxidation reaction (Hazelwood et al., 2008, Sentheshanmuganathan, 1960).

Chapter 3, Chapter 4 and Chapter 5 determine the potential of sourdough addition to compensate the reduction of NaCl in yeasted wheat bread. Based on previous research three functional lactic acid bacteria strains were considered; the antifungal strain *Lactobacillus amylovorus* DSM19280 (Axel et al., 2015, Ryan et al., 2011), the exopolysaccharide (EPS) producer *Weisella cibaria MG1* (Wolter et al., 2014, Galle et al., 2011, Galle et al., 2010) and the strain *Lactobacillus reuteri* FF2 which is the first multifunctional strain published with a combination of both functionalities; antifungal activity and production of EPS. The antifungal strain *L. amylovorus* DSM19280 had the potential to replace artificial preservatives and fully compensate their impact on microbial shelf life of bread. The *W. cibaria* MG1 strain improved bread loaf volume as well as bread crumb structure due to the EPS which was produced during fermentation. The multifunctional characteristics of the *L. reuteri* FF2 strain made it possible to compensate the lack of sodium chloride in low-salt bread using only one type of sourdough. The impact of the different sourdoughs on the dough and bread quality was analysed. Rheological analyses were performed to determine changes to the dough samples based on sourdough addition. The changes of the different loaf characteristics such as loaf volume, crumb structure, water activity, shelf life and flavour profile were evaluated to optimise the quality of low-salt bread.

Chapter 6 determines the impact of NaCl on the yeast metabolism in bread dough during fermentation and its influence on the overall aroma of the bread. More specifically, the unsaturated aldehydes (E)-non-2-enal (*fatty*) and (E,E)-deca-2,4-dienal (*fatty*), and the alcoholic compound phenylethan-2-ol (*rose-like*) were investigated as the key aroma compounds in bread based on preliminary analyses and supported by reports in the literature (Birch et al., 2013, Schuh and Schieberle, 2006, Schieberle, 1996, Gassenmeier and Schieberle, 1995, Frasse et al., 1992, Schieberle and Grosch, 1992, Schieberle and Grosch, 1991). A complementary analytical approach using two-dimensional high-resolution gas chromatography-mass spectrometry (2D-HRGC-MS) and proton-transfer-reaction mass spectrometry (PTR-MS) was used to quantify and monitor the generation of the selected aroma compounds in bread crumb samples with different levels of salt and yeast. Sensory analysis was performed on the bread crumb samples to determine their

odour characteristics and assess the impact of salt reduction on bread crumb based on a discrimination triangle test.

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Chapter 2

The Impact of Salt Reduction in Bread: A Review

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2.1 Abstract

The dietary intake of sodium chloride has increased considerably over the last few decades due to changes in the human diet. This higher intake has been linked to a number of diseases including hypertension and other cardiovascular diseases. Numerous international health agencies, as well as the food industry, have now recommended a salt intake level of about 5-6 g daily, approximately half the average current daily intake level. Cereal products, and in particular bread, are a major source of salt in the diet. Therefore, any reduction in the level of salt in bread would have a major impact on global health. However, salt is a critical ingredient in bread production, and its reduction can have a deleterious effect on the production process. This includes an impact on dough handling, as well as final bread quality characteristics, including shelf-life, bread volume and sensory characteristics, all deviating from the bakers' and consumers' expectations. This review describes the effect of salt reduction during bread production and the resulting problems, both technological and qualitative, as well as evaluating some techniques commonly used to replace sodium chloride.

2.2 Introduction

Recently, there has been much focus on the effect of sodium chloride (table salt) in the human diet. It has been widely reported that high daily salt intake results in hypertension as well as numerous cardio-vascular diseases and other health problems (du Cailar et al., 2002, Elliott et al., 1996). This results in a serious strain on health systems and has a negative impact on society. The importance of sodium chloride reduction in the diet had been highlighted by several legislations introduced by the European Union (European Parliament and the Council of the EU, 2006), Canada (Canadian Food Inspection Agency, 2004) and the United States of America (FDA, 2005), allowing nutrition and health claims for reduced sodium contents in food.

Different studies pointed out that the vast majority of processed foods have significant levels of sodium added during the manufacturing process, leading to excessive sodium intake (He et al., 2001). Bread is classed as a staple food worldwide and has been found to be a major source of dietary sodium (James et al., 1987, Greenfield et al., 1984), being responsible for an average of 30% of the daily salt intake (Girgis et al., 2003). Irish bread contains an average of 1.2% salt (Lynch et al., 2009, Gormley and Morrissey, 1993). The daily bread consumption in Ireland averages at around 173 g of bread which equals 4 slices of bread and contributed with 26% to the mean daily salt intake in Ireland in 2003 (FSAI, 2015, IUNA, 2001). In 2013, the contribution of bread products was significantly lower with 22% mainly due to a reduction of salt in bread. Due to the fact, that bread is a leading source of sodium, the Food Standards Agency of UK (FSA) and the Food Safety Authority of Ireland (FSAI) required a reduction in the salt level in bread. Hence, the salt reduction programme was started by the FSAI in 2003. The periodic monitoring over the last 12 years showed that the concentration of salt in processed food were reduced. For white bread, the salt was reduced by 18% from 1.35% in 2003 to 1.1% in 2011. While the first target of 6 g per day was defined in 2005, the mean dietary salt intake still exceeded 6 g per day in 2011 (FSAI, 2015).

However, reduction of salt in foods influences many quality characteristics which are important for consumer acceptance and industrial suitability. These include the direct salty taste in foods as well as the enhancing effect salt has on other flavour constituents. Furthermore, salt serves as a preservative agent against microbial growth by reduction of

the water activity. The impact of salt on handling and processing properties of food must also not be underestimated (Beck et al., 2012, Hutton, 2002). In bread production, salt has a profound effect during processing and on the final product characteristics (Belz et al., 2012, Beck et al., 2012, Lynch et al., 2009, Kilcast and Angus, 2007).

2.3 Sodium Chloride

Sodium chloride (NaCl), commonly known as salt or table salt is widely used in food production (Man, 2007). Salt is an ionic combination of sodium and chlorine and every 2.54 g of salt consumed, yields 1 g of sodium. Pure salt is a transparent, colourless, hygroscopic and crystalline substance with a specific gravity of 2.165 (Man, 2007). Throughout the following review, the term ‘salt’ is used exclusively for sodium chloride.

For thousands of years salt was ubiquitously used to preserve foods. During the last century the invention of freezers and refrigerators resulted in salt losing its importance as a preservative and the daily salt uptake decreased. Recently, the daily uptake of salt has risen considerably, caused by the increasing consumption of processed, canteen and fast food.

This excessive salt intake has been correlated with hypertension, which is associated with an increased risk of cardiovascular diseases like heart attacks and strokes (Farquhar et al., 2015, AIB International, 2008, Gibson et al., 2000). After the assumption that approximately half of the deaths, caused by chronic diseases, may be attributable to cardiovascular diseases, the WHO and FAO (2003) outlined the necessity to lower the daily sodium intake. The impact of salt on hypertension and high blood pressure has been widely reported in a variety of different studies. These include epidemiological (Elliott et al., 1996), migratory (Poulter et al., 1990), interventional (Forte et al., 1989), treatment (He and MacGregor, 2002), animal (Denton et al., 1995), and genetic studies (Lifton, 1996). Elevated salt intake also has been linked to other diseases like albuminuria (du Cailar et al., 2002, Verhave et al., 2004), stomach cancer (Beevers et al., 2004, Tsugane et al., 2004), asthma (Carey et al., 1993, Mickleborough et al., 2005) and kidney diseases (Matkovic et al., 1995). Hence, the obvious need of salt reduction is urgent and undisputable from a health point of view.

Recently, it has been reported that only about 13% of the population in UK are aware of the important contribution of bread and other cereal products to the daily salt intake

(Angus, 2007). A reduction of salt in cereal products would already lead to a significant decrease of the daily salt intake resulting in an enormous impact on reducing the number of strokes, heart attacks and heart failure (He et al., 2007). Therefore, partnerships between the responsible organisations like food industries, governments and research institutions are beneficial.

2.3.1 Recommended Levels of Salt

Over the last 20 years, many developed countries have been confronted with the excessive average salt intakes of its populations. These countries have determined the current salt intake throughout the population and set up recommended levels for the daily salt intake, which vary slightly considering the different political, economic and scientific national aspects. The WHO has recommended a daily salt intake of 5 g per day as a worldwide guideline. Within the EU, most of the member states recommended 2.4 g sodium (6 g salt) daily (Table 1); the same level is promoted in the United States. Recently, the WHO highlighted again their recommendation of 5 g per day during the 61st Regional Committee for Europe (WHO, 2011). However, the amount of sodium required to ensure normal body function and regulation of extracellular fluid is about 1.5 g (~3.8 g salt) per day (Commision of the European Communities, 1993). All these targets represent a substantial reduction in salt intake; this can be achieved only by cooperation with food manufacturers. In Ireland, the FSAI has set up a wide range of agreements with food companies and industry bodies to support a national salt reduction policy. The first target was defined in 2005 as an average salt intake of 6 g per day in adults by 2010 (FSAI, 2005). In 2011, the data presented by the Irish University Nutrition Alliance (IUNA, 2011) showed that the mean dietary salt intake still exceeded the set target of 6 g per day. For 18-65 year olds, the mean daily salt intake was 7.4 g (8.5 g for men and 6.2 g for women).

Finland and the UK were the only two European countries who recommended specific nutritional intakes of salt for babies (European Commission, 2008). The importance of restricted salt intake for infants was shown by Hofman et al. (1983) and Geleijnse et al. (1997). In these studies, a comparison was performed between babies with reduced salt intake and regular salt intake over the first six months of life. The mothers were allowed to breast feed and received formula milk depending to which group they belonged. They were

also advised to start with solid foods 13 weeks after birth which was provided as part of the study. After six months, the study was discontinued and all participants resumed the usual daily salt intake. On examination of the subjects, 15 years later, significant lower blood pressures were observed compared to participants without a low-salt diet in their first six months of life.

Table 1: Values of current national salt intake averages and recommended national salt intake targets. The countries presented were chosen by the availability of data only. As far as our research could determine the presented values are the newest available data of each country.

Country	Organisation	year	daily salt intake		
			current		target
			male	female	
	WHO and FAO	2003	-		<5.0 g
Australia	Food Standards Australia New Zealand	2007	9.0 g		<6.0 g by 2012
Canada	Canadian Food Inspection Agency	2004	8.0 g		5.8 g by 2016; final target of 3.8 g
European Union*	European Commission (2008)	2008	7.0-18.0 g **		5.0-8.7 g**
Finland	National Institute for Health and Welfare	2004	10.0 g	8.0 g	6.0 – 7.0 g / <2 years 0.5 g
France	French Food Safety Authority	2007	7.7 g		8.0 g
Germany	Federal Institute of Risk Assessment and German Nutrition Society	2009	8.2 g	6.0 g	<6.0 g recommended
Hungary	Rodler, Bíró et al. (2004)	2004	18.0 g	16.0 g	5.0 g
Ireland	Food Safety Authority of Ireland	2015	8.5 g	6.2 g	6 g
Netherlands	National Institute for Public Health and the Environment	2006	9.7 g	7.6 g	6.0 g
Portugal	Martins et al. (2009); Portuguese Society of Hypertension	2006	11.9 g		6.0 g
Spain	Spanish Food Safety Agency	2010	9.7 g		8.5 g by 2014; final target of 5.0 g
United Kingdom	Medical Research Council Human Nutrition Research	2004	11.0 g	8.0 g	6.0 g by 2010
Iceland	Steingrimsdottir et al. (2002); Icelandic Nutrition Council	2002	10.0 g	7.0 g	6.0-7.0 g by 2010
Japan	Japanese Hypertension Society	2008	11.0 g		6 g
Korea	Korean Food and Drug Administration	2007	13.5 g		no target
Norway	Ministry of Health and Care Service	2007	10.0 g		5.0 g
South Africa	Charlton et al. (2008)	2007	8.0 g		6.0 g
Switzerland	Federal Office of Public Health (2009)	2007	10.0-13.0 g		5.0 g
Turkey	Turkish Society of Hypertension and Renal Diseases	2008	19.3 g	16.8 g	no target
United States of America	Institute of Medicine of the National Academies (2010)	2008	9.0 g		6 g

*The EU gives only general regulations and supporting guidelines. The member states have to act themselves.

**based on the data of the member states available in 2008

2.3.2 Levels of Salt in Foods

Salt plays a role in human physiology, nutrition and health and has also a multi-functional role in processed foods and drinks influencing e.g. flavour, shelf-life and several technological characteristics. As salt is a relatively cheap product with versatile beneficial impacts it is used extensively in food processing (MacGregor and de Wardener, 1998). In 2000, approximately 75% of daily salt intake in Northern Ireland originated from processed foods (Gibson et al., 2000). Over 60% of this intake came from two major food groups. Group one was meat/fish products including processed meats, providing almost 30% of total sodium intake. The second group consisted of cereals and cereal products including bread as well as breakfast cereals, biscuits, cakes, pastries and confectionaries. This product group contributed approximately 35% to the daily intake of sodium in the UK in 2007 (IUNA, 2011, Angus, 2007). Bread and rolls contributed up to 26% of this daily sodium intake level (Table 2). Ten years later, the contribution to the daily sodium intake of both product groups still summed up to 52% (IUNA, 2011) of which 22% came from bread.

Table 2: Mean daily Sodium Intake from Foods in Irish Adults Aged 18-64 Years by Food Group* (FSAI, 2015, IUNA, 2011 + 2001)

	2003		2013		Sodium change
Food/Food category	**g/day**	**% total**	**g/day**	**% total**	**in %**
Meat & fish	**0.97**	**29.8**	**0.97**	**33.4**	**+0.01**
Cured/processed meats	0.67	20.5	0.58	19.9	-14.0
Meat/meat dishes	0.23	6.9		n/d	
Fish/fish dishes	0.08	2.4		n/d	
Bread & rolls	**0.84**	**25.9**	**0.64**	**22.0**	**-23.8**
Milk & milk products	**0.27**	**8.5**	**0.25**	**8.6**	**-7.4**
Cheese	0.12	3.5	0.09	3.1	-26.4
Soups, sauces & miscellaneous foods	**0.23**	**7.0**	**0.15**	**5.2**	**-36.2**
Spreading fats	**0.19**	**5.9**	**0.16**	**5.5**	**-14.3**
Biscuits/cakes/pastries/confectionary	**0.15**	**4.5**	**0.11**	**3.8**	**-30.0**
Breakfast cereals	**0.14**	**4.2**	**0.10**	**3.4**	**-30.9**
Ready-to-eat breakfast cereals	0.13	4.1	0.07	2.4	-44.5
Other	trace	0.1	0.03	1.0	+51.0
Vegetables/processed vegetables	**0.13**	**4.0**	**0.20**	**6.8**	**+54.0**
Processed vegetables/vegetable dishes	0.04	1.1		n/d	
Savouries (e.g. pizza, ready meals etc.)	**0.095**	**2.9**	**0.07**	**2.4**	**-26.7**
Egg/egg dishes	**0.049**	**1.5**		**n/d**	
Desserts	**0.035**	**1.1**		**n/d**	
Other foods	**0.15**	**4.7**	**0.26****	**8.9**	**n/a**
Total	3.25	100.0	2.91	100.0	-10.4

* Estimated from the Northern/South Ireland Food Consumption Survey (IUNA, 2001 + 2011) for Republic of Ireland only (n=776), excluding under-rep and FSAI (2015)

**includes egg dishes and desserts

2.3.2 Regulations for Low-salt Claims

Rising awareness of the negative health effects of high salt intake levels has caused an increased interest in low-salt foods. This can be seen by the dramatic increase in low-salt products currently available on supermarket shelves. Due to the rising costs in health systems and increasing numbers of cardiovascular diseases, political institutions like the European Union have taken action and are now actively promoting a reduction in salt levels.

Nearly ten years after the Codex Alimentarius was introduced giving guidelines on how to manage nutrition and health claims, regarding sodium with guideline CAC/GL 23-1997 (Codex Alimentarius, 1997), the European Parliament and the Council of the EU (2006) passed the Regulation (EC) No 1924/2006 (2006) on "Nutrition and health claims made on foods". Amongst others, this document allows the use of nutrition claims regarding the content of sodium/salt and regulates the following labels in low sodium/salt, very low sodium/salt and sodium-free/salt-free as outlined below:

Low sodium/salt: A claim that a food is low in sodium/salt, and any claim likely to have the same meaning for the consumer, may only be made where the product contains no more than 0.12 g of sodium, or the equivalent value for salt (0.30 g), per 100 g or per 100 ml.

Very low sodium/salt: a claim that a food is very low in sodium/salt, and any claim likely to have the same meaning for the consumer, may only be made where the product contains no more than 0.04 g of sodium, or the equivalent value for salt (0.10 g), per 100 g or per 100 ml.

Sodium-free or salt-free: a claim that a food is sodium-free or salt-free, and any claim likely to have the same meaning for the consumer, may only be made where the product contains no more than 0.005 g of sodium, or the equivalent value for salt (0.0125 g), per 100 g.

2.4 Technological impact of salt on bread production

Bread is one of the world's oldest foods (Orth and Shellenberger, 1988) and is usually produced by mixing flour, water, yeast and salt, followed by fermentation and baking. While the average amount of salt in Irish bread was still 1.2% in 2003 (Lynch et al., 2009, FSAI, 2005, Gormley and Morrissey, 1993), the Irish salt reduction programme resulted in

a current average of about 1.0% in Irish bread (IUNA, 2011). Salt, while present in low amounts has quite a high impact on the quality characteristics of bread.

In general, the functions of salt in bread are summarised as (Man, 2007):

- Sensory effect by imparting flavour
- Control of yeast growth and fermentation rate
- Improvement of product texture
- Reduction of spoilage, particularly mould spoilage.

While excess salt use is problematic from a nutritional point of view, it has been shown to positively influence the technological process of every stage of bread production including: (i) mixing, (ii) fermentation, (iii) baking as well as on (iv) final bread quality characteristics.

Three main technological effects of salt on the process of bread baking are summarised as (Cauvain, 2007):

- The development of gluten-structures in the mixing of dough is promoted.
- Yeast activity is inhibited during the fermentation step, which results in a lower gas release.
- Water activity is controlled in the baked products.

An important fourth technological effect of salt is the formation of fine elastic crumb during baking (Sluimer, 2005).

2.4.1 Mixing

Wheat dough is a complex mixture of starch, proteins, water, fibres, fat, salt and minor ingredients. The first step of bread production is the development of dough by mixing. The ingredients are incorporated and dispersed to form dough with unique viscoelastic properties (Kilborn and Tipples, 1972). It has been widely reported that the quality characteristics of bread are mainly dependent on the quality of the dough. This is primarily based on structural improvement and gas incorporation at this stage of production (Dobraszczyk and Morgenstern, 2003, Naeem et al., 2002, Dobraszczyk et al., 2000, Bloksma, 1990, Hoseney and Rogers, 1990).

Salt influences the physical requirements for dough development due to its ionic nature. It is also known to cause changes in water absorption of flour as well as the necessity of changes in production settings like mixing time, mixing intensity and relaxation time (Lynch et al., 2009, Larsson, 2002, Wehrle et al., 1997, Preston, 1981). Other parameters like temperature, duration and work input (speed and energy) (Kilborn and Tipples, 1972) and the ratio of flour to available water (Seetharaman et al., 2004) are also essential for the formation of the unique viscoelastic network associated with wheat dough. To date, numerous studies have evaluated the effect of salt on dough; however, numerous methods of evaluation have been used leading to some disparity in the reported results.

Characterising dough requires the use of rheological measurements to obtain the extent of changes, which occur during the mixing process. Rheology is the study of the flow and deformation of matter. Two independent and unrelated types of rheological tests (empirical and fundamental) are performed, which meet the various knowledge requirements of bakers as well as food scientists. A controlled and well-defined deformation or strain is applied to a material over a given time to measure rheological behaviour and the resulting force response (or *vice versa*). This gives an indication of material parameters such as stiffness, modulus, viscosity, hardness, strength or toughness (Dobraszczyk and Morgenstern, 2003).

Industrialised production of low-salt bread requires precise description of the rheological changes in dough to secure machinability and proper handling. Therefore, rheological analyses can help to understand what happens at different salt levels (Lynch et al., 2009, Salvador et al., 2006, Wehrle et al., 1997).

2.4.2 Empirical Rheology

Empirical measurements of rheological properties have a long history within the cereals industry to evaluate performance during processing and for quality control. Instruments such as the farinograph, extensograph and alveograph are robust, easy to handle and have provided a great deal of information on the quality and performance of cereal products such as e.g. consistency, hardness and texture (Dobraszczyk and Morgenstern, 2003). Several rheological investigations have now accurately characterised the phenomena experienced by artisanal and craft bakers during low-salt bread production.

A comparison of farinograms of doughs produced with salt levels of 0 % and 2 % resulted in a significant increase of dough consistency for the salted dough (Wehrle et al., 1997). Interestingly, these authors also showed that the addition of salt resulted in a softer dough. These phenomena were most likely related to the restriction of water mobility by salt interaction with macromolecules in the dough (Salvador et al., 2006, Angioloni and Rosa, 2005, Dobraszczyk, 1997a, Dobraszczyk and Roberts, 1994, Oosten, 1979). In fact, less water is available for the development of the gluten network and dough development time is increased (Hlynka, 1962). Furthermore, a decrease in water absorption with increasing salt level has been demonstrated by Hlynka (1962) and Preston (1989).

Besides the farinograph, the extensograph is used commonly for empirical rheological analyses measuring resistance and extensibility of dough. Tanaka et al. (1967) showed a threefold increase in resistance to extension in dough containing 3% compared to 0% salt. These results correlate with those recently reported by Lynch et al. (2009). These authors found that at lower salt levels between 0.0 and 1.2%, an increase of dough resistance correlated with an increasing amount of salt. However, only the 0% and 1.2% doughs were significantly different (Lynch et al., 2009). Different results were presented by Preston (1989) with a significant increase in dough resistance being found between samples with 0.3, 0.5, 0.75, and 1.0% salt. This is most likely due to variations in the ingredients properties as well as testing methods utilised in both studies. This highlights the importance of having standardised methods of evaluation to characterise rheological properties.

2.4.3 Small Scale Deformation Oscillatory Rheology

In spite of the advantages of using empirical rheology these methods do not fulfil the requirements for a fundamental rheological test since the sample geometry is variable and not well defined, the stress and strain states are uncontrolled, complex and non-uniform. Therefore, it is impossible to define any rheological parameters such as stress, strain, strain rate, modulus or viscosity. Thus, these empirical tests are purely descriptive and hardly comparable among themselves as well as to other test methods (Dobraszczyk and Morgenstern, 2003). Closing this gap of imprecision, fundamental rheological analyses using controlled stress and strain rheometers give the possibility to obtain results under defined conditions. Usually a controlled and well-defined stress or strain is applied to a material over a given time to measure the physical response. This gives an indication of

descriptive material parameters such as elastic, complex and viscous modulus as well as phase angle and viscosity. All these parameters describe the ability of the material to store and dissipate energy as well as its hardness, strength or resistance to deformation (Dobraszczyk and Morgenstern, 2003) .

Wehrle et al. (1997) reported that the phase angle (a measure of solid to liquid behaviour) was significantly lower for a dough with 2% salt than for one with 0% salt during a frequency sweep between 0.01 Hz to 10 Hz. This indicates that the dough was more elastic with increased addition of salt. These findings correlate well with results obtained for the elastic modulus (G') indicating that salt addition results in a more elastic dough (Lynch et al., 2009, Salvador et al., 2006, Angioloni and Rosa, 2005). In contrast to this, Larsson (2002) observed a significant decrease for the elastic modulus G', which might be ascribed to the use of different flour varieties. However, the same trend for the viscous modulus G'' was reported by all authors. G'' was found to decrease with a reduction in the amount of salt added (Lynch et al., 2009, Larsson, 2002, Wehrle et al., 1997).

2.4.4 Interpretation of Rheological Findings on Dough Properties

In summary, both empirical and fundamental rheological experiments showed that salt reduction results in dough with reduced elasticity, which is less machinable than dough with the standard amount of salt (≥ 1.2%). Furthermore, salt reduced dough is firmer at the beginning of the mixing process but less stable, resulting in weak doughs which have a high risk of becoming over-mixed. Wehrle et al. (1997) reported that the most profound change of rheological characteristics with regard to an industrial process is the reduction in dough consistency in the absence of salt. This change has been found to have a deleterious effect on the dough handling/machinability due to an increase in the stickiness of dough. Dobraszczyk (1997b) reported that the stickiness of wheat dough can be discussed as a function of rheological and surface properties. The lack of a universally accepted standard for stickiness measurements in food industry makes it impossible to date to quantify the impact of salt on dough stickiness.

One reason for these rheological changes is the ionic nature of sodium chloride resulting in extensive interaction with the water as well as macromolecules. These interactions are responsible for a reduced availability of water in the dough and for changes of moisture content and water activity in bread. Hence, salt influences the development of a gluten

network through the restriction of the availability of water (Cauvain and Young, 2001). As a result, reduction of salt leads to a decreased dough development time. The delayed addition of salt is one way, bakers use, to avoid long dough development times. Initially, the gluten network is developed, having the pure water available. At the end of the mixing process, the salt is quickly incorporated, which happens easily due to its readily soluble nature. This procedure is essential processing flours with poor gluten-forming potential (Cauvain, 2007).

A further impact of salt is the competition for hydrogen bonding sites in the gluten by the sodium ions (Hutton, 2002, Matz, 1992). This competition leads to the formation of an electrostatic shielding of ionic amino acids residues on the gluten surface which reduces the electrostatic repulsion between individual gluten proteins. Furthermore, sodium chloride increases the electrostatic free entropy associated with the exposure of apolar residues. Thus, stronger inter-protein hydrophobic interactions are induced, resulting in an increased aggregation (Preston, 1981, Bernardin, 1978). This is also a further explanation for the increasing dough development time, caused by addition of salt. Longer time is needed to reduce these aggregates and to obtain the optimum in dough development. These explanations are underlined by the fact that gluten proteins, in particular glutenin, from strong flours interact strongly through hydrophobic interactions (Huebner and Wall, 1980, Kobrehel, 1980, Caldwell, 1979, Chung and Pomeranz, 1979).

2.4.5 Fermentation

The fermentation process is subdivided into two sections: a phase before moulding, called “intermediate proofing” and one after moulding, called “final proofing”. During the fermentation process the dough development is continued by stretching the gas cell membranes continuously in a biaxial way. This causes a hardening of the gluten network which dramatically affects the protein structure. Proofing is also performed to increase the specific volume of the dough piece and to obtain the required bread properties during the baking process. Furthermore, the whole fermentation process is important for flavour development (Sluimer, 2005).

The initial growth of gas nuclei developed during the mixing process involves the following processes (Sluimer, 2005):

- Disappearance of oxygen leading to an anaerobic dough
- Diffusion of CO_2 from yeast cells to gas nuclei
- Change of CO_2 from a dissolved to a gaseous state
- Excess gas pressure in the gas cells necessary to get dough expansion

Lynch et al. (2009) using the Rheofermentometer, observed a significant increase in the maximum dough height as the salt level decreases. This suggests that the reduction of salt resulted in an increase of the total volume of released gas. In combination with this increase in gas production, the gluten network is weaker due to the reduced level of salt. Therefore, the dough piece cannot hold all the gas produced and thus, as demonstrated by Lynch et al. (2009), a higher amount of CO_2 is lost from the dough piece. This result is supported by the retention coefficient indicating the capability of dough to retain gas as a percentage of the total amount of gas produced. This was found to decrease as the salt level was reduced.

The inhibiting effect of salt on yeast and the resulting limited total gas production is used in the baking process to control the yeast. Due to this inhibitory effect, the proofing time has to be adapted, whenever the salt level is changed (Hutton, 2002). The reason for the inhibiting effect of salt on yeast in dough systems is not fully understood. Matz (1992) reported that doughs with an inadequate amount of salt led to excessive fermentation. This causes gassy and more acidic doughs resulting in loaves with poor texture and an open grain. Kawai et al. (1999) showed that small amounts of salt up to 2%, in dough as well as in liquid culture medium, stimulate the fermentation activity of yeast. Amounts of salt higher than 2% resulted again in a decrease of leavening ability. Unfortunately, this was only shown for a liquid medium system and not for dough.

It has to be considered that dough consists of approximately 40% of water, whereas the vast majority is bound by macromolecules. Hence, the salt concentration in the free water is more than double than its percentage related to the whole amount of dough. Therefore, yeast is exposed to significantly higher salt concentrations in dough, containing standard amounts of salt, than the 3% used in liquid culture medium by Kawai et al. (1999). Yeast is also influenced by many other ingredients changing conditions such as osmotic pressure, availability of free sugars and enzyme inhibition potential (Oda and Tonomura, 1993, Watson, 1970). Due to these various environments, yeast behaviour cannot be compared easily and further investigations seem to be necessary.

2.4.6 Baking Process

The final stage of the industrial or artisanal process in which bread takes its recognised form is baking. Baking is a process which involves heating up the fermented dough at temperatures between 200° and 250° C for a time of 20 to 45 min depending on the bread type. These relatively high temperatures result in a profound change in taste, flavour and texture of breads (van Boekel, 2006). During baking a number of physical changes occur in bread including the rapid expansion of CO_2 and water vapour as well as starch gelatinisation and protein denaturation (Sluimer, 2005). These phenomena play a key role in the formation of the two sections of bread, the crust and crumb. Crumb formation involves the change of the gas fraction in dough, which is a disperse phase like foam, into a sponge structure where the gas cells are interconnected. At the beginning of baking the temperature rises up to about 100 °C and maintains there as long as the outer layer contains moisture. The moisture evaporates or diffuses to the interior (Sluimer, 2005). As soon as the temperature rises above 110 °C the formation of Maillard Reaction products starts (Mondal and Datta, 2008). The structure of the outer dough layer is essential for an optimal browning (Sluimer, 2005). Hence, the reduction of salt influences crust texture due to its impact on the formation of the gluten network and crust flavour and crust colour due to the impact of salt on yeast activity. This is due to the fact that increased yeast activity leads to a reduction in the amount of free reducing sugars remaining for Maillard Reaction.

With respect to the crumb, Lynch et al. (2009) could show that bread without salt results in a smaller amount of larger gas cells when compared to salt containing bread. The effects of salt on the gluten proteins described above are lowered in salt reduced bread. This leads to the assumption that salt reduction contributes to a decrease of strain hardening of the gluten proteins and hence, to a weaker gluten network. In addition, the increased amount of leavening gas contributes to an uneven crumb.

2.5 Influence of salt on final bread quality

Breads with reduced levels of salt (0.3 and 0.6%) were found to have no significant technological differences to breads containing 1.2% salt with respect to specific volume, moisture content and bake-loss (Lynch et al., 2009). The omission of salt did, however, result in significant changes regarding crumb structure and level of staling after 5 days of

storage (Lynch et al., 2009). Regarding flavour, crust formation and shelf-life of low-salt breads, significant changes were determined (Lynch et al., 2009, Kilcast and Angus, 2007, Sluimer, 2005).

2.5.1 Bake-loss and Bread Volume

The specific volume, given by the ratio of bread volume to bread weight, is a common measurement to assess bread quality. The final weight of bread depends on the original amount of dough and the loss of weight during the baking process. Theoretically, reduction of salt leads to excessive gas production and therefore, to a greater volume. However, Lynch et al. (2009) and Okano and Mizutani (1995) reported no significant difference in the specific volume of bread at reduced salt levels. A trend towards a slight increase of specific bread volume was reported (Lynch et al., 2009, Okano and Mizutani, 1995). In contrast to these findings, Czuchajowska et al. (1989) observed a slight decrease of the specific bread volume in the absence of salt. This might be due to differences in the recipe used by Czuchajowska et al. (1989) including more ingredients than the ones used by Lynch et al. (2009) and Okano and Mizutani (1995).

2.5.2 Microbial Shelf-life

Doerry (1990) demonstrated that microbes are the main spoilage agents in intermediate and high moisture food products. Bread is known as a high moisture product with a_w values between 0.96 - 0.98 (Smith et al., 2004). In bread, salt acts as a preservative agent, due to its ability to reduce the water activity. The increased osmotic pressure causes cells to loose water to the environment, thus inhibiting cell metabolism and inhibiting microbial growth (Yigit and Korukluoglu, 2007). Furthermore, sodium ions and chloride ions can promote specific changes in metabolisms inside the cells (Hutton, 2002). On the contrary, Samapundo et al. (2010) reported no significant differences in the growth of *Penicillium roqueforti* on standard white bread, bread containing 30% less salt and bread with salt replacers.

2.5.3 Sensory and Changes during Storage

Three of the four main sensory characteristics of bread, namely texture, flavour and colour, are influenced by salt addition. To date, odour as the fourth sensory characteristic is not

known to be influenced significantly by salt (Lynch et al., 2009). However, due to its reported influence on yeast (Watson, 1970), some effects on bread odour can be assumed. During the storage of bread, staling changes the quality characteristics. The two factors mainly affected are texture and flavour. These changes are mainly based on water activity and changes of water distribution inside the bread (Gray and Bemiller, 2003).

2.5.4 Texture

Texture is a critical factor for respect to the consumer acceptance of bread. This important parameter can be evaluated in a number of ways. Typically, texture profile analyses as well as sensory panels are utilised. As mentioned earlier, salt has an important impact on the development of the gluten network during the dough mixing process. Accordingly, salt is necessary for an even crumb structure, which is expected by the consumers (Matz, 1992). The crumb grain of bread without salt is described as uniform but 'heavily walled' (Czuchajowska et al., 1989). Lynch et al. (2009) showed with the help of image analysis that bread without salt has a significantly increased cell to total area ratio and thus a smaller number of larger air cells when compared to bread containing salt.

One of the main problems associated with the storage of bread is the process of starch retrogradation, commonly called staling. This complex process involves a change of texture and is associated with the change of the moisture content (Pateras, 2007, Gray and Bemiller, 2003). Lynch et al. (2009) demonstrated, using a trained sensory panel, that bread without any salt showed the greatest textural differences between 18 h and 72 h post-baking. Breads, 18 h post-baking, were described as "soft" and as having high "crust resistance" both by hand and mouth assessment. Breads, tested 72 h post-baking, were described as having "dry" texture by hand and mouth assessment and "adhesive" in mouth. After storage for 18 h, the texture of bread, containing the common salt amount of 1.2%, was still acceptable, whereas breads containing reduced amounts of salt were unacceptable in texture. This trend could not be shown for the breads stored for 72 h. No significant change could be determined for the hardness of breads with different levels of salt, using the texture profile analyser, after 2 h and 50 h of storage. Only bread with 0% salt was significant harder measured after 120 h of storage (Lynch et al., 2009).

2.5.5 Colour

Maillard reaction products are known to be formed during heating processes in food. The reaction involves proteins and reducing carbohydrates and contributes to colour and flavour of processed food. During crust formation, melanoidins are formed by polymerisation during the final stage of the Maillard Reaction (Belitz et al., 2008). Other reactions such as caramelisation and carbonisation do not significantly contribute to the colouring of the crust (Sluimer, 2005). As reported by Czuchajowska et al. (1989), bread baked without salt has a lighter coloured crust. The reason for a decreased Maillard Reaction is the reduced amount of free sugars due to the fact that yeast is more active in the absence of salt and therefore, metabolises much more sugar, which is missing for the purpose of crust colouring during baking (Skobranek, 1998).

2.5.6 Flavour

The influence of salt on the characteristic flavour of certain food products is widely acknowledged. Miller and Hoseney (2008) reported that bread baked without any salt has an insipid taste. However, salt addition also has an effect on the flavour of other ingredients. Ugawa et al. (1992) could show that small amounts of 0.17% salt addition enhanced the sweetness of the amino acids glycine, alanine and serine significantly. Furthermore, it has been shown that sodium-containing compounds reduce the sensation of bitterness (Breslin and Beauchamp, 1995). These flavour-enhancing effects seem to be related to the effect of salt on water activity. The water restriction by salt results in the concentration of flavour molecules in solution according to the ratio of free available and bound water and affects their volatility (Costa-Corredor et al., 2009). In addition, the increase of ionic strength, caused by the presence of salt, influences the binding of compounds within the food system and therefore, the flavour sensation (Hutton, 2002).

The extent to which reduction of salt impacts the flavour of bread depends on the various ingredients which are used for bread production. Girgis et al. (2003) could show that the standard salt content of bread of 1.2% can be reduced by 25% without detection. Wyatt (1983) reported that the salt content of white or whole wheat bread could be even reduced by 50% without any change in flavour and overall acceptability. On the contrary, Lynch et al. (2009) showed that a reduction of salt by 50% significantly influenced the flavour. However, various ingredients might be responsible for the different results. In the absence

of salt, bread was described as “yeasty”, “sour” or “acidic” and having “sour dough” type characteristics (Lynch et al., 2009). These results confirm earlier reports by Breslin and Beauchamp (1995), that salt addition has an effect on bitterness. The mentioned influence of salt reduction on crust development has an important impact on the flavour of bread. The melanoidins formed by the Maillard Reaction contribute essentially to the flavour composition of bread (Belitz et al., 2008). The light crust of salt reduced bread is caused by a lack of melanoidins. Hence, the contribution of crust to the whole flavour composition of bread is reduced and the bread tastes insipid and stale.

An evaluation of Irish consumer preferences showed a significant preference for the bread with the highest available salt level, which was 1.2%. Breads with levels of 0.4% of salt still had acceptable sensory properties (Gormley and Morrissey, 1993). A sensory evaluation of bread regarding the influence of salt on changes of flavour during the staling process showed no significant differences between breads with salt levels of 0.3, 0.6 and 1.2% (Lynch et al., 2009). The omission of salt resulted in more acidic taste and yeasty flavour after 72 h than compared to 18 h post-baking.

2.6 Possible alternatives for salt

This review has shown the importance of salt in bread and that its reduction requires a modification in the ingredients or technologies utilised during production. These changes in bread and bread dough can be classified into three distinct classes, namely technological, organoleptic and shelf-life.

Regarding the shelf-life of bread, a considerable reduction of salt may lead to a decrease in the mould free shelf life of bread. This reduction in salt can be compensated by the use of a number of alternative methods including modified atmosphere packaging, pasteurisation of packaged bread, irradiation and the addition of chemical additives like propionic acid and its salts (Legan and Voysey, 1991). Propionic acid has previously been shown to inhibit moulds and *Bacillus* spores but not yeast, which is essential for the bread baking process (Pateras, 2007, Legan, 1993). Therefore, it is used generally as a chemical preservative in bread.

The replacement of salt with potassium chloride does not result in any significant production disadvantages but has an adverse impact on the flavour of bread once the

substitution level rises above 10% of sodium chloride. Kilcast and Angus (2007) reported that the lower the level of sodium chloride the more noticeable the distinctive 'metallic' taste of potassium chloride became. Further restrictions are required due to the fact, that extensive intakes of potassium are known to influence potassium homeostasis seriously causing hyperpotassemia (Youn and McDonough, 2009). Considering that sodium reduction in bread is due to its impact on the vascular system, many of the target patients, e.g. suffering from diabetes mellitus, have already an increased risk to develop hyperpotassemia (Uribarri et al., 1990). Hence, the replacement of salt by potassium chloride has to be re-evaluated. Charlton et al. (2007) successfully replaced 32 % of sodium in bread using a mixture of magnesium chloride, potassium chloride and magnesium sulphate. These breads were found to be comparable to regular bread with respect to flavour and texture. Unfortunately, nothing was reported about the effect on the shelf-life and potential influences on potassium homeostasis. Recent trends in the food industry have included the desire to minimise the amount of chemical preservatives for high-quality food. Therefore, natural preservation systems are the main focus.

Sourdough, one of the oldest biotechnological processes in food production, is the natural preservation system for bread (Roecken and Voysey, 1995). As reviewed by Arendt et al. (2007) the ability of sourdough to improve bread quality and increase its shelf-life has been described extensively. Recently, Dal Bello et al. (2007) could show that the addition of sourdough fermented by the antifungal strain *Lactobacillus plantarum* FST 1.7 inhibited the outgrowth of *Fusarium spp.* in wheat bread. Hence, sourdoughs are able to increase the shelf-life of bread by inhibiting the growth of various microorganisms. Responsible for this phenomenon is the production of compounds like organic acids and dipeptides during the fermentation process (Ryan et al., 2009a, Ryan et al., 2009b). Ryan et al. (2008) has also shown that sourdough can be used to significantly reduce the level of chemical additives in bread. Furthermore, the use of sourdough has technological advantages, due to its impact on dough structure and the whole range of bread quality characteristics based on acidification (Wehrle et al., 1997, Collar et al., 1994, Kawamura and Yonezawa, 1982), optimal activity of various enzymes (Thiele et al., 2002, Schieberle, 1996), synergies with dough additives and application of exopolysaccharides produced by lactic acid bacteria (De Vuyst and Degeest, 1999, Laws and Marshall, 2001, Monchois et al., 1999).

A new approach was presented by Noort et al. (2010) who could demonstrate an enhanced perception of saltiness by inhomogeneous distribution of sodium chloride in bread. Encapsulated salt as well as combinations of different salt concentrations in different parts of the bread dough resulted in an enhanced saltiness perception of the sensory panel (Noort et al., 2012, Noort et al., 2010). The use of coarse-grained sodium chloride showed the same result (Konitzer et al., 2013), caused by the increased contrast of saltiness which is known to trigger salt perception (Busch et al., 2009). Pflaum et al. (2013) reported the direct impact of the crumb texture on the saltiness perception. The bread crumb was impacted by different conditions during fermentation and the various crumb cell structures resulted in enhanced saltiness perception the more coarse-pored the bread crumb was. Considering that the use of sourdough can also change the crumb texture, it shows another beneficial influence of sourdough addition on low-salt bread.

2.7 Conclusion

The target of a reduced daily salt intake of 6 g is recommended due to the detrimental impact on human health causing hypertension, which is linked with cardiovascular diseases. Since bread has the highest individual contribution to the daily salt intake, any reduction in the salt levels present in bread would equate to a significantly decreased sodium intake in society. Reduction of salt in bread involves several changes of quality characteristics, namely flavour, shelf-life and texture. Furthermore, the bread manufacturing process is affected by changes such as dough stickiness which can be compensated mostly by changing technological settings and composition.

Doughs with reduced salt levels have been shown to possess different rheological behaviour in comparison to regular dough. The reduced dough consistency as well as the decrease of the viscous and elastic moduli and the potential changes of dough stickiness must be considered during industrial bread manufacturing. Also the shortened dough development time in systems with reduced levels of salt require adjustments of mixing time in order to avoid overmixing. The earlier start of starch gelatinisation does not significantly influence the final bread quality characteristics. Technologically, the production of bread reduced in salt is feasible, but the sensory characteristics of bread have to be adjusted to meet the consumer’s expectations. The use of sourdough is promising since it has been shown to improve bread quality. In particular, its effect on flavour and the influence on

further sensory characteristics, like crumb texture, can be used to improve the quality of salt reduced bread (Arendt et al., 2007). All facts considered the effort which has to be put in to achieve a trouble-free production of high-quality low-salt bread is justifiable. Changes in both ingredients composition and processing, range in a dimension which can be managed regarding cost-effectiveness.

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Chapter 3

The effect of sourdough and calcium propionate on the microbial shelf-life of salt reduced bread

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3.1 Abstract

While cardiovascular health issues are related to increased intakes of sodium chloride (NaCl), the consumption of low-salt bread represents a potential way to improve public health. The reduction of NaCl influences the bread quality characteristics, in particular the shelf life. Calcium propionate (CP) is commonly used in bread as an antifungal agent. Alternatively, sourdough can be used as a natural preservative. This work addresses the feasibility of NaCl reduction in wheat bread focussing on shelf life and the compensation using sourdough as well as chemical preservatives. The impact of NaCl reduction and the addition of preservative agents in conjunction with different NaCl concentrations on the shelf life of bread were tested under "environmental" conditions in a bakery as well as using challenge tests against selected fungi. The challenge tests were performed using fungi commonly found in the bakery environment such as *Penicillium expansum, Fusarium culmorum* and *Aspergillus niger*. NaCl reduction decreased the shelf life by 1-2 days. The fungal challenge tests revealed differences in the determined shelf life between the different fungi based on their resistance. The addition of sourdough with antifungal activity prolonged the shelf life to 12-14 days whereas the addition of 0.3% calcium propionate prolonged the shelf life to 10-12 days only. Similar antifungal performance was observed in sourdough breads and calcium propionate breads when tested against the different indicator moulds. The findings of this study indicate that addition of sourdough fermented using a specifically selected antifungal *Lactobacillus amylovorus* DSM 19280 can replace the chemical preservative calcium propionate addition and compensate for the reduced level and therefore, guarantee the product safety of low-salt bread.

3.2 Introduction

Mean daily salt intakes of the population in developed countries are well in excess of their dietary needs (ca. 3-4 g salt/day) (European Food Saftey Authority 2005). One of the effects of the high salt intake is hypertension, which is seen as a causal factor for cardiovascular diseases (Elliott et al. 1996). Up to 35% of the daily NaCl intake is attributed to cereal products, where bread plays the most important role (Angus 2007). Hence, low-salt bread is one of the most efficient ways to decrease the daily NaCl intake. The technological process of bread baking as well as some of the final quality characteristics of bread, in particular shelf life is influenced by NaCl level. Bread is known as a high moisture product with a_w values between 0.96 - 0.98 (Smith et al. 2004). As demonstrated by Doerry (1990), microbial spoilage is the main cause for shelf life issues in intermediate and high moisture food products. Nowadays, mould growth is still a cause of high losses to the bread producing industry (Legan 1993; Corsetti et al. 1998; Smith et al. 2004; Pateras 2007). Many references show that fungal spoilage is not a current problem in baking industry but causing trouble and losses since decades (Jarvis 1972; Knight & Menlove 1961; Legan 1993; Filtenborg et al. 1996; Pateras 2007; Samapundo et al. 2010). Knight & Menlove (1961) could show that mould spores are killed during the baking process and the issue limiting the long-term shelf life of bread is a post-baking fungal contamination. Sodium chloride (NaCl), commonly called salt, acts as a preservative agent in bread, due to its ability to reduce the water activity (a_w). Increased osmotic pressure causes cells to loose water to the environment, thus, inhibiting cell growth. Up to date research on NaCl reduction was mostly focused on changes in flavour and the impact on dough properties influencing the production process. Hence, little is reported about influences on microbial shelf life of bread due to reduction of NaCl. The present work aims to determine the influence of NaCl reduction on the microbial shelf life of wheat bread as well as to present some possible feasible solutions.

3.3 Materials and Methods

3.3.1 Bacteria and growth conditions

The antifungal strain *Lactobacillus amylovorus*, deposited as a strain at the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von

Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany) with the number DSM 19280, was previously isolated from gluten-free sourdough Arendt et al. (2009). The bacteria were grown on MRS5 (Meroth et al. 2003) agar plates for 48 h at 30 °C and a single colony was transferred into MRS5 broth for about 16 h at 30 °C overnight.

3.3.2 Fungal cultures and preparation of the spore solution

The moulds *Fusarium culmorum* TMW 4.0754 (isolated from brewing barley and kindly provided by Prof. Rudi F. Vogel, TUM, Germany), *Aspergillus niger* FST 4.21 and *Penicillium expansum* LTH S46 (both isolated from cereal environment) were chosen as representative bread spoilage fungi (Moreau 1980). Moulds were grown on malt extract agar until sporulation occurred. Spores were transferred from this stock solution into a synthetic nutrient-poor medium (Nirenberg 1976). Vigorous stirring (200 rpm) for 8 days at room temperature provided a fungal cell and conidial suspension with a concentration of 5×10^7 CFU/ml. Mould spores were counted using a Thoma chamber haemacytometer.

3.3.3 Sourdough fermentation

For sourdough fermentation the overnight culture of *L. amylovorus* DSM 19280 was subcultured in 40 ml of MRS5 broth and incubated for 24 h at 30 °C resulting in a cell suspension containing approximately 5×10^9 CFU/ml. Cells were harvested by centrifugation at 3000 g for 10 min, washed twice with ringer solution and resuspended in 40 ml ringer solution. Sourdough was prepared with a dough yield of 200 and an inoculation of about 10^6 CFU/g of sourdough using 600 g of wheat flour, 600 ml of sterile distilled water and 500 µl of cellular suspension. After mixing with a Kenwood mixer (Kenwood KM020) using the batter attachment for 1 min at speed 1, the dough was covered and fermented at 30 °C for 48 h. At the end of fermentation, lactic acid bacteria cell counts were determined on MRS5 agar plates and the total titratable acid (TTA) and pH values were determined as described in following. For all sourdough samples TTA ≥ 14.0 ml, pH ≤ 3.90 and cell counts of about 2×10^8 CFU/ml were detected and used as quality parameter of the ready fermented sourdough.

3.3.4 Total titratable acids (TTA) and pH

For sourdough samples and bread samples both TTA and pH were determined according to Arbeitsgemeinschaft Getreideforschung e.V. (AGF) (1994). The frozen bread crumb samples were defrosted overnight at 4 °C and homogenised together with the respective amount of water, using a Kenwood KM020 with the blender devise for 2 min at speed 2.

3.3.5 Baking procedure

Wheat bread was prepared by mixing Baker's flour (Odlums, Ireland), distilled water (water level set to 64.7% (flour weight) using a Brabender farinograph), dry yeast (Puratos Group, Belgium) and 1.04, 0.52, 0.26 and 0.0% (w/w) of NaCl with a spiral mixer (Kenwood KM020). Considering an average bakeloss of 13.5% the NaCl concentrations in the final bread loaves resulted in 1.2, 0.6 and 0.3%. After a bulk fermentation of 15 min at 30 °C and 85% relative humidity (Koma Popular, Koma, Roermond, The Netherlands) 450 ± 1 g bread loaves were moulded with a moulding machine (Machinefabriek Holtkamp B.V., Almelo, Holland). The ready moulded loaves were placed in non-stick pans (180 mm x 120 mm x 60 mm) and proofed for 75 min under the same conditions used during bulk fermentation. Subsequently, the breads were baked for 35 min at 230 °C top and bottom heat. Ovens were pre-steamed (0.3 L) and steamed when loaded (0.7 L). Loaves were depanned and subjected to a 120 min cooling period on cooling racks at room temperature. The same breads were baked with addition of calcium propionate at 0.3% dough weight (DW) or with addition of sourdough at 23% DW. The recipes for the control breads (A), the sourdough breads (B) and the calcium propionate breads (C) are given in Table 3.

Table 3: Preparation of bread doughs

	Control bread			Sourdough breads			CP breads		
	g	% DW*	% FW**	g	% DW	% FW*	g	% DW	% FW*
Baker's flour	891.1	59.41	100	712.9	47.53	80	888.4	59.23	100
Water	572.7	38.18	64.3	394.5	26.3	44.3	571	38.07	64.3
Sourdough				356.4	23.76	40			
Baker's yeast	18.2	1.2	2.0	18.2	1.2	2.0	18.1	1.2	2.0
NaCl	0.0 - 18.0	0.0 - 1.2	0.0 - 2.0	0.0 - 18.0	0.0 - 1.2	0.0 - 2.0	0.0 - 18.0	0.0 - 1.2	0.0 - 2.0
Calcium propionate							4.5	0.3	0.5
Total amount	1500	100	168.3	1500	100	168.3	1500	100	168.8

* Dough weight
** Flour weight

3.3.6 Water activity

Water activity was measured using a water activity meter AquaLab Series 4TE from Decagon Devices, Inc. The a_w-meter was calibrated using the verification standards (distilled water; 0.5 M KCl; 6.0 M NaCl; 8.57 M LiCl; 13.41 M LiCl) from Decagon (NE Hopkins Ct. Pullman, WA – USA) at 25 °C. For each of the five water activity levels the values were determined within the required range of 0.003 (Decagon Devices 2009). The bread samples were measured in triplicate on 3 independent baking batches 2 h after baking at 25 °C.

3.3.7 Antifungal activity in bread

For the bakery-environmental contamination trials, breads were sliced and 10 slices of each type of bread were exposed to the bakery-environment for 10 min. For the fungi challenge tests, breads were sliced in sterile conditions and sprayed on each side with about 100 spores (0.1 ml of fungal suspension containing 1000 spores/ml). Each of the bread slices were packaged in polyethylene bags and heat sealed. Sterile air exchange was enabled by inserting two filter tips in each storage bag. During storage the temperature was kept constant at 20 °C. Storage took place up to 14 days observing the appearance of mould spoilage every day. As soon as spoilage appeared on one of the bread slices the previous day was determined as the microbial shelf life of that bread.

3.3.8 Statistics

Statistical analyses were performed using SigmaPlot 11.0 for Windows computerized statistical analysis package (Systat Software, Inc., Chicago, IL). Data were examined using one-way analysis of variance (ANOVA). Where an F-test showed significant differences ($p<0.05$), Fisher's least significant difference (LSD) test was used for multiple comparisons. Each result is the average of 3 separate experiments with 3 independent samples from each batch.

3.4 Results

In this study, the effect of NaCl reduction on the shelf life of bread was determined using different NaCl concentrations as regulated by the European Union (2006) of 0.6% NaCl

(reduced sodium, 0.2% sodium) and 0.3% NaCl (low sodium, 0.1% sodium). Furthermore, breads of 1.2% NaCl (Irish standard level) (Abdel-Aal & Wood 2005; Gormley & Morrissey 1993; Lynch et al. 2009) and bread with 0% of NaCl were produced and used as control breads. Two different preservative strategies were used to prolong the shelf life of bread: a chemical preservation using calcium propionate and natural preservation using sourdough fermented with a specifically selected antifungal *L. amylovorus* strain DSM 19280 (Arendt et al. 2009). As described by Arendt et al. (2009) several bioactive compounds, produced by the patented antifungal strain *L. amylovorus* DSM 19280, interfere with the metabolism of fungi, inhibiting the spoilage of the bread.

3.4.1 Water activity

NaCl has been well known for its hygroscopic nature and therefore, the level of NaCl added to bread will impact on the water activity which has previously been shown to affect microbial growth. The impact of NaCl reduction on the a_w-value of breads containing NaCl concentrations of 1.2%, 0.6%, 0.3% and 0.0% was measured and the results are depicted in Figure 1. For control breads containing 1.2% NaCl, an aw-value of 0.974 was determined according to the aw-values of 0.96 to 0.98 which are published in literature (Chirife & Favetto 1992; Seiler 1988). For breads without NaCl the a_w increased to 0.989.The water activity of the breads containing either sourdough or calcium propionate in combination with different concentrations of NaCl was also measured. In both cases, a high linear correlation between NaCl concentrations and a_w was determined ($R^2 > 0.996$). The breads containing sourdough in combination with the different NaCl concentrations showed slightly lower a_w than the breads containing only NaCl. For the NaCl concentration of 1.2% the a_w was even significantly different to the respective control bread. The addition of calcium propionate led to a significant reduction in a_w compared to the control breads.

3.4.2 TTA and pH of bread crumbs

The various bread types: control bread (A), sourdough bread (B), and 0.3% calcium propionate breads (C), each one containing different NaCl concentrations, resulted in significant differences in pH and TTA values (Table 4). The analyses of pH and TTA of the control breads resulted in values of pH = 6.0 ± 0.02 and TTA = 2.7 ± 0.0 ml which are

comparable to the results determined by Katina et al. (2009) for standard wheat bread containing 1.0% of NaCl.

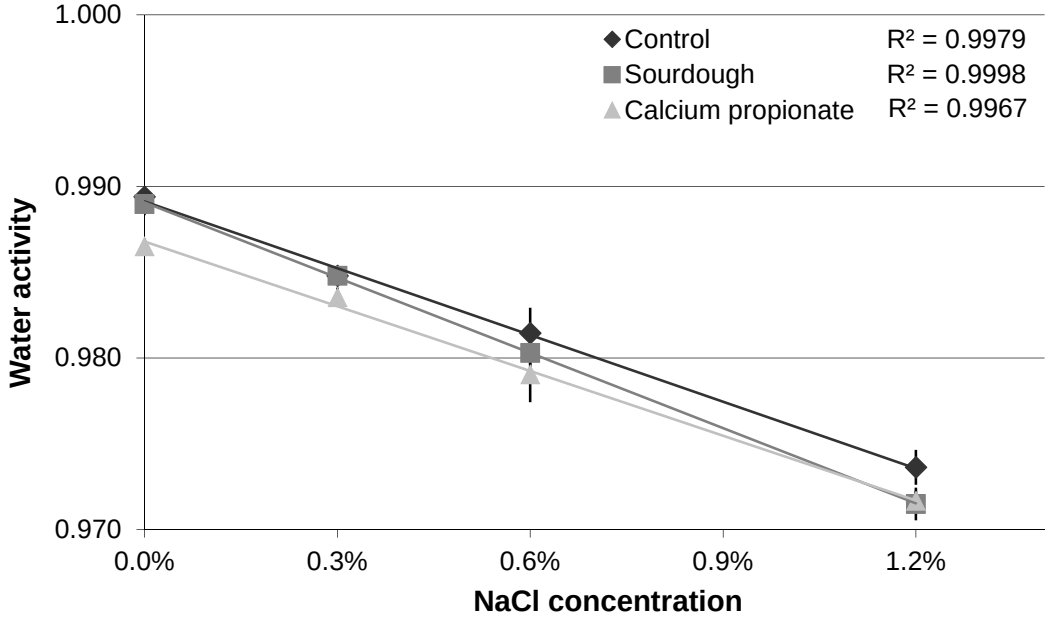


Figure 1: Water activity of control breads (♦), sourdough breads (■) and calcium propionate breads (▲) containing NaCl concentrations of 1.2%, 0.6%, 0.3% and 0.0%.

Table 4: The pH and TTA of bread crumb samples. Values followed by a different letter are significantly different ($p < 0.05$).

(A) Control breads	**pH**	**TTA**
1.2 % NaCl	$6.2 \pm 0.3^{a,d}$	2.7 ± 0.2^{a}
0.6 % NaCl	$6.0 \pm 0.1^{a,b,d}$	2.8 ± 0.2^{a}
0.3 % NaCl	$5.9 \pm 0.0^{b,d}$	2.7 ± 0.1^{a}
0.0 % NaCl	$5.9 \pm 0.0^{b,d}$	2.6 ± 0.2^{a}
(B) Sourdough breads		
1.2 % NaCl	5.0 ± 0.2^{c}	5.0 ± 0.5^{b}
0.6 % NaCl	5.0 ± 0.2^{c}	5.0 ± 0.7^{b}
0.3 % NaCl	4.9 ± 0.2^{c}	4.9 ± 0.8^{b}
0.0 % NaCl	4.9 ± 0.2^{c}	4.8 ± 0.7^{b}
(C) CP breads		
1.2 % NaCl	5.8 ± 0.0^{d}	3.0 ± 0.3^{a}
0.6 % NaCl	5.8 ± 0.0^{d}	2.8 ± 0.0^{a}
0.3 % NaCl	5.8 ± 0.0^{d}	2.8 ± 0.0^{a}
0.0 % NaCl	5.8 ± 0.0^{d}	2.7 ± 0.1^{a}

The values of pH and TTA were independent of the different NaCl concentrations used. Moreover, similar results were detected in the respective doughs (data not shown). The breads containing sourdough had a pH = 4.9 ± 0.02 and a TTA = 4.9 ± 0.01ml. The pH was significantly lowered by about 1.0 unit compared to the control breads. As expected, the breads containing 0.3% calcium propionate showed no significant differences in pH and TTA compared to the respective control breads.

3.4.3 Shelf life evaluation under bakery environmental conditions

The impacts of different NaCl concentrations as well as the addition of sourdough and calcium propionate respectively on the shelf life of bread were tested under the environmental condition of a pilot scale bakery. The results shown in Figure 2 reveal that control bread containing the standard concentration of 1.2% NaCl had a shelf life of 5 to 6 days. A reduction of NaCl to 0.6%, 0.3% and 0.0% led to a significant reduction in shelf life of approximately 2 days. No significant differences in shelf life could be observed between the different reduced concentrations of NaCl. The increase in shelf life for the bread without any NaCl compared to 0.3 and 0.6% NaCl can be explained with a minimum amount of NaCl which favours the growth metabolism of fungi.

Sourdough fermented by the antifungal strain *L. amylovorus* DSM 19280 was added at a level of 23.8% to the breads containing 0.0% to 1.2% of NaCl. The addition of sourdough resulted in a significantly prolonged shelf life up to 12 to 14 days compared to the control breads. The NaCl concentration did not influence the determined shelf life significantly. Currently, calcium propionate can be added to bread at a maximum concentration of 0.3% (European Union 1995). Based on results published by Guynot et al. (2005) the maximum concentration was chosen for the present work considering the determined a_w-values (Figure 1). Guynot et al. (2005) who could show that at 0.85 a_w, 0.03% of calcium propionate delayed the growth of *Aspergillus spp*. and *Penicillium spp*. significantly but a retarding effect at 0.90 a_w was only observed for 0.3% calcium propionate. The impact of the addition of this preservative agent in combination with different NaCl concentrations on shelf life is also shown in Figure 2. Neither for the control (calcium propionate only) nor for the combination with different NaCl concentration differences in mould inhibition was detected.

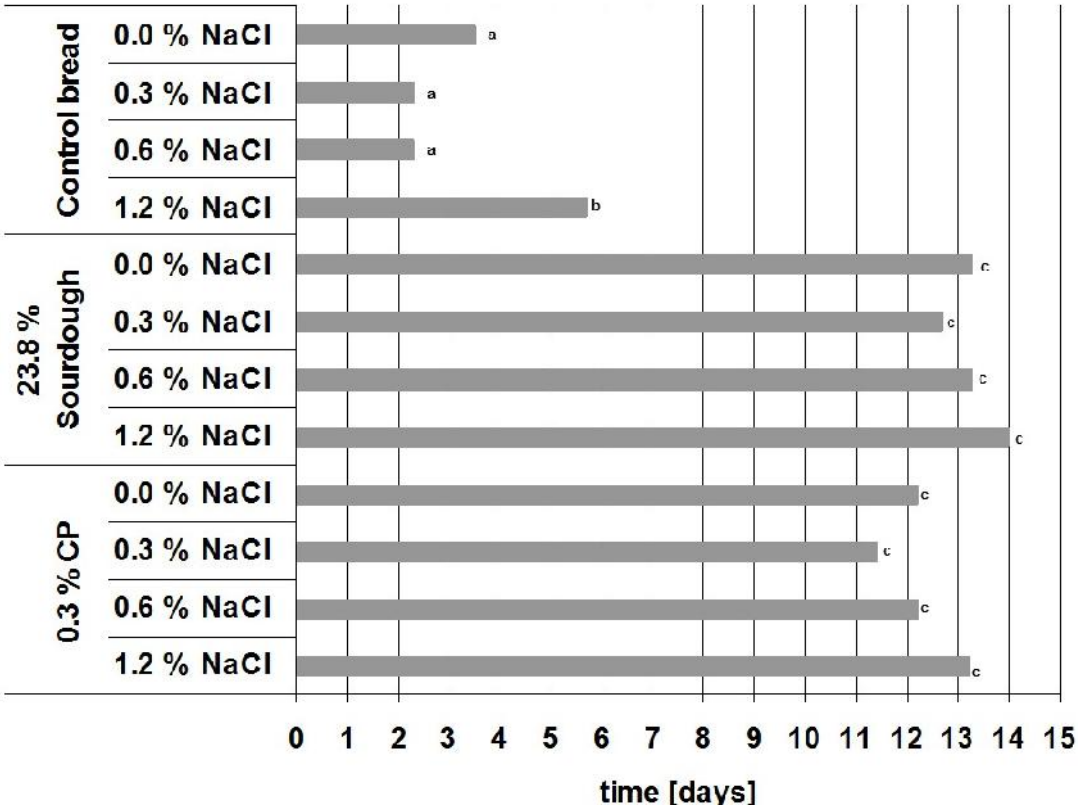


Figure 2: Shelf life of slices of control breads, sourdough breads and calcium propionate breads containing the different NaCl concentrations 0.0%, 0.3%, 0.6% and 1.2% contaminated in a pilot scale bakery environment. Mean values of at least three replicates measuring point ± standard deviation; values followed by a different letter are significantly different ($p < 0.05$).

3.5 Fungi challenge tests

In addition to the environmental shelf life tests, "challenge tests" were performed using fungi which are commonly found in the bakery environment. For these trials three different fungal species, namely *P. expansum*, *F. culmorum* and *A. niger*, were selected. These challenge tests were performed to evaluate the risk associated with NaCl reduction in a heavily contaminated bakery environment. In these experiments, approximately 100 spores of the various fungi were applied on the bread slices. A detailed outline of the influence of the NaCl concentration on bread shelf life is depicted in Figure 3. An example of the visual appearance of the bread slices contaminated with the three fungi is given in Figure 4 – 6. These pictures show the differences of the area of mould growth of the various fungi dependent on the NaCl concentration.

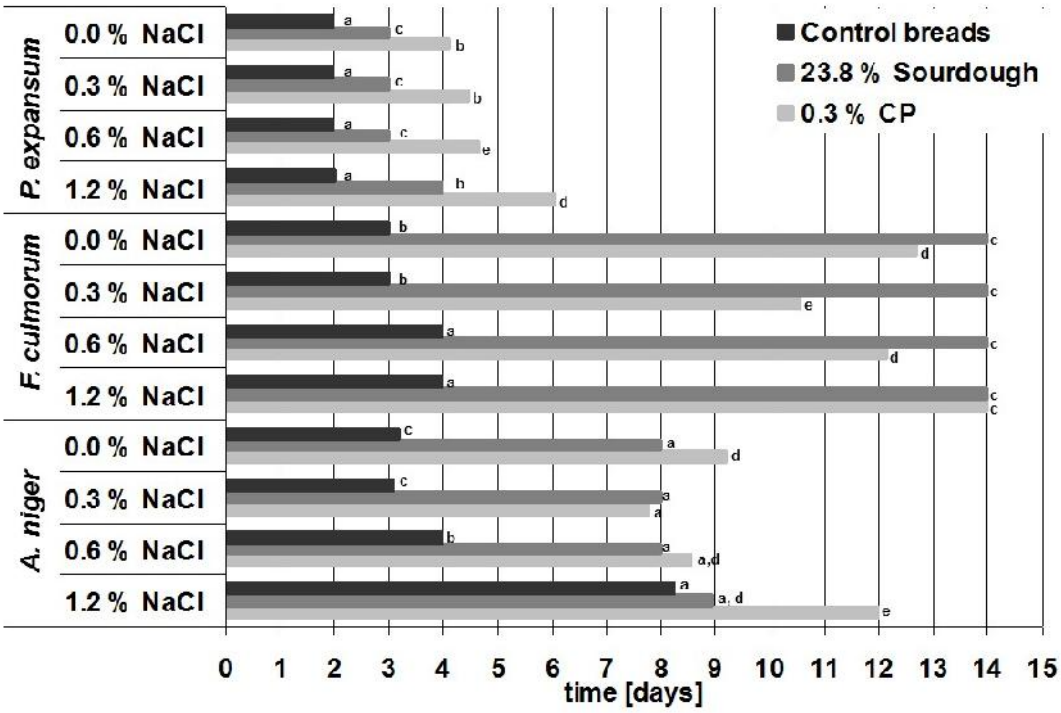


Figure 3: Shelf life of slices of control breads, sourdough breads and calcium propionate breads containing the different NaCl concentrations 0.0%, 0.3%, 0.6% and 1.2% contaminated with ca. 100 spores per slice and side using spores of *P. expansum*, *F. culmorum and A. niger*. Mean values of at least three replicates measuring point ± standard deviation; values followed by a different letter in the experiment concerning the same fungus are significantly different ($p < 0.05$).

3.5.1 Challenge tests with *Penicillium expansum*

Penicillium species *are* known to cause most of the spoilage in cereal and baking industry (Legan 1993). In the present study, *P. expansum* was chosen due to its frequent occurrence on wheat (Carter & Young 1950) and other cereals (Panasenko 1967). For the control breads challenged against *P. expansum* a shelf life of 2 days was observed independent of the NaCl concentration (Figure 3). Independent of the determined shelf life, the area of mould growth after 4 days was larger the lower the NaCl concentration (Figure 4) correlating with the a_w results (Figure 1).

The addition of the sourdough prolonged the shelf life significantly by 2 days for a NaCl concentration of 1.2%, whereas the shelf life was significantly prolonged by 1 day only for the reduced/no NaCl breads ($p < 0.05$). In comparison to the control breads as well as the sourdough breads a significant increase in shelf life was determined adding 0.3% calcium propionate.

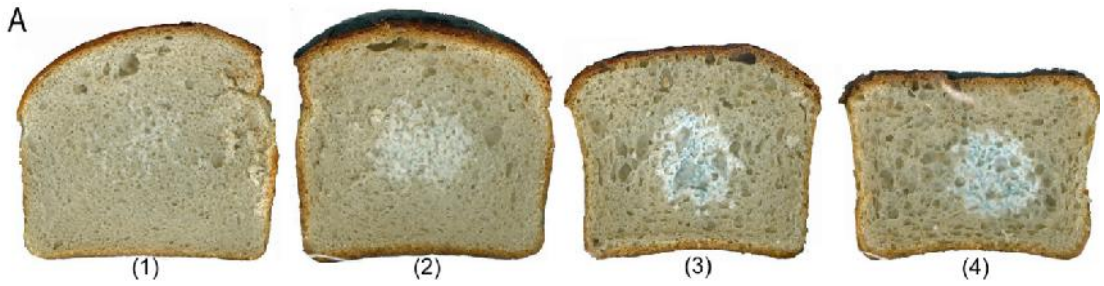


Figure 4: Slices of control breads containing the NaCl concentrations (1) 1.2%; (2) 0.6%; (3) 0.3%; (4) 0.0% challenged with about 100 spores of *P. expansum* after 4 days. Different slice heights shown in the picture is due to the convex shape of the bread loaf top and not due to differences in bread volume.

3.5.2 Challenge tests with *Fusarium culmorum*

Fusarium species are often found on cereal grains as well as in milling and bakery environments and known to be involved in mycotoxin production (Filtenborg et al. 1996; Corsetti et al. 1998; Samson et al. 2000). For the present study, *Fusarium culmorum* was chosen to challenge bread slices since it is known to produce deoxynivalenol, one of the predominant mycotoxins found on cereals in Europe (Eriksen & Alexander 1998). For the control breads containing different NaCl concentrations, a significant decrease of shelf life by one day could be determined between the two higher (1.2%, 0.6%) and the two lower NaCl concentrations (0.3%, 0.0%) (

Figure 3: 3).

The visual appearance of the area of mould growth of bread slices challenged against *F. culmorum* showed visible decrease only for the control bread containing 1.2% of NaCl (Figure 5). Although 0.6% NaCl retarded the initial appearance of mould spoilage as well

as 1.2% of NaCl resulting in a shelf life of 4 days, *F. culmorum* could spread on bread containing 0.6% NaCl as quick as on bread containing lower concentrations (0.3%, 0.0%).

The antifungal compounds of the sourdough inhibited *F. culmorum* completely over the 14 days of observation (Figure 3). The addition of 0.3% calcium propionate resulted in a shelf life between 10 and 14 days. With increasing NaCl concentrations from 0.3% to 1.2% the inhibition of the fungus was increased due to an increased synergistic impact of NaCl and calcium propionate against the *F. culmorum* growth.

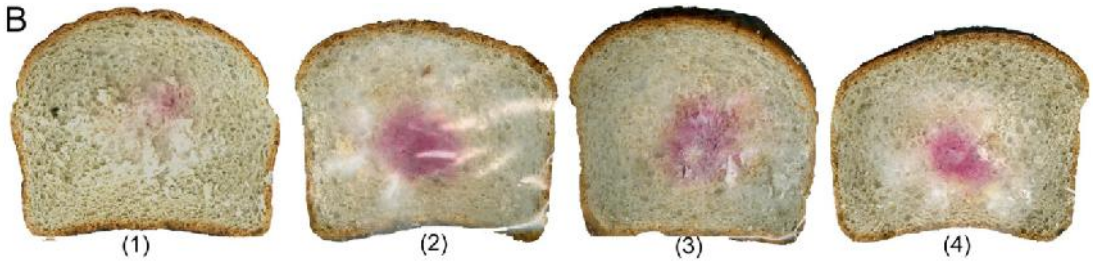


Figure 5: Slices of control breads containing the NaCl concentrations (1) 1.2%; (2) 0.6%; (3) 0.3%; (4) 0.0% challenged with about 100 spores of *F. culmorum* after 6 days. Different slice heights shown in the picture is due to the convex shape of the bread loaf top and not due to differences in bread volume.

3.5.3 Challenge tests with *Aspergillus niger*

Aspergillus spp. are known to appear frequently on cereals such as wheat and corn (Carter & Young 1950; Marin et al. 2002; Panasenko 1967). Thus, *Aspergillus niger* was used in this study for one of the challenge tests. For the control bread containing the standard concentration of NaCl of 1.2% a shelf life of about 8 days was determined. Any reduction (0.6% and 0.3%) as well as the omission of NaCl shortened the shelf life significantly by 4-5 days (Figure 3). The increase of a_w from the NaCl concentration 1.2% (0.974 a_w) to 0.6% (0.981 a_w) seems to exceed the crucial inhibition concentration of NaCl for *A. niger* in the bread environment. Further decreases of the NaCl concentration (0.3% and 0.0%) decreased the shelf life significantly by 1 more day only (Figure 3). In Figure 6 the visual appearance of control bread slices challenged against *A. niger* after 9 days is shown. The area of mould growth correlated with the NaCl concentration and a_w respectively (Figure 1). Again, the extent of mould growth was independent of the determined shelf life.

The shelf life of about 9 days determined for the sourdough bread containing 1.2% NaCl did not change significantly compared to the respective control bread (Figure 3). On the contrary, the addition of sourdough to the breads containing reduced amounts of NaCl (0.6%, 0.3%, 0.0%) resulted in a shelf life of 8 days; 3 to 4 more days compared to the control breads.

For the breads containing 1.2% NaCl, the use of 0.3% of calcium propionate as chemical preservative resulted in a significant increase of shelf life up to 12 days compared to all the other breads challenged against *A. niger*. The reduction of NaCl let to a significantly shorter shelf life of about 8-9 days observing one significant difference between the calcium propionate breads containing 0.0% and 0.3% NaCl. In conclusion, the addition of 0.3% calcium propionate prolonged the shelf life for each NaCl concentration significantly compared to the respective control breads.



Figure 6: Slices of control breads containing the NaCl concentrations (1) 1.2%; (2) 0.6%; (3) 0.3%; (4) 0.0% challenged with about 100 spores of *A. niger* after 9 days. Different slice heights shown in the picture is due to the convex shape of the bread loaf top and not due to differences in bread volume.

3.6 Discussion

3.6.1 Shelf life evaluation under bakery environmental conditions

NaCl acts as a preservative in bread and any reduction of NaCl decreased the shelf life of bread significantly. A 4 to 5 day shelf life for standard bread under summer conditions, presuming a common NaCl concentration of about 1.2%, was previously reported by Seiler (1988). The one day difference compared with the results of a 5 to 6 days shelf life

determined in the present study during winters time is based on the different environmental conditions between summer and winter time (Legan 1993). While the storage temperature was always kept at 20 °C, the micro load of the bakery environment was proven to be higher in summer time than in winter time. The reduced shelf life of reduced/no salt breads can be explained by the impact of NaCl on the osmotic pressure causing an increased water activity (a_w). The reduction of NaCl coincides with high linear correlation to the reduction of the water activity ($R^2 = 0.997$) (Figure 1) as previously shown by Samapundo et al. (2010).

Sourdough fermented with the selected antifungal strain as well as calcium propionate has the potential to compensate the lack of NaCl. The presence of antifungal agents in the sourdough breads produced by *L. amylovorus* DSM 19280 (Arendt et al. 2009) resulted in a further a_w-lowering predominating the inhibition of mould over the osmotic effect of NaCl lowering the a_w. The reduction of shelf life also correlated with the determined values of pH and TTA. The preservative effect of sourdough can be attributed in parts to the reduction of the pH-value due to the production of organic acids formed by *L. amylovorus* DSM 19280 during sourdough fermentation as investigated by Arendt et al. (2009). But in this particular case, the formation of specific antifungal agents produced by *L. amylovorus* DSM 19280 mainly contributed to the preservative effect.

The significant lower a_w for breads containing calcium propionate reveal that calcium propionate significantly increased the shelf life of bread to approximately 10 days. Surprisingly, the NaCl concentration did not significantly effect the action of calcium propionate even that the a_w of the breads were significantly different. The results presented in this study show that at NaCl concentrations between 0.0% and 1.2%, usually used in bread, mould inhibition by 0.3% calcium propionate is efficient, independent of the amount of NaCl.

The antimicrobial effect of calcium propionate is known since 1913 (Kiesel 1913). The uncharged protonated propionic acid can readily diffuse across the cell membrane and dissociate in the higher pH environment of the cytosol (Piper et al. 2001). This leads to a decrease of the cytosolic pH which inhibits many metabolic functions (Krebs et al. 1983) as well as to the accumulation of the propionate anion inside the cell (Piper et al. 2001).

The detailed mechanism is still under investigation but up to date it was shown that in a first step the propionate anion is transformed to propionyl-Coenzyme A which is discussed to inhibit crucial enzymes of the glucose metabolism such as adenosine triphosphate citrate lyase and succinyl-Coenzyme A synthases (Brock & Buckel 2004). The independence of the inhibition by calcium propionate from the NaCl concentration was previously published by Razavi-Rohani & Griffiths (1999) who investigated the impact of NaCl concentration (3 – 10%) on mould growth (*Candida* spp., *Penicillium* spp., *Sporothrix schenkii, Paecilomyces niveus and Aspergillus spp.*) tested on potato dextrose agar in the presence of 3176 µg/ml calcium propionate at a pH of 5.6.

All breads without NaCl showed a trend of a prolonged shelf life compared to the respective breads containing 0.3% NaCl (Figure 2). As shown by Cuppers et al. (1997) each fungus requires a specific concentration of NaCl which leads to a tendency of longer shelf life for bread without any salt.

3.6.2 Fungi challenge tests

The high resistance of *P. expansum* against NaCl was previously determined in a similar experiment by Samapundo et al. (2010) challenging bread against *P. roqueforti* reducing the concentration of NaCl from 1.3% to 0.9%. The extended shelf life of 1 to 2 days for the combination of sourdough and 1.2% of NaCl can be explained by a synergistic effect of NaCl, the antifungal compounds and the lowered pH of 5.0 on the growth of *P. expansum* which occurs apparently below a certain a_w only. A similar synergistic effect was determined for NaCl and calcium propionate which prolonged significantly the shelf life of the calcium propionate bread containing 1.2% NaCl by 1 to 2 days compared to the calcium propionate breads contained reduced concentrations of NaCl. This is in contrast to the results obtained for the environmental contamination where calcium propionate acted independently of the NaCl concentration. The unspecific contamination under environmental conditions concerning amount and nature of spores is the reason for the descript differences. For a controlled challenge against *P. expansum* spores the combined hurdles using NaCl and calcium propionate resulted in a synergy regarding the shelf life.

In comparison, *F. culmorum* showed less resistance against NaCl than *P. expansum*. This confirms the results published by Tresner & Hayes (1971) who determined an outstanding

higher resistance of *Penicillium* spp. (273 strains of 124 species tested) and *Aspergillus* spp. (196 strains of 81 species tested) against NaCl compared to any of the other 349 genera of fungi studied. This finding confirm the previous results published by Arendt et al. (2009) who determined that *F. culmorum* was one of the most sensitive fungi inhibited by the antifungal compounds produced by *L. amylovorus* DSM 19280. The higher resistance of *F. culmorum* on the CP bread containing no NaCl compared to the respective one containing 0.3% NaCl leads to the assumption that *F. culmorum* might need a physiological amount of NaCl to be able to resist against calcium propionate (Cuppers et al. 1997).

A. niger showed least resistance for 1.2% NaCl compared to the two other fungi and no additional shelf life could be obtained using sourdough. These results reveal that the antifungal compounds of the sourdough fermented with the *L. amylovorus* DSM 19280 acted independently of the NaCl concentration resulting in a shelf life of 8-9 days for all sourdough breads challenged against *A. niger*.

The addition of 0.3% calcium propionate prolonged the shelf life for each NaCl concentration significantly compared to the respective control breads due to the antimicrobial effect of calcium propionate (Piper et al. 2001). In accordance to the results obtained for the challenge against *P. expansum*, a synergistic effect was determined for the addition of calcium propionate in bread with 1.2% NaCl prolonging the shelf life by 3 days. Again, a significant longer shelf life of about 1 day was determined for bread without any NaCl compared to 0.3% NaCl which is based on an enhancing effect of a specific physiological NaCl concentration where fungi grow best. Cuppers et al. (1997) determined an optimum NaCl concentration of 3.47% in malt extract agar for *A. niger*. The different environments comparing wheat bread crumb against malt extract agar need to be considered explaining the differences in optimum NaCl concentrations.

These results outline in particular the potential of sourdough with its antifungal compounds. For the use of sourdough the NaCl concentration hardly influenced the preservation effect due to the wide range of antifungal compounds produced by *Lactobacillus amylovorus* DSM 19280 (Arendt et al. 2009) which predominated the antifungal effect shown by NaCl. Compared to the respective sourdough breads, the addition of calcium propionate prolonged the shelf life of breads only when challenged

against *P. expansum* (all NaCl concentrations) and against *A. niger* (1.2% NaCl). Under the environmental contamination conditions of a pilot scale bakery, bread slices were mould-preserved to the same extend by the antifungal sourdough than by calcium propionate. Hence, sourdough is the preferred option and gives furthermore an improvement of flavour and texture (Arendt et al. 2007). Moreover, sourdough is recognised as a natural "green" preservative and is more widely accepted by the consumer. The shelf life prolonging of low-salt bread is a novel approach which will ensure the microbial quality of low-salt bread in future.

3.7 Acknowledgement

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Chapter 4

Improvement of taste and shelf life of yeasted low-salt bread containing functional sourdoughs using *L. amylovorous* DSM 19280 and *W. cibaria* MG1

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4.1 Abstract

The challenge remains for the baking industry to reduce salt levels in yeasted bread as directed by governments, retailers and consumers around the world. The two main problems associated with the reduction of salt are a lack of salty taste and the reduction in shelf-life. Both of these issues are addressed in the presented work. A range of breads containing different levels of salt (0.0%, 0.3% and 1.2% of NaCl) in combination with various levels of sourdough (0%, 6%, 12%, 18%, 24%) were produced. The different doughs were analysed for their rheological behaviour using fundamental rheology. The bread quality characteristics such as loaf volume, crumb structure, staling rate and microbial shelf life were also determined. The sourdoughs were analysed for their different metabolites: organic acids, sugars, exopolysaccharides (EPS), antifungal compounds, pH and TTA. A trained sensory panel was used to perform descriptive analysis of the bread samples. The object of this paper is to use functional sourdoughs, containing *Lactobacillus amylovorus* DSM 19280 and *Weisella cibaria* MG1 to compensate for the quality problems that occur when salt is reduced in yeasted bread. The application of functional sourdoughs containing exopolysaccharides and/or antifungal substances in salt reduced breads improved the quality significantly. The application of functional sourdoughs allows the reduction of salt to a level of 0.3%.

Keywords: Antifungal, Exopolysaccharide, Low-Salt Bread, Salt Reduction, Functional Sourdough, Descriptive Sensory

4.2 Introduction

The excessive intake of sodium by populations in developed countries is linked to several health related issues. In particular, cardiovascular diseases were determined as the most concerning impact on human health. (Farquhar et al., 2015, O'Donnell et al., 2015, Brown et al., 2009, Angus, 2007, Stamler, 1997, Reddy and Marth, 1991, Freis, 1976). Hence, a reduction of salt in the western diet has been targeted in all industrialised countries. Global organisations (WHO and FAO, 2003) as well as national authorities (German Nutrition Society (DGE), 2009, European Commission, 2008, Korean Food and Drug Administration, 2007, FSAI, 2005, SACN, 2003) have put salt reduction on their agenda. The intake of sodium increased mainly due to the increase in the processed food being consumed (Reddy and Marth, 1991, Gibson et al., 2000, SACN, 2003). Bread as a staple food, is one of the main food products targeted by health organisations and can contribute up to 40% of the dietary sodium depending on the country and the respective culinary tradition (Thomson, 2009, Cauvain, 2007, Angus, 2007).

The health related necessity of salt reduction in bread leads to a number of challenges for bakers and the bread industry. A range of bread characteristics are affected by the salt reduction, such as taste profile (Lynch et al., 2009), crumb structure (Beck et al., 2012c) and shelf-life (Samapundo et al., 2010, Pateras, 2007, Filtenborg et al., 1996). Breads with reduced amounts of NaCl were described as more sour/acidic and yeasty (Lynch et al., 2009). Beck et al. (2012b) reported significant changes in crumb structure based on different amounts of NaCl. As previously reported (Belz et al., 2012), salt reduction from 1.2% of NaCl to a low-salt concentration of 0.3% NaCl reduces the shelf-life of bread by approximately two days.

The present paper focuses on the determination of the minimum amount of sourdough needed to compensate for the reduction of NaCl in yeasted wheat bread. Based on previous research two functional lactic acid bacteria strains were considered; the antifungal strain *Lactobacillus amylovorus* DSM19280 (Axel et al., 2015, Belz et al., 2012, Ryan et al., 2011) as well as the exopolysaccharide producer *Weisella cibaria* MG1 (Wolter et al., 2014, Galle et al., 2011, Galle et al., 2010). The impact of the sourdough on the dough and bread quality was analysed. Rheological analyses were performed on the dough samples

based on sourdough addition. The changes of the different bread characteristics were also evaluated to optimise the quality of low-salt bread.

4.3 Materials and Methods

4.3.1 Bacteria and growth conditions

The exopolysaccharide (EPS) producing strain *W. cibaria* MG1 was previously isolated from gluten-free buckwheat sourdough (Moroni et al., 2011a). The antifungal strain *L. amylovorus* DSM 19280, was previously isolated from gluten-free sourdough (Arendt et al., 2009). The bacteria were grown on MRS5 (Meroth et al., 2003) agar plates for 48 h at 30 °C and a single colony was transferred into MRS5 broth for about 16 h at 30 °C.

4.3.2 Sourdough fermentation

For sourdough fermentation, a pre-culture of the respective strains was subcultured in 40 ml of MRS5 broth and incubated for 24 h at 30 °C; *W. cibaria* MG1 and *L. amylovorous* (DSM19280) resulted in a cell suspension containing approximately 5 x 10^9 CFU/ml. Cells were harvested by centrifugation at 3000 g for 10 min, washed twice with Ringer's solution and re-suspended in 40 ml of the same solution. Sourdough was prepared with a dough yield of 200 and an inoculation of about 10^7 CFU/g of sourdough using 600 g of wheat flour, 560 ml of sterile distilled water and 40 ml of cellular suspension. After mixing with a Kenwood mixer (Kenwood KM020) using the batter attachment for 1 min. at speed 1, the dough was covered and fermented at 30 °C for 24h for the *W. cibaria* MG1 sourdough and at 30 °C for 48 h for the *L. amylovorus* DSM19280 sourdough. At the end of fermentation, lactic acid bacteria cell counts were evaluated on MRS5 agar plates and the total titratable acid (TTA) and pH values were determined according to Arbeitsgemeinschaft Getreideforschung e.V. (AGF, 1994). For the *W. cibaria* MG1 sourdough samples TTA = 7.6 ± 0.2 ml, pH = 4.26 ± 0.04 and for the *L. amylovorus* DSM19280 sourdough samples TTA = 17.8 ± 0.9 ml, pH = 3.75 ± 0.04 were detected. Both strains showed cell counts of about 5x10^8 ± 10^3 CFU/g. These parameters were used as the "stability" parameter of sourdough fermentation.

4.3.3 Baking procedure and loaf analysis

Wheat bread was prepared by mixing Baker's flour (Odlums, Ireland), distilled water (water level set to 64.7% (flour weight) using a Brabender farinograph), dry yeast (Puratos Group, Belgium) and NaCl at levels of 1.04, and 0.26% (w/w) with a spiral mixer (Kenwood KM020). Considering an average bakeloss of 13.5% the NaCl concentrations in the final bread loaves resulted in 1.2 and 0.3%. Sourdough levels of 6, 12, 18, 24% were added to the different samples for sourdough fermented with both; *L. amylovorus* DSM19280 and *W. cibaria* MG1. After a bulk fermentation of 15 min. at 30 °C and 85% relative humidity (Koma Popular, Koma, Roermond, The Netherlands) 450 ± 1 g bread loaves were moulded with a moulding machine (Machinefabriek Holtkamp B.V., Almelo, Holland) and put into pans. The loaves were proofed for 75 min under the same conditions used during bulk fermentation. Subsequently, the breads were baked for 35 min at 230 °C top and bottom heat in a deck oven (MIWE condo, Arnstein, Germany). Ovens were pre-steamed (0.3 L) and steamed when loaded (0.7 L). Loaves were depanned cooled for 120 min at room temperature. The breads were baked with the different addition levels of sourdough and NaCl as shown in

Table 5. The specific loaf volume was measured using a Volscan Profiler (Stable Micro Systems, UK). A TA-XT2i texture analyser (Stable Micro Systems, Surrey, UK) equipped with a 25 kg loading cell and a cylindrical aluminium probe (diameter of 35 mm) was used to analyse the texture profile of the bread crumbs. Crumb grain was described by the following parameters: slice brightness, number of cells, porosity expressed as the area of cells (the total area of cells as a percentage of the total slice area), wall thickness (the average thickness of cell walls) and the average cell volume, using a C-cell bread imaging system (Calibre Control International Ltd., UK).

Table 5: Overview of the prepared bread recipes

	Control breads (no sourdough)						Sourdough breads (low-salt)											
	Standard salt			Low-salt			6% Sourdough			12% Sourdough			18% Sourdough			24% Sourdough		
	% FW*	% DW**	g	% FW*	% DW**	g	% FW*	% DW**	g	% FW*	% DW**	g	% FW*	% DW**	g	% FW*	% DW**	g
Baker's flour	100.0	59.5	1785	100.0	60.0	1799	95.0	57.0	1709	90.0	54.0	1619	85.0	51.0	1529	80.0	48.0	1439
Baker's yeast	2.0	1.2	36	2.0	1.2	36	2.0	1.2	36	2.0	1.2	36	2.0	1.2	36	2.0	1.2	36
Water	64.3	38.2	1147	64.3	38.5	1156	59.3	35.6	1067	54.3	32.5	976	49.3	29.6	887	44.3	26.6	797
NaCl	1.75	1.04	31.2	0.44	0.26	7.9	0.44	0.26	7.9	0.44	0.26	7.9	0.44	0.26	7.9	0.44	0.26	7.9
Sourdough	0.0	0.0	0	0.0	0.0	0	10.0	6.0	180	20.0	12.0	360	30.0	18.0	540	40.0	24.0	720
Total amount	168.0	100.0	3000	166.7	100.0	3000	166.8	100.0	3000	166.7	100.0	3000	166.7	100.0	3000	166.7	100.0	3000

* Flour weight ** Dough weight

4.3.4 Analyses of organic acids and sugars

An Agilent 1260 high performance liquid chromatography (HPLC) 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 acid and sugar analyses freeze-dried sourdough samples were extracted with water and proteins were precipitated with Carrez solutions. After centrifugation (2000 x *g*, 20 min) and filtration (0.2 µm), 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 mm, Agilent, Cork, Ireland), equipped with a guard column (50 x 7.7 mm, 8 mm, 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 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 mL.

4.3.5 Analyses of antifungal compounds

The sample preparation was performed as described by Brosnan (2015). Sourdough samples were freeze-dried and ground to a fine powder. These freeze-dried sourdough samples (2.0 g ± 0.01g) were weighed into individually labelled polypropylene tubes (50 mL) and H_2O (10 mL) was added and vortexed for 30 seconds. The samples were then fortified with deuterated internal standard (100 µL) and left to stand for 15 minutes. Ethyl acetate (EA) (10 mL) with 0.1% formic acid (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 dimethyl sulfoxide (DMSO) and evaporated under nitrogen at 50 ^{o}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 ultra high pressure liquid chromatography (UHPLC)-MS/MS system.

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 ^{o}C and the pump was operated at a flow rate of 0.6 mL min^{-1}. 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 acetonitrile. 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 the 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 MassLynx^{TM} software and data were processed using TargetLynx^{TM} 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 ^{o}C, respectively. Nitrogen was employed as the desolvation and cone gases and was set at 1000 and 50 L h^{-1}, respectively. Argon was employed as the collision gas at a flow rate of 0.21 µg mL^{-1} – which typically gave pressures of 3.52 x 10^{-3} mBar. The MS conditions were optimised by teed infusion of 10 µg mL^{-1} standard solutions into 50% mobile phase A and B at a flow rate of 20 µL min^{-1} and 0.2 mL min^{-1}, respectively. A validation of the method was performed in compliance with the EC (EU, 2002) and ICH (ICH, 2005) guidelines taking into account specificity, linearity, limits of detection and quantitation, trueness and precision. The validation was completed by analysing standard concentrations (1 ppm, 5 ppm, 10 ppm, 15 ppm, 20 ppm, 25 ppm, 50 ppm) in triplicate on the first 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 the first 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).

4.3.6 Evaluation of microbial shelf-life and water activity

For the microbial shelf life tests the “natural” bakery-environment was used for contamination. Breads were sliced and 10 slices of each type of bread were exposed to the bakery-environment for 10 min. Each of the bread slices were packaged in polyethylene bags and heat sealed. The sterile air exchange was enabled by inserting two filter tips in each storage bag. During storage the temperature was kept constant at 20 °C. The storage took place up to 14 days observing the appearance of mould spoilage every day. As soon as spoilage appeared on one of the bread slices, the previous day was determined as the shelf-life of that bread (Belz et al., 2012). Water activity was measured using a water activity meter AquaLab Series 4TE from Decagon Devices, Inc. The a_w-meter was calibrated using the verification standards (distilled water; 0.5 M KCl; 6.0 M NaCl; 8.57 M LiCl; 13.41 M LiCl) from Decagon (NE Hopkins Ct. Pullman, WA – USA) at 25 °C. For each of the five water activity levels the values were determined within the required range of 0.003 (Decagon Devices 2009). The bread samples were measured in triplicate on 3 independent baking batches 2 h after baking at 25 °C.

4.3.7 EPS analysis

EPS were detected with a refractive index detector (RID) and their molecular weight was estimated using two dextrans; low molecular weight (LM) dextran with a relative molecular weight $M_r = 10^5 - 2 \times 10^5$ and high molecular weight (HMW) dextran ($M_r = 5 \times 10^6 - 4 \times 10^7$) and inulin from chicory ($M_r = 10^4$) for calibration (all obtained from Sigma Aldrich, Oakville, Canada). EPS size was determined by asymmetrical flow field-flow-fractionation (FFF) coupled to multi-angle light scattering (MALS) and a refractive index (RI) detector (Postnova, Salt Lake City, UT) as described by Galle et al. (2010 and 2011). The channel dimensions were 335 x 60 x 40 mm (Postnova) with a molecular weight cut-off of 10 kDa. A polyetherketone pre-column filter unit placed between channel and detectors contained a 2 µm pore-size and a Teflon microfilter (0.1 µm poresize) of regenerated cellulose filter paper (all Postnova). The samples were injected onto the channel at a flow rate of 0.2 ml/min and a cross flow of 2 ml/min for 1 min. After injection, the cross flow rate of 2 ml/min decreased exponentially to 0.1 ml/ min over 30 min and was then maintained at 0.1 ml/min for 30 min. The molecular weight was measured by static light scattering data as processed by the AF 2000 software (Postnova, Salt Lake City,

UT) with the RI signal as concentration detector. A dn/dc value of 0.147 was used for light scattering calculations of EPS. Polystyrolsulphonate standards (Postnova) and bovine serum albumin (Sigma Aldrich) were used for calibration of detectors. Sugars were analysed with a Carbopac PA20 column (Dionex, Oakville, Canada) using water (A), 200 mM NaOH (B) and 1 M Na-acetate (C) as solvents at a flow rate of 0.25 mL/ min with the following gradient: 0 min. 30.4% B, 1.3% C, 22 min. 30.4% B, 11.34% C followed by washing and regeneration. Sucrose, glucose, fructose, maltose, panose, isomaltose, isomaltotriose (all obtained from Sigma Aldrich) and isomaltooligosaccharides (IMO, from Bionutra Inc., Edmonton, Canada) were used as external standards.

4.3.8 Fundamental Rheology

Freshly prepared dough pieces (14g) were sealed in airtight containers and allowed to rest for 10 min before loading. Measurements were conducted using a controlled stress/strain rotational rheometer (MCR301 Anton Paar GmbH, Germany). Parallel serrated plates with a diameter of 25 mm were used set at a temperature of 30 °C. The measurements were performed at a final gap of 1.000 mm after a relaxation rest of 10 min (Addo et al., 2001). Fundamental rheological properties were determined within the linear viscoelastic range (LVR). Frequency sweeps were conducted in the range of 1-100 Hz at a constant strain of 0.01%. The complex modulus (G*) data were obtained from the frequency sweeps and the weak gel model was applied using the equation $G^*(\omega)=A_F \cdot \omega^{1/z}$ according to Gabriele et al. (2001). All results are averages of two measurements of three independent samples.

4.3.9 Sensory

Descriptive sensory analysis was carried out by a panel of eight assessors, recruited according to international standards (ISO, 2012). At the start of the study, the panel took part in a number of focus groups for the development and refinement of a lexicon to describe the sensory characteristics of the breads. During these focus groups, all bread samples included in the study were examined and a list of attributes describing their odour, appearance, texture and flavour characteristics were generated. These terms were refined in subsequent sessions and the most representative terms describing the samples were retained. Descriptive sensory analysis was carried out using a final vocabulary of 5 odour (“roasted”, “sourdough”, “cheesy”, “doughy”, “yeasty”), 1 appearance (“density”), 1

texture (“density”) and 6 flavour (“yeasty”, doughy”, “musty/stale”, “roasted”, “salt”, “bitter”) terms which showed a level of significance of $p<0.05$.

The descriptive testing took place over a period of two days with a total of 11 breads analysed in duplicate. The order of tasting was balanced to account for the order of presentation and carry-over effects (MacFie et al., 1989). Ahead of the assessment, the assessors were provided with deionised water, a list of the defined vocabulary and instructed to cleanse their palate between tastings. Breads were scored for attributes on unstructured 100 mm line scales labelled at both ends with extremes of each attribute. The intensity of each of the descriptive terms was recorded for each sample using the Compusense ® five V. 4.0 sensory data acquisition programme (Guelph, Ontario, Canada). The descriptive analysis yielded duplicate data matrices consisting of 8 assessors by 13 sensory attributes by 11 breads. The mean panel scores from the duplicate descriptive sensory analysis were subjected to one-way analysis of variance (ANOVA, SPSS v 14.0 SPSS Inc. Chicago, Illinois, USA) to determine which terms were effective at providing discrimination amongst the breads at $p \leq 0.05$. All descriptive terms significantly discriminated ($p < 0.05$) between the samples and were included in subsequent analyses. Data were averaged across replicates, standardised (1/standard deviation) and analysed by means of principal component analysis (PCA) using Guideline +7.5 (CAMO ÅS, Trondheim, Norway). The way in which each principal component (PC) discriminated between all bread samples was determined by performing an ANOVA (SPSS v 14.0 SPSS Inc.) on the PCA scores prior to averaging across replicates. The final number of components for interpretation was based on the discriminating ability ($p \leq 0.05$) of each PC and a visual inspection of explained validation variance (to indicate whether additional PCs were modelling information or noise).

4.3.10 Statistics

Statistical analyses were performed using MiniTab 16 for Windows computerised statistical analysis package (MiniTab Ltd., Coventry, UK). Data were examined using the one-way analysis of variance (ANOVA). Where an F-test showed significant differences ($p<0.05$), Fisher’s least significant difference (LSD) test was used for multiple comparisons. Each result is the average of at least three separate experiments with three independent samples from each batch.

4.4 Results and Discussion

4.4.1 Sourdough metabolites analyses

Sourdoughs were prepared as described fermenting Baker's flour with two selected lactic acid bacteria strains *Weisella cibaria* MG1 and *Lactobacillus amylovorus* DSM19280. For both strains, the typical sourdough characteristics were determined – such as sugars and organic acids using HPLC analyses, total titratable acids (TTA) and pH. Furthermore, exopolysaccharides and antifungal compounds were determined based on the specific functionalities of both selected strains.

The unfermented wheat flour contained no single sugars or acids, 45.5 mmol sucrose and 48 mmol maltose per 1 kg baker's flour. With a TTA of 2.2 ml and a pH of 6.1 the flour had a standard quality as described by Souci et al. (2000). The sourdough fermented with the heterofermentative *Weisella cibaria* MG1 strain contained fructose as well as glucose as intermediate metabolites. All the measured concentrations of metabolites (

Table 6) are in line with the results published by Galle et al. (2010). The added sucrose was completely metabolised. The acidification level was low resulting in a TTA = 7.6 ml, pH = 4.6 based on the concentrations of acetic acid (73 mmol kg^{-1} flour) and lactic acid (139 ± 4). The presence of maltose, initially in the flour and during the fermentation process released by the amylase activity, favoured the metabolism of sucrose and lead to an increased EPS synthesis as described by Galle et al. (2010). Furthermore, the analyses on exopolysaccharides (EPS) resulted in 4.5 ± 0.3g kg^{-1} sourdough as previously described by Galle et al. (2010).

The fermentation with the *Lactobacillus amylovorus* (DSM19280) strain resulted in an highly acidified sourdough with a pH = 3.75 ± 0.04 and a TTA = 17.8 ± 0.9 (Ryan et al., 2011). Due to the homofermentative characteristics of *Lactobacillus amylovorus* strains lactic acid was the only acid which could be determined. All sucrose originating from the wheat flour was metabolised. The amount of maltose increased significantly due to the high amylase activity of the *L. amylovorus bacteria* (Axel et al., 2015). No EPS could be determined in the sourdough containing *L. amylovorus*.

Table 6: Sourdough metabolites sugars, lactate, acetate, TTA, pH and exopolysaccharides (EPS).

Sourdough Metabolites [mmol kg^{-1}]/ Flour	**Fructose**	**Glucose**	**Sucrose**	**Maltose**	**Lactate**	**Acetate**	**TTA [ml]**	**pH**	**EPS [g kg-1]/ Sourdough**
Wheat flour, unfermented	n/d	n/d	45.5 ± 0.4	48 ± 2	n/d	n/d	2.2 ± 0.0	6.1 ± 0.1	1.7 ± 0.4
Weisella cibaria (MG1)	23 ± 2	49 ± 4	n/d	198 ± 4	139 ± 4	73 ± 3	7.6 ± 0.2	4.26 ± 0.03	4.5 ± 0.3
L. amylovorus (DSM19280)	25 ± 2	13 ± 2	n/d	181 ± 5	360 ± 7	n/d	17.8 ± 0.9	3.75 ± 0.04	n/d

4.4.2 Quantification of antifungal acid compounds

The selected strain *L. amylovorus* (DSM19280) had proven to compensate the reduced shelf life of bread due to reduced levels of salt (Belz et al., 2012). The antifungal acidic compounds were determined for both types of sourdough using HPLC-MS/MS analyses (Figure 7). The determined amount of quantified compounds was significantly higher for the *L. amylovorus* DSM19280 strain than for the *W. cibaria* MG1 with the exception of vanillic, caffeic and hydro-p-coumaric acid (phloretic acid). This correlates with the determined antifungal impact of the *L. amylovorus* strain on the bread samples prolonging their shelf life. As previously reported for different fermented substrates such as grass silage inoculated with *L. plantarum* (Broberg et al., 2007) or wort fermented with a *L. brevis* (Axel et al., 2014), the concentrations of the various compounds were too low to be effective based on the known minimum inhibition concentrations (MIC) (Ryan et al., 2011, Aziz et al., 1998). But complex synergistic effects can explain the determined antifungal activities in the various studies. The analyses resulted in 9 antifungal compounds detected for the *L. amylovorus* sourdough sample while 15 different compounds had been identified previously in cell free supernatant (Ryan et al., 2011). The discrepancy between the two studies can be explained with the different substrates being sourdough and cell free supernatant. Vermeulen et al. (2006) showed that the production of antifungal compounds is substrate specific. While caffeic acid has been reported to show no antifungal activity as an isolated compound (Bisogno et al., 2007, Kim et al., 2004), all of the other determined compounds are known for their antifungal activity (Axel et al., 2015, Guo et al., 2012, Broberg et al., 2007). The compounds 3-phenyllactic acid and 4-hydroxyphenyllactic acid have previously been detected as antifungal substances produced by *L. plantarum* and several studies have proven the antifungal effect in sourdough and sourdough breads (Ryan et al., 2009, Broberg et al., 2007, Dal Bello et al., 2007, Lavermicocca et al., 2003, Ström et al., 2002). Leucic acid was reported to inhibit the growth of *Candida* and *Aspergillus* species in broth (Sakko et al., 2014) and also the azelaic acid had been isolated from *L. reuteri* as an antifungal compound by Guo et al. (2012).

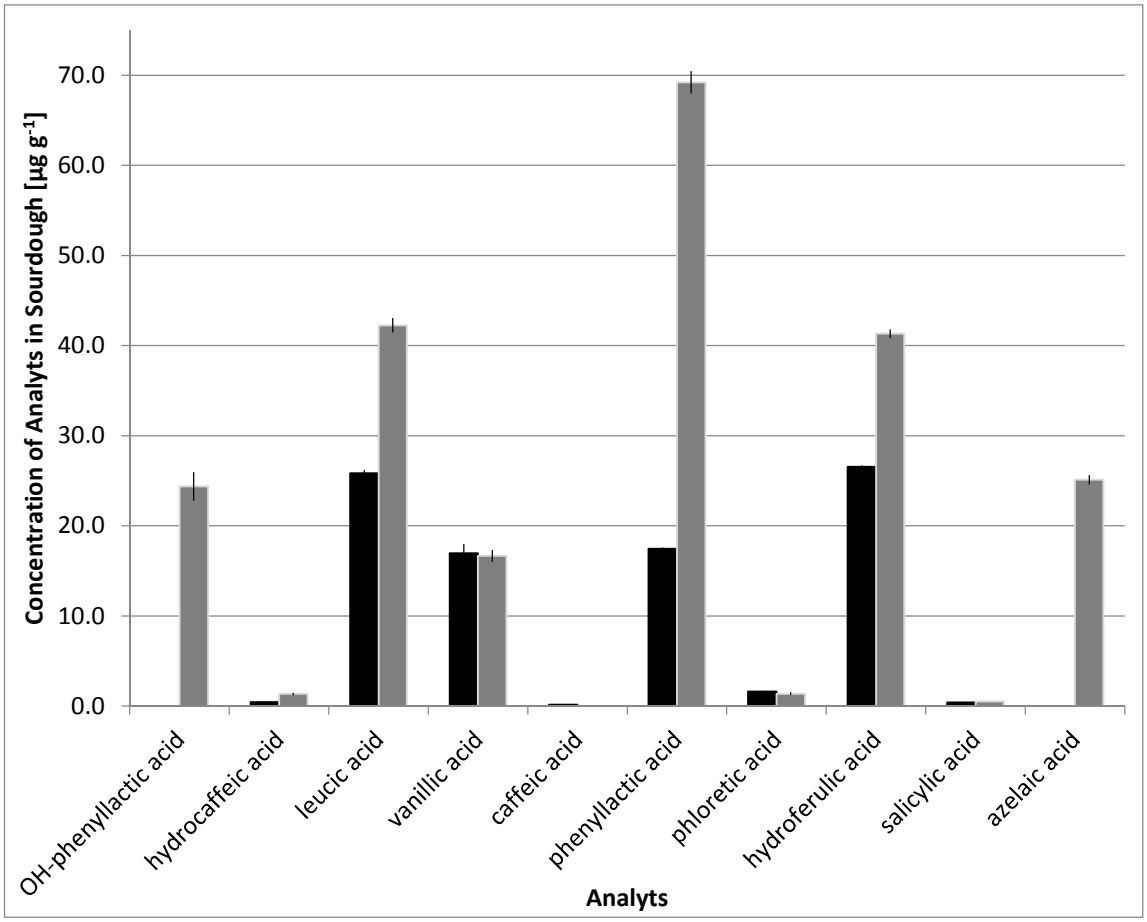


Figure 7: Quantification of antifungal acid compounds in sourdough fermented using *L. amylovorus* (DSM19280) (■) and *W. cibaria* MG1 (■). The analyses were performed using a UPLC-MS/MS.

4.4.3 Fundamental rheology

Rheological frequency sweeps were performed to determine the impact of the sourdough addition on the microstructural characteristics of the bread dough samples (Table 7). The weak gel model was applied to determine the resistance to deformation A_F and the network connectivity z of the dough samples over the frequency range of ω = 1 – 100 Hz. This model was introduced by Gabriele et al. (2001) and is based on the power equation $G^* \omega = A_F \cdot \omega^{\frac{1}{z}}$. The frequency sweeps of all dough samples showed a viscoelastic behaviour which means all measured elastic moduli (G') were higher than the corresponding viscous moduli (G'') (Song and Zheng, 2007, Dobraszczyk and Morgenstern, 2003, Hoseney and Rogers, 1990). The reduction of NaCl from standard salt

to low-salt led to a significant increase of dough strength of 29% as well as to a significant increase in network connectivity. On the contrary, Beck et al. (2012a) described a decrease of dough strength for reducing amounts of NaCl for large scale deformation using a farinograph. It was explained by the interaction of sodium and chloride ions with the positive and negative side chains of the gluten protein molecules favouring the network formation between the protein molecules. Our result can be explained by the known differential behaviour between small-scale and large-scale deformation rheological properties of wheat dough (Mann et al., 2005, Tronsmo et al., 2003, Lynch et al., 2009). In actual fact, there is still a gap of knowledge concerning how to relate small-scale deformation and large-scale deformation. For all low-salt samples fermented with *W. cibaria* the resistance to deformation decreased significantly with a linear correlation to the sourdough concentration. The fermentation with *L. amylovorus* resulted in a similar picture whereby the values were insignificantly lower. The stronger acidification of the *L. amylovorus* strain led to a more intense degradation of the gluten network resulting in a decreased dough strength. The network connectivity *z* did not differ between the two sourdoughs for lower addition levels. Only for the addition of 24% of sourdough the network connectivity differs significantly making an impact to the dough network. There are two main reasons for those changes of dough properties upon sourdough addition: firstly, being a positive net charge due to the presence of the organic acids which caused an unfolding of the gluten proteins (Galal et al., 1978) and secondly, an activation of the endogenous proteinase naturally present in wheat flour during fermentation of the sourdough (Thiele et al., 2002, Bleukx et al., 1997). Both effects led to a weaker integrity of the gluten network (Bleukx and Delcour, 2000). Comparing the samples containing the two different sourdoughs, it shows clearly that the acidification and the degradation of the flour polymers are the cause for the drop in dough strength and the EPS has no measurable impact on it. On the contrary, the presence of EPS caused a significant reduction of the network connectivity while the organic acids did not change the network integrity significantly. According to the weak gel model the EPS impacts on the network connectivity reducing the interactions between starch and protein molecules (Moroni et al., 2011b, Gabriele et al., 2001).

Table 7: Dough strength towards deformation (A_F) and network connectivity (z) are shown for bread dough samples containing *W. cibaria* MG1 and *L. amylovorus* (DSM19280). The frequency sweep was performed at an angular frequency range of ω = 1 – 100 Hz and a constant target strain of γ = 0.01% (after the weak gel model of Gabriele et al. (2001)). Values followed by a different letter are significantly different ($p < 0.05$)

NaCl conc.	Dough samples	Dough strength A_F	ΔA_F [%]	Network connectivity *z*	R^2
1.04%	Standard salt control	4122 ± 115^{a,c}		3.8 ± 0.1^{a}	0.996 ± 0.002
0.26%	Low-salt control	5298 ± 418^{b}	29%[1]	4.60 ± 0.10^{c}	0.99990 ± 0.00004
	6% *W. cibaria* MG1	4663 ± 231^{a}	-12%[2]	4.46 ± 0.08^{c}	0.9998 ± 0.0001
	12% *W. cibaria* MG1	3934 ± 150^{c,d}	-26%[2]	4.40 ± 0.40^{b,c}	0.996 ± 0.006
	18% *W. cibaria* MG1	3480 ± 260^{d}	-34%[2]	4.3 ± 0.2^{a,b,c}	0.9997 ± 0.0004
	24% *W. cibaria* MG1	2590 ± 304^{e}	-51%[2]	4.05 ± 0.05^{a,b}	0.99991 ± 0.00003
	6% *L. amylovorus* (DSM19280)	4354 ± 190^{a,c}	-18%[2]	4.45 ± 0.05^{b,c}	0.99993 ± 0.00002
	12% *L. amylovorus* (DSM19280)	3974 ± 272^{a,c,d}	-25%[2]	4.40 ± 0.08^{b,c}	0.99995 ± 0.00002
	18% *L. amylovorus* (DSM19280)	3437 ± 285^{d}	-35%[2]	4.42 ± 0.07^{b,c}	0.9992 ± 0.0007
	24% *L. amylovorus* (DSM19280)	2399 ± 382^{e}	-55%[2]	4.30 ± 0.10^{b,c}	0.9997 ± 0.0002
	6% L. amylovorus + 18% W. cibaria	3346 ± 311^{d}	-35%[2]	4.34 ± 0.06^{a,b,c}	0.9998 ± 0.0004

4.4.4 Bread analyses

The baked bread loaves were analysed measuring the specific loaf volume as well as the specific characteristics of the crumb grain structure; namely slice brightness, number of cells, porosity, wall thickness and cell volume. Salt reduction caused significant differences for crumb porosity, wall thickness and cell volume. This is in line with previous research published by Lynch et al. (2009) who could show a significant increase of porosity between 1.2% and 0.3% NaCl. The reduction of NaCl resulted to an increased yeast activity in bread dough during fermentation and hence, causing a more open and porous crumb structure (Cauvain, 2007). The increased wall thickness is based on a smaller number of larger cells surrounded by heavier cell walls (Czuchajowska et al., 1989).

The increasing addition of *W. cibaria* sourdough caused significant increases of loaf volume (Table 8). As little as the interaction of EPS, gluten and starch is understood, the positive impact of EPS on the specific bread volume has been proven in several research papers (Di Monaco et al., 2015, Wolter et al., 2014, Galle et al., 2010, Katina et al., 2009). On the contrary, the *L. amylovorus* sourdough showed the optimum addition level at 18%

which translates to about 6 mmol of lactic acid per 100g of bread dough. Addition levels of 6% (ca. 2 mmol lactic acid per 100g) and 12% (ca. 4 mmol of lactic acid per 100g) did not influence the volume significantly and the reduction of loaf volume for the highest addition level of 24% (ca. 9 mmol of lactic acid per 100g) can be traced back to the high amount of acid in the bread dough. The type of acids and the acid concentration are the determining factors whether the impact on bread quality is beneficial or contra-productive (Wehrle et al., 1997). This effect is based on the technological impact of acids on the baking process (Arendt et al., 2007, Moore et al., 2008). While small amounts of acid are known to improve bread quality characteristics such as specific volume, higher additions of sourdough and therefore, higher acid concentrations, reduce the specific volume but improve freshness and shelf-life (Komlenic et al., 2010, Rozylo et al., 2015a, Rozylo et al., 2015b). As the *W. cibaria* sourdough is only a low acidified sourdough, the acid addition had for all addition levels positive impact on the loaf volume in combination with the known beneficial impact of EPS on yeasted wheat bread (Wolter et al., 2014, Galle et al., 2010, Katina et al., 2009).

The total numbers of cells per slice did not differ between the samples. Only for the highest addition level of *L. amylovorus* sourdough the cell number was significantly higher, with an average of 4142 ± 118 cells per slice. The fact that this sample had the smallest loaf volume but also the highest number of cells resulted, with 7.9 mm^3 in the smallest cell volume of all the samples as well as the thinnest cell walls. While the loaf volume increased for increasing amounts of *W. cibaria* sourdough porosity, wall thickness and cell volume also increased (Table 8).

The combination of both sourdoughs at 6% of *L. amylovorus* and 18% of *W. cibaria* resulted in a loaf volume and bread crumb characteristics which were not significantly different to the sample containing 18% *W. cibaria* sourdough. The low addition level of 6% *L. amylovorus* sourdough did not impact on the loaf volume or the crumb quality as previously determined for the same addition level to the low-salt control.

Table 8: Specific bread loaf volume and bread crumb characteristics. Values followed by a different letter are significantly different ($p < 0.05$).

NaCl conc.	Dough samples	Specific bread volume [ml/g]	Number of cells	Porosity (area of cells) [%]	Wall Thickness [mm]	Cell Volume [mm^3]
1.04%	Standard salt control	2.7 ± 0.2^{a}	3620 ± 148^{a}	53.8 ± 0.4^{a}	$0.466 \pm 0.006^{a,b}$	$8.2 \pm 0.5^{a,b}$
0.26%	Low-salt control	$3.0 \pm 0.3^{a,b}$	3564 ± 103^{a}	$54.8 \pm 0.3^{b,c}$	$0.488 \pm 0.005^{c,d}$	$9.5 \pm 0.4^{c,d}$
	6% *W. cibaria* MG1	$3.1 \pm 0.2^{b,d}$	3827 ± 158^{a}	$54.9 \pm 0.5^{b,c,d}$	$0.477 \pm 0.008^{b,c}$	$8.9 \pm 0.4^{a,b,c}$
	12% *W. cibaria* MG1	$3.1 \pm 0.2^{b,d}$	3831 ± 99^{a}	$54.9 \pm 0.3^{c,d,e}$	$0.482 \pm 0.006^{c,d,e}$	$9.1 \pm 0.3^{c,d,e}$
	18% *W. cibaria* MG1	3.4 ± 0.2^{c}	3703 ± 80^{a}	$55.9 \pm 0.4^{e,f}$	$0.499 \pm 0.005^{d,e}$	$10.2 \pm 0.5^{d,e}$
	24% *W. cibaria* MG1	4.1 ± 0.1^{d}	3712 ± 90^{a}	56.8 ± 0.3^{f}	0.505 ± 0.004^{e}	10.7 ± 0.3^{e}
	6% *L. amylovorus* (DSM19280)	3.0 ± 0.3^{b}	3378 ± 118^{a}	$55.0 \pm 0.6^{a,b,c,d,e}$	$0.493 \pm 0.007^{c,d,e}$	$10.2 \pm 0.8^{b,c,d,e}$
	12% *L. amylovorus* (DSM19280)	$3.0 \pm 0.2^{a,b}$	3556 ± 76^{a}	$55.5 \pm 0.3^{c,d,e}$	$0.498 \pm 0.007^{d,e}$	$10.4 \pm 0.5^{d,e}$
	18% *L. amylovorus* (DSM19280)	3.1 ± 0.3^{d}	3686 ± 102^{a}	$55.9 \pm 0.3^{d,e}$	$0.491 \pm 0.008^{c,d,e}$	$10.0 \pm 0.8^{c,d,e}$
	24% *L. amylovorus* (DSM19280)	2.8 ± 0.0^{a}	4142 ± 194^{b}	$54.1 \pm 0.8^{a,b}$	0.459 ± 0.011^{a}	7.9 ± 0.8^{a}
	6% L. amylovorus + 18% W. cibaria	3.4 ± 0.3^{c}	3642 ± 174^{a}	$55.1 \pm 0.3^{c,d,e,f}$	$0.497 \pm 0.006^{d,e}$	$10.0 \pm 0.3^{c,d,e}$

Crumb hardness was measured over a storage period of five days. NaCl reduction resulted in a softer crumb after two and five days of storage but no difference could be detected on the baking day (Figure 8). Increasing amounts of *W. cibaria* sourdough led to a softer bread crumb. For the addition of 24% *W. cibaria* sourdough (= 11 mg EPS per 100g bread dough) the bread after 5 days storage was as soft as the control breads on the baking day. Similar effects were reported by Wolter et al. (2014) and Galle et al. (2012) who could show a significant increase of crumb softness as well as significantly reduced staling rates in breads made out of different flours containing 20% of the *W. cibaria* sourdough. The addition of *L. amylovorus* sourdough increased the crumb hardness only for the maximum addition level of 24% having the highest staling rate of all breads. The low-salt control and the *L. amylovorus* sample at 6% addition level showed no difference in staling over the five days of storage. With increasing addition levels of *W. cibaria* sourdough the staling was delayed and the bread crumb became softer. The *W. cibaria* sample with 24% sourdough resulted in the softest crumb followed by the sample with an additional level of 18% sourdough which had also the optimal specific bread volume. The delayed staling and softer crumb was most likely based on the retarded starch crystallisation by EPS and not by the increased loaf volume (Davidou et al., 1996, Galle et al., 2012). For the combination

of both sourdoughs the crumb hardness and staling behaviour were not significantly different to the *W. cibaria* samples with addition level of 12% and 18%. The small amount of organic acid added with the *L. amylovorus* sourdough could be compensated with the presence of EPS and hence, has no negative influence on crumb hardness.

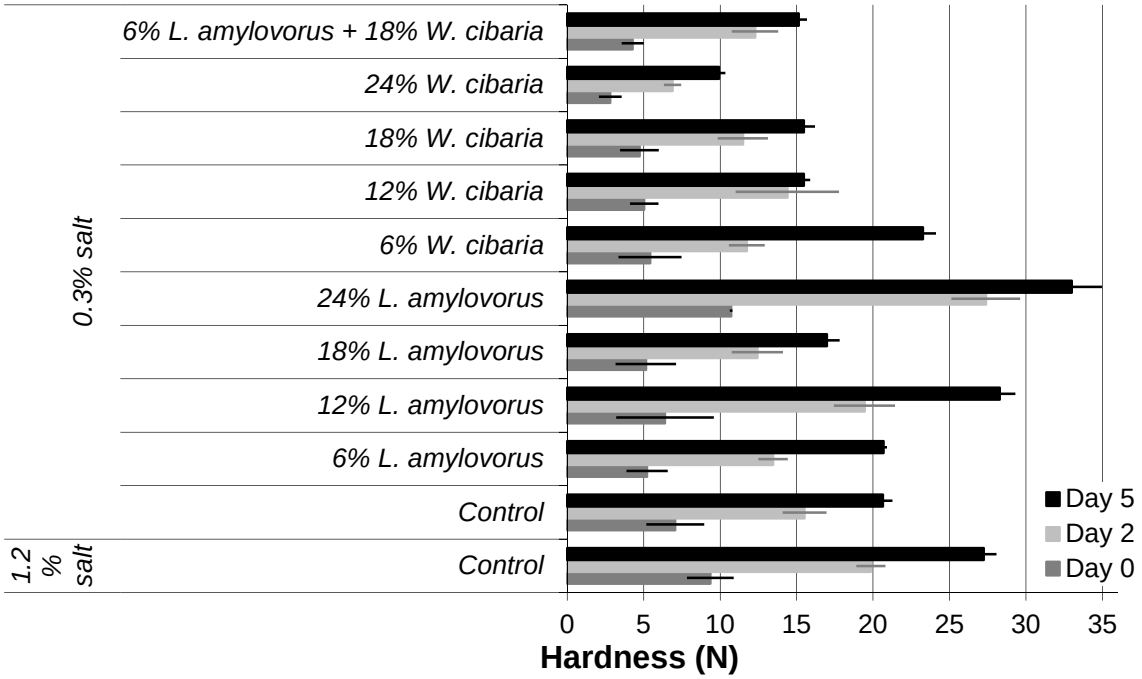


Figure 8: Hardness of bread crumb on day 0, 2 and 5 after baking. Control breads were analysed as well as sourdough bread containing *W. cibaria* or *L. amylovorus* at concentrations of 6, 12, 18 and 24% and the combination of both sourdoughs at optimum levels.

4.4.5 Microbial shelf-life and water activity

The microbial shelf life of all bread samples was assessed exposing the different samples of bread slices to the bakery environment for a defined time of 10 min. The reduction of NaCl in bread without sourdough addition from standard salt to low-salt resulted in a reduced shelf life of about two days as previously reported (Belz et al., 2012, Samapundo et al., 2010). The loss of shelf life of two days based on NaCl reduction could be compensated with the addition of 6% sourdough containing the antifungal strain *L. amylovorus* DSM19280 to low-salt bread. Each additional 6% of sourdough extended the shelf life by a further two days resulting in approx. 13 days for 24% sourdough addition.

On the contrary, the addition of sourdough fermented with *W. cibaria* MG1 did not result in a significant change of the shelf life for low-salt breads (Figure 9). These results correlate with the fact that the *W. cibaria* bacteria are a low acidifying strain and an insignificant amount of antifungal acid compounds could be detected (Figure 7). The combination of both sourdoughs resulted in a shelf life of 6-7 days which was based on the antifungal effect of the addition of 6% *L. amylovorus* sourdough. The additional 18% *W. cibaria* sourdough had no influence on the bread shelf life.

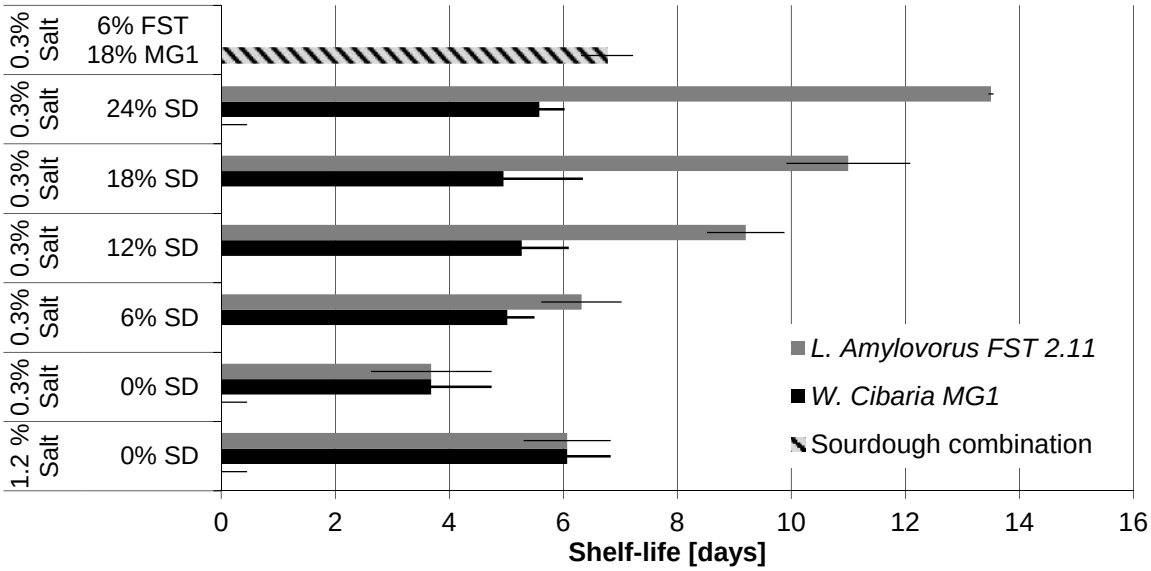


Figure 9: Microbial shelf-life of bread slices exposed to the bakery environment. The following bread samples were considered: standard salt and low-salt breads without SD, sourdough breads at low-salt level with sourdough addition of 6%, 12%, 18% and 24% for both sourdough; 6% *L. amylovorus* (DSM19280) and 18% *W. cibaria* MG1.

The water activity of the bread crumb samples was measured and resulted in a direct correlation to the added amounts of sourdough (Figure 10). The sourdough fermented with *L. amylovorus* DSM19280 lead to a lower a_w compared to *W. cibaria* but only at the highest addition level of 24% sourdough the a_w was significantly different with $a_w = 0.982 \pm 0.002$ for *W. cibaria* MG1 compared to 0.979 ± 0.002 for *L. amylovorus* DSM 19280. The water binding nature of EPS resulted in a higher water activity for the bread samples containing *W. cibaria* MG1. None of the sourdough breads with 0.3% NaCl reached the water activity

of the standard salt bread of $a_w = 0.974 \pm 0.002$. The fact that the water activity did not correlate with the determined shelf life can be explained with the antifungal compounds found in the sourdough fermented with the *L. amylovorus* DSM19280 (Figure 7).

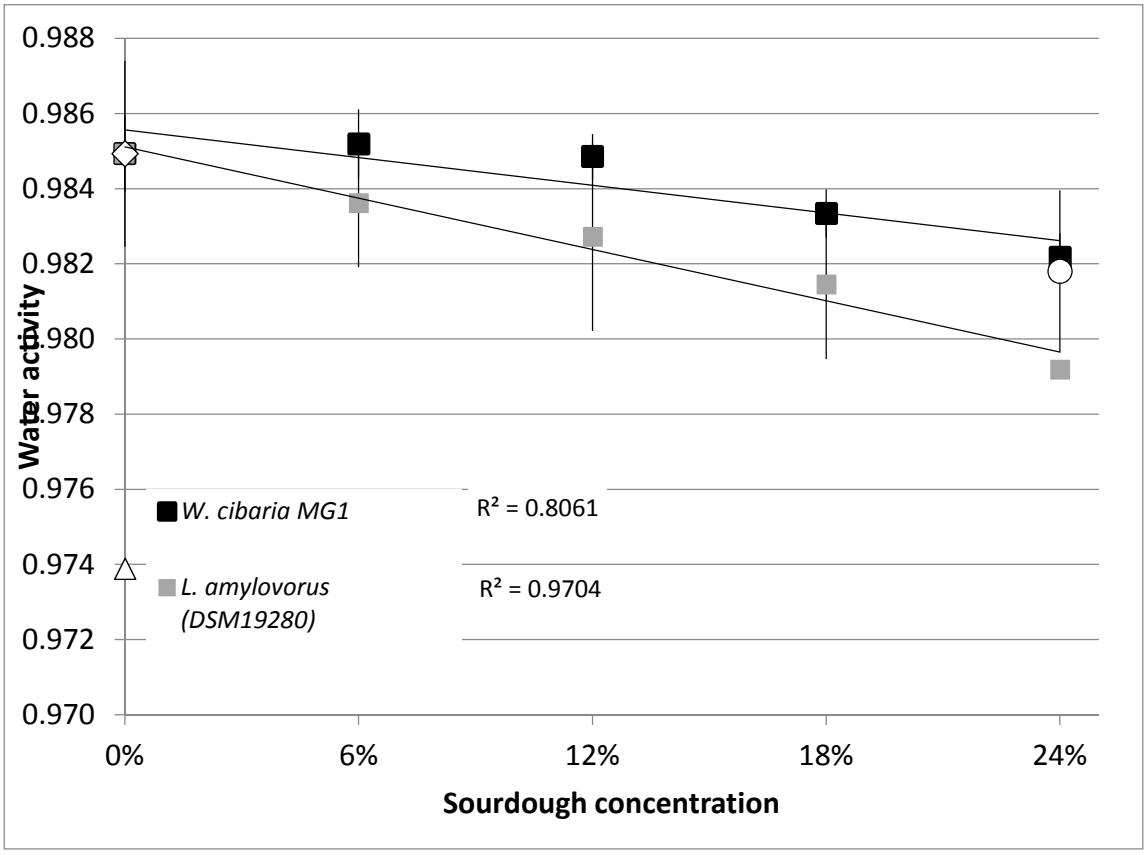


Figure 10: Water activity of control breads at standard salt (filled circle) and low-salt levels (empty square), *L. amylovorus* breads (grey squares) and *W. cibaria* breads (black squares) at low-salt level.

4.4.6 Sensory evaluation

The set of breads selected for the descriptive sensory analysis section of the research was based on the best performance of the quality characteristics of the bread loaves. For the samples containing *L. amylovorus*, a level of 6% was chosen, based on the shelf life of 6 days for the low salt level of 0.3% NaCl. The samples containing *W. cibaria* resulted in the best quality loaves with respect to volume and crumb structure at an addition of 18%, and was also chosen for the descriptive sensory analyses. While a level of 24% additional sourdough resulted in an even higher bread loaf volume, the crumb texture of this loaf was too porous and open to be chosen. Furthermore, for comparison reasons three more control

samples were added without any NaCl. Table 9 shows the average panel scores for the sensory characteristics measured. The assessors were able to differentiate between the samples for all attributes measured. Increasing amounts of NaCl let to a higher bread crumb density independent of the addition of sourdough. This was found for both appearance and texture density. Based on the inhibition of yeast activity by NaCl the increased density was caused by a reduced gas production by the baker's yeast during proofing (Lynch et al., 2009). For the olfactory attributes "roasted" and "sourdough" no significant impact could be determined. The aroma note "cheesy" was significantly more intense adding the *L. amylovorus* sourdough. A reduced NaCl concentration resulted in an additional significantly higher cheesy aroma. The PCA generated from all the attributes measured was used to summarize the relationship between the samples and these attributes, allowing these to be visualized easily. PC1 accounted for 67% of the determined variances and it distinguished the samples mainly between a "yeasty" flavour and odour on the one side and a "salty" and musty/stale flavour on the other side. PC2 separated the samples mainly between "sour" and "bitter" flavours which was not relevant for the presented work. Across PC3 which accounts for 8% the samples were split between a "cheesy, sourdough" like flavour and a "salty" flavour. The PCA (Figure 11) was generated using PC1 and PC3. Hence, most samples with a NaCl concentration of 1.2% can be found in the right bottom quadrant. The one exception is the sample also containing 6% of the *L. amylovorus* sourdough, which significantly showed a cheesy/sourdough like note independent of the NaCl concentration (Table 9). The samples were distinguished across the PC3 (Figure 11) between "cheesy" and "salty" which shows that both attributes compete against each other. The yeasty attributes were influenced mostly by the NaCl concentration resulting in an increased yeast perception for reducing amounts of NaCl. This correlates again with the descript yeast activity being influenced by NaCl. The reverse effect was determined for the doughy attributes. The higher the NaCl concentration the higher the doughy perception. Salt is known to enhance other flavours (Kare, 2012, Gillette, 1985) resulting in a higher recognition of the doughy compounds originating from the wheat flour in combination with water and yeast. Independent of the use of sourdough, no difference of the perception of salt was recognised between 0.0% and 0.3% NaCl but significantly different for the standard amount of 1.2%. For the omission of salt the assessors determined a significant increase of bitterness. As previously reported sodium suppresses bitterness (Breslin and

Beauchamp, 1995b, Breslin and Beauchamp, 1995a) enhancing other flavours (Breslin and Beauchamp, 1997). However, the addition of sourdough avoided an increase of bitterness.

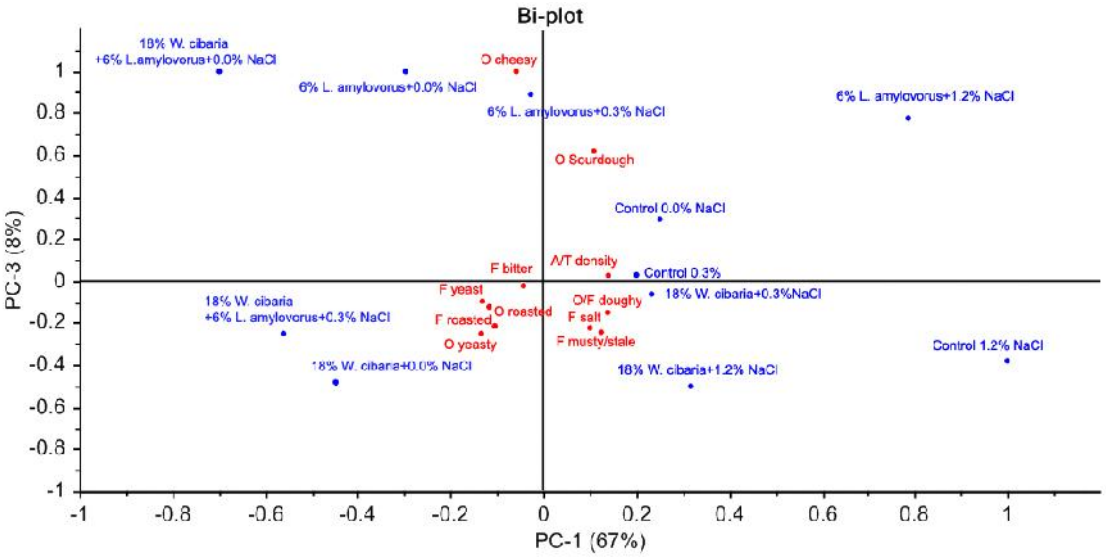


Figure 11: Principle component analysis of sensory attributes of bread samples containing different amounts of NaCl (1.2%; 0.3%; 0.0%) and different sourdoughs (6% sourdough fermented with *L. amylovorus* and 18% sourdough fermented with *W. cibaria*). O = odour; F = flavour; A = appearance; T = texture.

Hence, all samples containing *L. amylovorus* sourdough are located in the upper half showing the typical cheesy note of this particular sourdough. The attributes “density” correlate with the determined specific volumes of the bread loaves. The addition of sourdough fermented with *W. cibaria* increased the volume significantly. As previously reported, a more porous crumb with lower density leads to a more intense recognition of salt and possibly of other flavours too (Pflaum et al., 2013, Konitzer et al., 2013). Both NaCl containing samples with 18% *W cibaria* sourdough (1.2% and 0.3% NaCl) were described as slightly saltier than the respective control samples with 1.2% and 0.3% NaCl. Hence, the increased loaf volume, caused by the added EPS with the *W. cibaria* sourdough, resulted in a coarser crumb texture and thus, the salt perception was more intense for the assessors compared to the control breads. In addition, the lactic and acetic acids enhanced the saltiness perception of the bread crumb (Reddy and Marth, 1991). The combination of both sourdoughs led for 0.0% NaCl to the sample described as most “yeasty” and “cheesy”. The increased loaf volume enhanced the cheesy flavour of the *L. amylovorus* sourdough

and increased the perception. The addition of 0.3% NaCl changed the flavour profile significantly away from the cheesy flavour towards a more salty one.

Table 9: Mean panel description sensory score for the attributes determined by the descriptive sensory categorised into the four main sensory responses: appearance (A), texture (T), olfactory (O) and flavour (F). Values followed by a different letter are significantly different ($p < 0.05$).

	Appearance	**Texture Response**	**Olfactory Responses**					**Flavour Responses**					
Product	**A Density**	**T Density**	**O Roasted**	**O Sourdough**	**O Cheesy**	**O Doughy**	**O Yeasty**	**F Yeast**	**F Doughy**	**F Musty/Stale**	**F Roasted**	**F Salt**	**F Bitter**
Control 0.0% NaCl	60.5 e f	48.7 e,f	4.4 a,b	13.9 b,c	0.00 a	31.3 b,c,d	26.8 a,b	29.0 a,b,c,d	28.2 a,b,c	8.5 a,b,c	0.1 a	5.6 a	2.6 b,c
Control 0.3% NaCl	59.6 e f	45.5 d,e,f	4.9 a,b	9.7 a,b	0.75 a	28.6 a,b,c,d	29.9 a,b,c	26.5 a,b,c,d	33.1 c,d	6.6 a,b,c	0.3 a	7.6 a	1.8 a,b,c
Control 1.2% NaCl	83.8 f	70.7 g	2.3 a,b	12.0 a,b	0.04 a	45.4 e	21.3 a	21.6 a	44.4 e	18.2 d	0.2 a	58.4 b	1.4 a,b
6% *L. amylovorus* 0.0% NaCl	44.3 a b c d e	35.3 b,c,d,e	16.6 b,c	7.7 a,b	15.88 c	16.1 a	29.9 a,b,c	28.8 a,b,c,d	23.0 a,b	6.1 a,b,c	3.4 a,b,c	4.1 a	0.5 a
6% *L. amylovorus* 0.3% NaCl	58.9 e,f	45.3 d,e,f	17.1 b,c	9.4 a,b	7.80 b	21.3 b,c	28.1 a,b,c	24.5 a,b	27.6 a,b,c	6.9 a,b,c	3.3 a,b,c	9.3 a	2.0 b,c
6% *L. amylovorus* 1.2% NaCl	68.7 e f	59.8 f,g	1.3 a,b	19.7 c	5.06 b	38.1 d,e	21.1 a	23.7 a,b	39.2 d,e	11.4 c	0.2 a	63.8 b	1.4 a,b
18% *W. cibaria* 0% NaCl	26.0 a b	25.0 a,b,c	11.7 a,b,c	6.1 a	0.03 a	16.1 a	41.0 d,e	36.4 d,e	28.0 a,b,c	6.3 a,b,c	1.2 a,b	5.2 a	3.1 c
18% *W. cibaria* 0.3% NaCl	52.0 c d e	45.1 d,e,f	4.5 a,b	11.0 a,b	0.03 a	31.9 c,d,e	29.6 a,b,c	30.1 a,b,c,d,e	30.7 a,b,c,d	10.7 b,c	0.2 a	12.5 a	1.1 a,b
18% *W. cibaria* 1.2% NaCl	57.6 d e	51.9 e,f	5.9 a,b	9.7 a,b	0.00 a	33.2 c,d,e	33.2 b,c,d,e	30.2 a,b,c,d,e	32.3 b,c,d	11.5 c	1.2 a,b	64.2 b	1.3 a,b
18% *W. cibaria* +6% *L. amylovorus* 0.0% NaCl	18.6 a	15.9 a	16.7 b,c	10.6 a,b	15.16 c	16.0 a	44.0 e	38.7 e	21.9 a	3.3 a	1.5 a,b,c	3.6 a	3.2 c
18% *W. cibaria* +6% *L. amylovorus* 0.3% NaCl	33.8 a b c d	28.1 a,b,c,d	33.5 d	6.1 a	7.34 b	19.3 a,b,c	42.1 d,e	34.6 c,d,e	28.5 a,b,c	6.3 a,b,c	4.0 b,c	12.3 a	1.9 b,c
P Value	0.0012	0.0002	0.0013	0.0293	0.0000	0.0028	0.0025	0.0123	0.0029	0.0043	0.0398	0.0000	0.0370

4.5 Conclusion

The beneficial impact of sourdough addition on low-salt bread was investigated using two functional lactic acid bacteria strains; the antifungal strain *L. amylovorus* DSM19280 and the EPS producing strain *W. cibaria* MG1. The use of *L. amylovorus* DSM19280 had previously been shown to prolong the microbial shelf life significantly (Axel et al., 2015, Belz et al., 2012). The present study determined the minimum amount of 6% sourdough addition to compensate the reduced shelf life caused by the salt reduction. The antifungal effect was mainly based on the determined antifungal compounds 3-phenyllactic acid, 4-hydroxyphenyllactic acid, leucic acid and azelaic acid. The shelf life of a low salt bread of about 4 days could be prolonged to the same shelf life of a standard salt bread of about 6 days. The EPS producing strain *W. cibaria* MG1 was found to increase the bread volume and the bread crumb porosity as well as delaying the bread staling significantly with an optimum addition level of 18%. The fundamental rheology analyses showed a significant dough softening with increasing acidification due to sourdough addition. Enhanced enzymatic activity due to a lowered pH during the fermentation process caused partial starch degradation as well as the weakening of the gluten network by increased protease activity. Hence, the use of sourdough will change the dough handling, and processing may need to be adapted to a softer dough. The descriptive sensory analyses distinguished mainly between a more “roasted” and “yeasty” versus a more “salty” and “dough” perception. The samples at no/lower NaCl levels and the more acidified samples mainly based on the addition of *L. amylovorus* sourdough tended to have a more “yeasty” and “roasted” profile whereas the standard salt levels as well as the control breads and the low acidified samples mainly based on the addition of *W. cibaria* resulted in a more “salty” and “dough” perception. The combination of both sourdoughs lead to a low-salt bread with improved quality characteristics. The beneficial characteristics of both functional lactic acid bacteria strains could be combined resulting in an improved shelf life, a softer bread crumb, increased bread volume and an improved sensory profile.

The presented work could demonstrate that the use of functional sourdoughs can compensate the amount of salt reduced in low-salt bread. High product quality for low-salt bread can be achieved in a natural way without any additives, matching standard salt bread closely. Considering reports about adaptation of the saltiness perception based on a gradual

reduction of salt in food, the achieved result would be a natural alternative for the long-term goal of a low-salt bread.

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Chapter 5

The multifunctional strain *L. reuteri* FF2hh2 and its beneficial use for yeasted low-salt bread

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5.1 Abstract

The intake of sodium has been related to cardiovascular diseases influencing public health significantly around the world. Thus, the overall reduction of the daily sodium intake has been made a global target. Bread contributes a significant amount of the daily sodium intake which is the reason for many national and international initiatives to reduce salt (sodium chloride) in bread. The application of sourdough is a natural way to compensate the lack of sodium chloride. The lactic acid bacteria strain *Lactobacillus reuteri* FF2hh2, isolated from a porcine source, is the first multifunctional strain which has been described. Beside a determined antifungal activity against *Fusarium culmorum* and *Penicillium roqueforti*, the *L. reuteri* strain FF2hh2 produced also a significant amount of exopolysaccharides, which have been characterised the first time for this strain as a high molecular dextran. The use of sourdough fermented with *L. reuteri* FF2hh2 improved the quality characteristics of low salt bread. Loaf volume, bread crumb and shelf life improved significantly. The antifungal compounds have been identified for the first time in wheat sourdough fermented with this strain and could be related to the extended bread shelf life measured, when sourdough was added at 6 – 18% to compensate for the lack of sodium chloride. A descriptive sensory study evaluated the impact of sourdough addition in low salt bread. The addition of sourdough enhanced the salty perception significantly. The results showed that a low-salt bread at 0.3% NaCl can be significantly improved in a natural way by the addition of sourdough fermented with *L. reuteri* FF2hh2 regarding all quality characteristics.

Keywords: *L. reuteri* FF2hh2, Antifungal, Exopolysaccharide, Low-Salt Bread, Salt Reduction, Functional Sourdough, Descriptive Sensory

5.2 Introduction

The excessive intake of sodium of the populations in developed countries is linked to several health related issues. In particular, cardiovascular diseases were determined as the most concerning impact on human health as well as the most expensive impact on the world economy (Farquhar et al., 2015, O'Donnell et al., 2015, Brown et al., 2009, Angus, 2007, Stamler, 1997, Reddy and Marth, 1991, Freis, 1976). Hence, salt reduction of the average daily Western diet has been targeted in all industrialised countries. Global organisations (WHO and FAO, 2003) as well as national authorities (German Nutrition Society (DGE), 2009, European Commission, 2008, Korean Food and Drug Administration, 2007, FSAI, 2005, SACN, 2003) have put salt reduction on their agenda. The intake of sodium increased mainly because of the increase in processed food being consumed (Reddy and Marth, 1991, Gibson et al., 2000, SACN, 2003). Bread as a staple food is in the main focus because it contributes up to 40 % of the dietary sodium depending on the country and the respective culinary tradition (Thomson, 2009, Cauvain, 2007, Angus, 2007). The reduction of salt in bread and other baked goods has become the main focus, along with other types of food (Belz et al., 2012b, Brown et al., 2009, EU Salt, 2008, He and MacGregor, 2003, Gibson et al., 2000). The health related necessity of salt reduction in bread has caused several challenges for bakers and the bread industry. Several bread characteristics are impacted, such as taste profile (Lynch et al., 2009), crumb structure (Beck et al., 2012c) and shelf-life (Belz et al., 2012a, Samapundo et al., 2010, Pateras, 2007, Filtenborg et al., 1996). Breads with reduced amounts of NaCl were described as more sour/acidic and yeasty (Lynch et al., 2009). Beck et al. (2012b) reported significant changes in crumb structure based on different amounts of NaCl. Several approaches have been published to overcome the impact of salt reduction either with special technologies such as the inhomogeneous distribution of NaCl (Noort et al., 2012, Noort et al., 2010) and usage of sourdough (Belz et al., 2012a) or the partial replacement of NaCl with other salts such as potassium chloride, magnesium sulfate or calcium chloride (Charlton et al., 2007, Samapundo et al., 2010).

The present work focused on the use of sourdough fermented with the lactic acid bacteria strain *L. reuteri* FF2hh2 to compensate for the reduction of NaCl in yeasted wheat bread. As part of the research published by Moroni et al. (2011), the strain *L. reuteri* FF2hh2 was isolated form a porcine surce. The two functional strains *W. cibaria* MG1 and *L. amylovorus* (DSM19280) had been used together (Chapter 4) combining both

functionalities; antifungal activity and production of EPS. The aim was to evaluate if the *L. reuteri* FF2hh2 strain with its multifunctional characteristics could compensate for the lack of sodium chloride in low-salt bread. The impact of the sourdough on the dough and bread quality was analysed. Rheological analyses were performed to determine changes to the dough samples based on sourdough addition. The changes of the different loaf characteristics were evaluated to optimise the quality of low-salt bread.

5.3 Materials and Methods

5.3.1 Bacteria and growth conditions

The strain *L. reuteri* FF2hh2 is a multifunctional strain which is known to produce exopolysaccharide (EPS) as well as antifungal compounds. The strain was previously isolated from a porcine source. The bacteria were grown on MRS5 (Meroth et al., 2003) agar plates for 24 h at 37 °C and a single colony was transferred into MRS5 broth for about 16 h at 30 °C under anaerobic conditions.

5.3.2 Antifungal plate assays

The antifungal activity of *L. reuteri* FF2hh2 against the spoilage moulds *Penicillium roqueforti* and *Fusarium culmorum* was investigated using the spraying method described by Dal Bello et al. (2007). The screening was performed on MRS 4 agar plates which were buffered to pH 6.5 using a 75 mmol KH_2PO solution. Cell spots of *L. reuteri* FF2hh2 were placed on the plates and incubated at 37 °C for 48 h in anaerobic jars. To investigate antifungal activity, 1 ml fungal spore solution (approx.10^4 spores/ml) was sprayed by nebulisation on the surface of plates. Plates were then incubated at room temperature for 3 days. The inhibitory activity was scored as follows: -, no inhibition; +, very weak inhibition around the colonies; ++, low inhibition with little clear zones around the colonies; +++, strong inhibition with detectable zones around the colonies; ++++, very strong inhibition with large clear zones and nearly no growth around the colonies.

5.3.3 Sourdough fermentation

For the sourdough fermentation, a pre-culture of the strain *L. reuterii* FF2hh2 was subcultured in 40 ml of MRS5 broth and incubated for 24 h at 30 °C resulting in a cell

suspension containing approximately 5×10^9 CFU/ml. Cells were harvested by centrifugation at 3000 g for 10 min, washed twice with Ringer's solution and re-suspended in 40 ml Ringer's solution. Sourdough was prepared with a dough yield of 200 and an inoculation of about 10^7 CFU/g of sourdough using 540 g of wheat flour, 60g of sucrose, 560 ml of sterile distilled water and 40 ml of cellular suspension. After mixing with a Kenwood mixer (Kenwood KM020) using the batter attachment for 1 min at speed 1, the dough was covered and fermented at 37 °C for 48 h. At the end of fermentation, lactic acid bacteria cell counts were determined on MRS5 agar plates and the total titratable acid (TTA) and pH values were determined according to Arbeitsgemeinschaft Getreideforschung e.V. (AGF, 1994). The *L. reuteri* FF2hh2 sourdough samples showed values for TTA = 15.9 ± 0.1 ml and pH = 3.95 ± 0.01. The determined cell counts resulted in 5×10^8 CFU/g. These parameters were used as the "stability" parameter of sourdough fermentation.

5.3.4 Baking procedure and loaf analysis

Wheat bread was prepared by mixing Baker's flour (Odlums, Ireland), distilled water (water level set to 64.7% (flour weight) using a Brabender farinograph), dry baker's yeast (Puratos Group, Belgium) and NaCl at levels of 1.04, and 0.26 % (w/w) with a spiral mixer (Kenwood KM020). Considering an average bakeloss of 13.5% the NaCl concentrations in the final bread loaves resulted in 1.2 and 0.3%. Sourdough levels of 6, 12, 18, 24% were added to different dough. After a bulk fermentation of 15 min at 30 °C and 85 % relative humidity (Koma Popular, Koma, Roermond, The Netherlands) 450 ± 1 g bread loaves were moulded with a moulding machine (Machinefabriek Holtkamp B.V., Almelo, Holland) and put into tins. The loaves were proofed for 75 min. under the same conditions used during bulk fermentation. Subsequently, the breads were baked for 35 min at 230 °C top and bottom heat in a deck oven (MIWE condo, Arnstein, Germany). Ovens were pre-steamed (0.3 L) and steamed when loaded (0.7 L). Loaves were depanned cooled for 120 min. at room temperature. The breads were baked with the different addition levels of sourdough and NaCl as shown in Table 10. The specific loaf volume was measured using a Volscan Profiler (Stable Micro Systems, UK). A TA-XT2i texture analyser (Stable Micro Systems, Surrey, UK) equipped with a 25 kg loading cell and a cylindrical aluminium probe (diameter of 35 mm) was used to analyse the texture profile of the bread crumbs.

Crumb grain was described by the following parameters: slice brightness, number of cells, porosity expressed as the area of cells (the total area of cells as a percentage of the total slice area), wall thickness (the average thickness of cell walls) and the average cell volume, using a C-cell bread imaging system (Calibre Control International Ltd., UK).

Table 10: Overview of the prepared bread recipes

	Control breads (no sourdough)						Sourdough breads (low-salt)											
	Standard salt			Low-salt			6 % Sourdough			12 % Sourdough			18 % Sourdough			24 % Sourdough		
	% FW*	% DW**	g	% FW*	% DW**	G	% FW*	% DW**	g	% FW*	% DW**	g	% FW*	% DW**	g	% FW*	% DW**	g
Baker's flour	100.0	59.5	1785	100.0	60.0	1799	95.0	57.0	1710	90.0	54.0	1619	85.0	51.0	1529	80.0	48.0	1439
Baker's yeast	2.0	1.2	36	2.0	1.2	36	2.0	1.2	36	2.0	1.2	36	2.0	1.2	36	2.0	1.2	36
Water	64.3	38.2	1147	64.3	38.5	1156	59.3	35.6	1067	54.3	32.5	976	49.3	29.6	887	44.3	26.6	797
NaCl	1.75	1.04	31.2	0.44	0.26	7.9	0.44	0.26	7.9	0.44	0.26	7.9	0.44	0.26	7.9	0.44	0.26	7.9
Sourdough	0.0	0.0	0	0.0	0.0	0	10.0	6.0	180	20.0	12.0	360	30.0	18.0	540	40.0	24.0	720
Total amount	168.0	100.0	3000	166.7	100.0	3000	166.7	100.0	3000	166.7	100.0	3000	166.7	100.0	3000	166.7	100.0	3000

* Flour weight ** Dough weight

5.3.5 Analyses of organic acids

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 acid and sugar analyses freeze-dried sourdough samples were extracted with water and proteins were precipitated with Carrez solutions. After centrifugation (2000 x *g*, 20 min) and filtration (0.2 µm), 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 mm, Agilent, Cork, Ireland), equipped with a guard column (50 x 7.7 mm, 8 mm, 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 ml.

5.3.6 Analyses of antifungal compounds

The sample preparation was performed as described by Brosnan (2015). Sourdough samples were freeze-dried and ground 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. The 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 ^{o}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.

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 ^{o}C and the pump was operated at a flow rate of 0.6 mlmin. 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 acetonitrile. 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 the 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 MassLynx^{TM} software and data was processed using TargetLynx^{TM} 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 ^{o}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^{-1} – 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. A validation of the method was performed in compliance with the EC (EU, 2002) and ICH (ICH, 2005) guidelines taking into account specificity, linearity, limits of detection and quantitation, trueness and precision. The validation was completed by analysing standard concentrations (1 ppm, 5 ppm, 10 ppm, 15 ppm, 20 ppm, 25 ppm, 50 ppm) in triplicate on the first 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 the first 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).

5.3.7 Evaluation of microbial shelf-life and water activity

For the evaluation of the microbial shelf life, the “natural” bakery-environment was used for contamination. Breads were sliced and 10 slices of each type of bread were exposed to the bakery-environment for 10 min. Each of the bread slices were packaged in polyethylene bags and heat sealed. The sterile air exchange was enabled by inserting two filter tips in each storage bag. During storage the temperature was kept constant at 20 °C. The storage took place up to 14 days observing the appearance of mould spoilage every day. As soon as spoilage appeared on one of the bread slices, the previous day was determined as the microbial shelf-life of that bread (Belz et al., 2012a). Water activity was measured using a water activity meter AquaLab Series 4TE from Decagon Devices, Inc. The a_w-meter was calibrated using the verification standards (distilled water; 0.5 M KCl; 6.0 M NaCl; 8.57 M LiCl; 13.41 M LiCl) from Decagon (NE Hopkins Ct. Pullman, WA – USA) at 25 °C. For each of the five water activity levels the values were determined within the required range of 0.003 (Decagon Devices 2009). The bread samples were measured in triplicate on 3 independent baking batches 2 h after baking at 25 °C.

5.3.8 EPS analysis

EPS were detected with a refractive index detector and their molecular weight was estimated using two dextrans: low molecular weight (LM) dextran with a relative molecular weight ($M_r = 10^5 - 2 \times 10^5$) and high molecular weight (HMW) dextran ($M_r = 5 \times 10^6 - 4 \times 10^7$). Inulin from chicory ($M_r = 10^4$) was used for calibration (all were obtained from Sigma, Oakville, Canada). EPS size was determined by asymmetrical flow field-flow-fractionation (FFF) coupled to multi-angle light scattering (MALS) and a refractive index (RI) detector (Postnova, Salt Lake City, UT) as described by Galle et al. (2010 and 2011). The channel dimensions were 335 x 60x40 mm (Postnova, Salt Lake City, UT) with a molecular weight cut-off of 10 kDa. A polyetherketone pre-column filter unit placed between channel and detectors contained a 2 µm pore-size and a Teflon microfilter (0.1 µm poresize) of regenerated cellulose filter paper (all Postnova). The samples were injected onto the channel at a flow rate of 0.2 ml/min and a cross flow of 2 ml/min for 1 min. After injection, the cross flow rate of 2 ml/min decreased exponentially to 0.1 ml/ min over 30 min and was then maintained at 0.1 ml/min for 30 min. The molecular weight was measured by static light scattering data as processed by the AF 2000

software (Postnova, Salt Lake City, UT) with the RI signal as concentration detector. A dn/dc value of 0.147 was used for light scattering calculations of EPS. Polystyrolsulphonate standards (Postnova) and bovine serum albumin (Sigma, Aldrich) were used for calibration of detectors. Sugars were analysed with a Carbopac PA20 column (Dionex, Oakville, Canada) using water (A), 200 mM NaOH (B) and 1 M Na-acetate (C) as solvents at a flow rate of 0.25 mL/ min with the following gradient: 0 min. 30.4 % B, 1.3 % C, 22 min. 30.4 % B, 11.34 % C followed by washing and regeneration. Sucrose, glucose, fructose, maltose, panose, isomaltose, isomaltotriose (all obtained from Sigma, Oakville, Canada) and isomaltooligosaccharides (IMO, from Bionutra Inc., Edmonton, Canada) were used as external standards.

5.3.9 Fundamental Rheology

Freshly prepared dough pieces (14g) were sealed in airtight containers and allowed to rest for 10 min. before loading. Measurements were conducted using a controlled stress/strain rotational rheometer (MCR301 Anton Paar GmbH, Germany). Parallel serrated plates with a diameter of 25 mm were used set at a temperature of 30 °C. The measurements were performed at a final gap of 1.000 mm after a relaxation rest of 10 min (Addo et al., 2001). Fundamental rheological properties were determined within the linear viscoelastic range (LVR). Frequency sweeps were conducted in the range of 1-100 Hz at a constant strain of 0.01 %. The complex modulus (G*) data were obtained from the frequency sweeps and the weak gel model was applied using the equation $G^*(\omega)=A_F \cdot \omega^{1/z}$ according to Gabriele et al. (2001). All results are averages of two measurements of three independent samples.

5.3.10 Sensory

Descriptive sensory analysis was carried out by a panel of eight assessors (ISO, 2012). At the start of the study, the panel took part in the development of a descriptive vocabulary to compile a list of attributes associated with the breads. These terms were refined in subsequent sessions. Descriptive sensory analysis was carried out using a final vocabulary of 3 odour (“roasted”, “cheesy”, “doughy”), 1 appearance (“density”), 1 texture (“density”) and 4 flavour (doughy”, “musty/stale”, “roasted”, “salt”) terms which showed a level of significance of $p<0.05$. Ahead of the assessment, the assessors were provided with deionised water, a list of the defined vocabulary and instructed to cleanse their palate

between tastings. Breads were scored for attributes on unstructured 100 mm line scales labelled at both ends with extremes of each attribute. The intensity of each of the descriptive terms was recorded for each sample using the Compusense five ® V. 4.0 sensory data acquisition programme (Guelph, Ontario, Canada). The descriptive testing took place over a period of two days with a total of 11 breads analysed per day. The order of tasting was balanced to account for the order of presentation and the carry-over effects (MacFie et al., 1989). The descriptive analysis yielded duplicate data matrices consisting of 10 assessors by 9 sensory attributes for 11 breads. The performance of the assessors was checked using standard procedures prior to averaging results for the breads. The mean panel scores from the duplicate descriptive sensory analysis were then subjected to a one-way analysis of variance (ANOVA, SPSS v 14.0 SPSS Inc. Chicago, IL 60611, USA) to determine which terms were effective at providing discrimination amongst the breads at $p \leq 0.05$. Data were averaged across replicates, standardised (1/standard deviation) and analysed by means of principal component analysis (PCA) using Guideline +7.5 (CAMO ÅS, Trondheim, Norway). The way in which each principal component (PC) discriminated between all bread samples was determined by performing an ANOVA (SPSS v 14.0) on bread scores prior to averaging across replicates. The final number of components for interpretation was based on the discriminating ability ($p \leq 0.05$) of each PC and a visual inspection of explained validation variance (to indicate whether additional PCs were modelling information or noise).

5.3.11 Statistics

Statistical analyses were performed using MiniTab 16 for Windows computerised statistical analysis package (MiniTab Ltd., Coventry, UK). Data were examined using the one-way analysis of variance (ANOVA). Where an F-test showed significant differences ($p<0.05$), Fisher's least significant difference (LSD) test was used for multiple comparisons. Each result is the average of at least three separate experiments with three independent samples from each batch.

5.4 Results and Discussion

The antifungal activity of *L. reuteri* FF2hh2 was tested in vitro on MRS agar plates against *P. roqueforti* and *F. culmorum* (Figure 12). The antifungal challenge test against

P. roqueforti resulted in a very strong inhibition (++++) whereas the antifungal challenge test against *F. culmorum* resulted in a strong inhibition (+++). Several authors have proven for different *L. plantarum* strains that a significant antifungal effect does not originate from the organic acids but from antifungal compounds (Guo et al., 2012, Dal Bello et al., 2007).

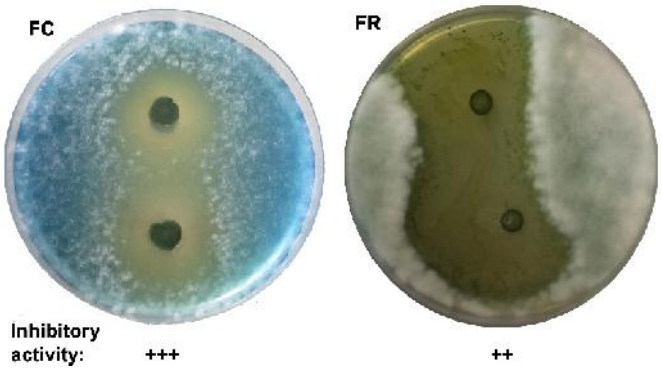


Figure 12: Antifungal effect of *L. reuteri* FF2hh2 (F) challenged against *P. roqueforti (R)* and *F. culmorum* (C) on MRS agar.

5.4.1 Sourdough metabolites analyses

The sourdough was prepared as described fermenting Baker's flour with the multifunctional lactic acid bacteria strain *L. reuteri* FF2hh2. The typical sourdough characteristics were determined – such as sugars and organic acids using HPLC analyses, total titratable acids (TTA) and pH. Furthermore, exopolysaccharides and antifungal compounds were determined based on the specific functionalities of the selected strain.

The unfermented wheat flour contained no single sugars or acids, 45.5 mmol sucrose and 48 mmol maltose per 1 kg baker's flour. With a TTA of 2.2 ml and a pH of 6.1 (Table 11) the flour had a standard quality as described by (Souci et al., 2000). The sourdough fermented with the heterofermentative *L. reuteri* FF2hh2 strain contained fructose as well as a significantly higher amount of glucose. *L. reuteri* strains are known for their β–glucosidase activity, which is responsible for the high concentration of glucose (Hayek et al., 2013). The added sucrose was completely metabolised favouring the exopolysaccharide (EPS) production in the same way as described by Galle et al. (2010) for different *Weisella* strains. The presence of maltose, initially in the flour and during the fermentation process released by the amylase activity, favoured the metabolism of sucrose as previously described for *Weisella cibaria* strains resulting in an increased EPS production (Galle et

al., 2010). The EPS consisted mainly of high molecular dextran and minor amounts of low molecular dextran as shown in Figure 13. The acidification level was high resulting in a TTA = 15.9 ml, pH = 3.95 based on the concentrations of acetic acid (138 mmol kg^{-1} flour) and lactic acid (155 mmol kg^{-1} flour). The analyses of the sourdough for exopolysaccharides (EPS) using a size exclusion chromatography resulted in 5.2 ± 0.7g kg^{-1} sourdough

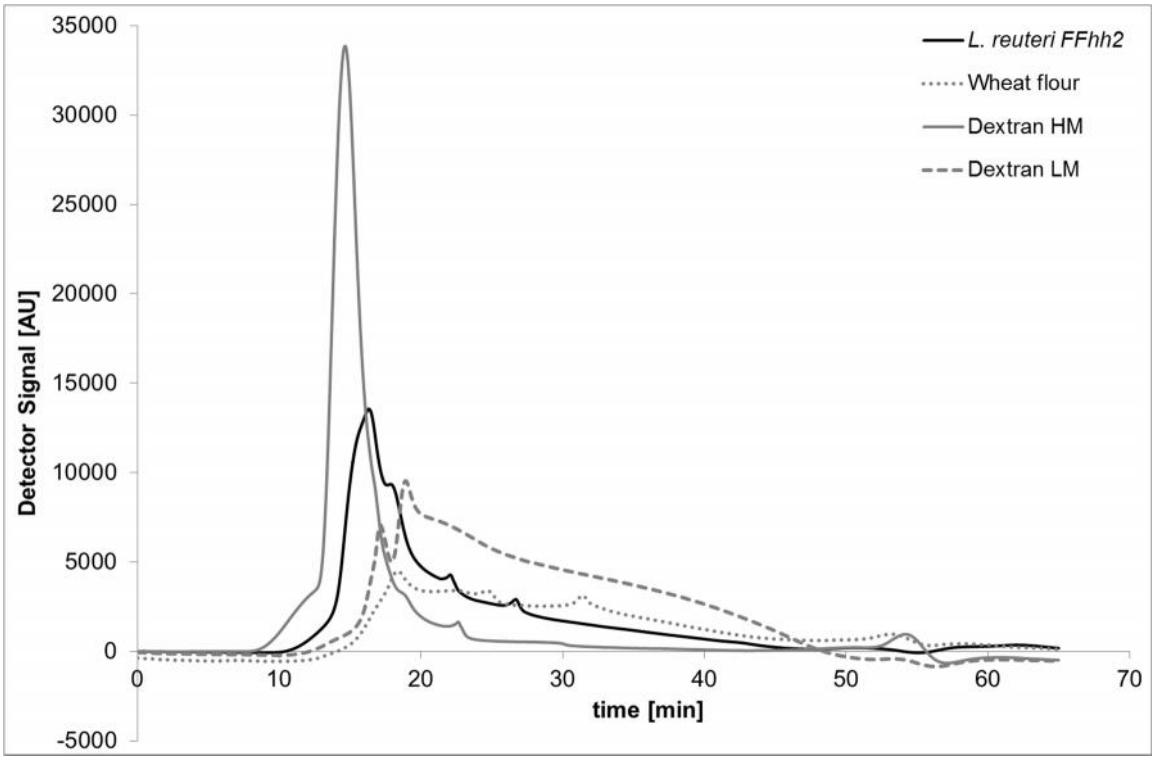


Figure 13: EPS synthesised by *L. reuteri* FF2hh2 during sourdough fermentation of wheat flour (10% sucrose addition) with a dough yield of 200. High molecular weight dextran (HM) with a relative molecular weight $M_r = 10^5 – 2 \times 10^5$, low molecular weight dextran (LM) with a $M_r = 5 \times 10^6 – 4 \times 10^7$ and inulin from chicory with a molecular weight of $M_r = 10^4$ was used for calibration.

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Table 11: Sourdough metabolites sugars, lactate, acetate, TTA, pH and exopolysaccharides (EPS).

Metabolites [mmol kg^{-1}]/ Flour	**Fructose**	**Glucose**	**Sucrose**	**Maltose**	**Lactate**	**Acetate**	**TTA [ml]**	**pH**	**EPS [g kg^{-1}]/ Sourdough**
Wheat flour, unfermented	n/d	n/d	45.5 ± 0.4	48 ± 2	n/d	n/d	2.2 ± 0.0	6.1 ± 0.1	1.7 ± 0.4
L. reuter (FF2hh2)	34 ± 3	328 ± 29	n/d	91 ± 8	155 ± 27	138 ± 11	15.9 ± 0.1	3.95 ± 0.01	5.2 ± 0.7

5.4.2 Quantification of antifungal acid compounds

The selected strain *L. reuteri* FF2hh2 had proven to have antifungal activity acting on agar plates against *P. roqueforti* and *F. culmorum*. The next step was to identify antifungal compounds in the sourdough. Based on previously published work (Brosnan et al., 2012), the sourdough fermented with *L. reuteri* FF2hh2 was analysed for the range of antifungal acidic compounds, identified by Ryan et al. (2011) in MRS broth after fermentation with *L. Amylovorus* DSM19280 (Figure 14). The determined antifungal compounds at significant concentrations were leucic acid, hydroxyphenyllactic acid, phenyllactic acid and vanillic acid. Leucic acid has been proven to have a fungicidal effect against *Candida* species as well as against certain *Aspergillus* species at minimum fungicidal concentration of 72 mg/ml (Sakko et al., 2014). The antifungal activity of the other 3 compounds was previously reported for different fermented substrates such as grass silage inoculated with *L. plantarum* (Broberg et al., 2007) or wort fermented with a *L. brevis* (Axel et al., 2014) or sourdough fermented with *L. plantarum* breads (Ryan et al., 2009, Broberg et al., 2007, Dal Bello et al., 2007, Lavermicocca et al., 2003, Strom et al., 2002) as well as on plate assays (Guo et al., 2012). Since the concentrations of each of the compounds in the analysed sourdough were too low to be responsible for any antifungal effect on its own, only a synergistic effect can explain the determined antifungal activities (Ryan et al., 2011, Aziz et al., 1998).

5.4.3 Fundamental rheology

Rheological frequency sweeps were performed to determine the impact of the sourdough addition on the microstructural characteristics of the bread dough samples (Table 12). The weak gel model was applied to determine the resistance to deformation A_F and the network connectivity z of the dough samples over the frequency range of ω = 1 – 100 Hz. This model was introduced by Gabriele et al. (2001) and is based on the power equation $G^*\ \omega\ = A_F \cdot \omega^{\frac{1}{z}}$. The frequency sweeps of all dough samples showed a viscoelastic behaviour which means all measured elastic moduli (G') were higher than the corresponding viscous moduli (G'') (Song and Zheng, 2007, Dobraszczyk and Morgenstern, 2003, Hoseney and Rogers, 1990).

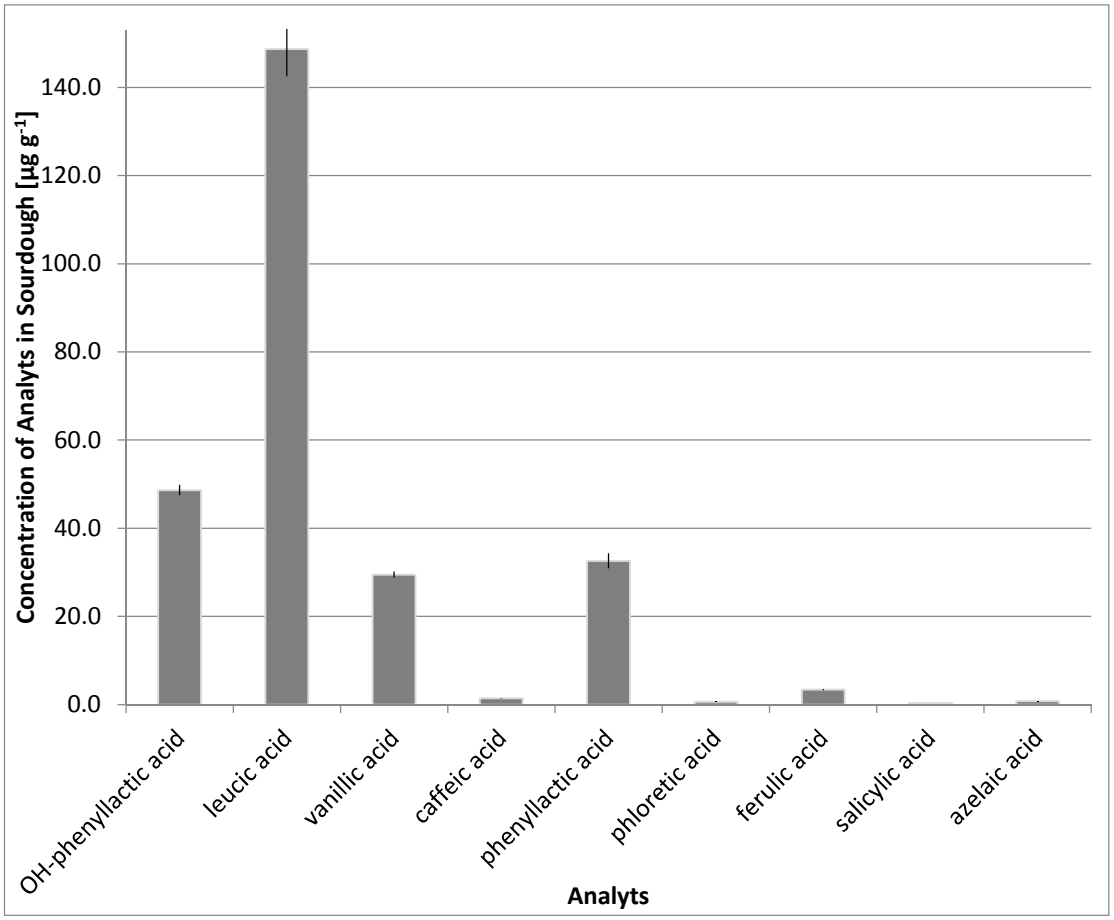


Figure 14: Quantification of antifungal acid compounds in sourdough fermented using *L. reuteri* FF2hh2. The analyses were performed using a UPLC-MS/MS.

The reduction of NaCl from standard salt to low-salt led to a significant increase of dough strength of 29 % as well as to a significant increase in network connectivity. On the contrary, Beck et al. (2012a) described a decrease of dough strength for reducing amounts of NaCl for large scale deformation using a farinograph. It was explained by the interaction of sodium and chloride ions with the positive and negative side chains of the gluten protein molecules favouring the network formation between the protein molecules. These results can be explained by the known differential behaviour between small-scale and large-scale deformation rheological properties of wheat dough (Mann et al., 2005, Tronsmo et al., 2003, Lynch et al., 2009). In actual fact, there is still a gap of knowledge how to relate small-scale deformation and large-scale deformation.

For the low-salt samples fermented with *L. reuteri* FF2hh2 the resistance to deformation decreased significantly with a direct linear correlation to the sourdough concentration. The strong acidification of the *L. reuteri* strain led to an intense degradation of the network polymers resulting in a significantly decreased dough strength. In contrast to the previously described sourdoughs, fermented with *W. cibaria* MG1 and *L. amylovorus* (DSM19280), the network connectivity z changed significantly with increasing amounts of sourdough added. Two main reasons are responsible for those changes of dough properties upon sourdough addition: firstly, being a positive net charge due to the presence of the organic acids which caused an unfolding of the gluten proteins (Galal et al., 1978) and secondly, an activation of the endogenous proteinase naturally present in wheat flour during fermentation of the sourdough (Thiele et al., 2002, Bleukx et al., 1997). Both effects led to a weaker integrity of the gluten network (Bleukx and Delcour, 2000). Although the fermentation with *L. reuteri* FF2hh2 resulted in an acidification with a TTA = 15.2 and pH = 3.95, which was less acidic than the sourdough fermented with *L. amylovorus* DSM 19280 with a TTA = 17.8 and pH = 3.75 (Chapter 4), the impact of the sourdough on the dough rheology was much stronger. This can be explained by the heterofermentative nature of *L. reuteri* FF2hh2 producing also acetic acid versus the homofermentative *L. amylovorus* DSM 19280 producing only lactic acid (Leenhardt et al., 2005, Boskov Hansen et al., 2002).

Table 12: Deformation (A_F) and elasticity (z) rheological measurements of bread dough samples with *L. reuteri* FF2hh2. The frequency sweep was performed at an angular frequency range of ω = 1 - 100 Hz and a constant target strain of γ = 0.01%. Values followed by a different letter are significantly different ($p < 0.05$).

NaCl conc.	Dough samples	A_F	ΔA_F [%]	z	R^2
1.04%	Standard salt control	4194 ± 154[a]		3.9 ± 0.1[a]	0.996 ± 0.002
0.26%	Low-salt control	5298 ± 418[b]	29%[1]	4.6 ± 0.1[b]	0.99990 ± 0.00004
	6% *L. reuteri* FF2hh2	4161 ± 697[a]	-21%[2]	4.60 ± 0.07[b]	0.9996 ± 0.0004
	12% *L. reuteri* FF2hh2	3969 ± 360[a,c]	-25%[2]	4.4 ± 0.2[c]	0.9997 ± 0.0005
	18% *L. reuteri* FF2hh2	3442 ± 200[c]	-35%[2]	4.17 ± 0.06[d]	0.99988 ± 0.00008
	24% *L. reuteri* FF2hh2	2205 ± 229[d]	-58%[2]	3.93 ± 0.06[a]	0.9996 ± 0.0002

[1] relating to standard salt control

[2] relating to low-salt control

5.4.4 Bread analyses

The baked bread loaves were analysed measuring the specific loaf volume as well as the specific characteristics of the crumb grain structure; namely slice brightness, number of cells, porosity, wall thickness and cell volume. Salt reduction caused significant differences for crumb porosity, wall thickness and cell volume. This is in line with previous research published by Lynch et al. (2009) who could show a significant increase of porosity between 1.2% and 0.3% NaCl. The reduction of NaCl resulted to an increased yeast activity in bread dough during fermentation and hence, causing a more open and porous crumb structure (Cauvain, 2007). The increased wall thickness is based on a smaller number of larger cells surrounded by heavier cell walls (Czuchajowska et al., 1989).

The addition of *L. reuteri* FF2hh2 sourdough caused, with increasing addition levels significant increases of loaf volume culminating in a maximum of the specific loaf volume for an addition level of 18% (Table 13). A further increase of the sourdough addition significantly reduced the loaf volume. The EPS had a positive influence on the loaf volume and could compensate the negative effect of organic acids up to a sourdough concentration of 18%. For the addition level of 24% sourdough the higher acidification could not be compensated by EPS and hence, resulted in a decreased loaf volume. As little as the interaction of EPS, gluten and starch is understood, the positive impact of EPS on the specific bread volume has been proven in by several authors (Di Monaco et al., 2015, Wolter et al., 2014, Galle et al., 2010, Katina et al., 2009). The type and amount of acids determine whether the impact on bread quality is beneficial or contra-productive (Wehrle et al., 1997). This effect is based on the technological impact of acids on the baking process (Arendt et al., 2007, Moore et al., 2008). While small amounts of acid are known to improve bread quality characteristics such as specific volume, higher additions of sourdough and therefore, higher acid concentrations, reduce the specific volume but improve freshness and shelf-life (Komlenic et al., 2010, Rozylo et al., 2015a, Rozylo et al., 2015b).

The total number of cells per slice increased significantly with increasing amounts of sourdough independent of the loaf volume. The highest sourdough concentration of 24% resulted in the highest number of cells which were smaller than for any other samples containing sourdough. While porosity increased significantly adding more sourdough the wall thickness did not change but at the highest level of sourdough the cell walls trended

to be the thinnest. The significantly higher acid concentrations strengthened the remaining gluten network and allowed small cells to exist although they have a higher the gas pressure compared to larger cells. A weaker gluten network will allow small cells to join to bigger ones while a strong network integrity will keep many small cells (Scanlon and Zghal, 2001, Sroan et al., 2009).

Table 13: Specific bread loaf volume and bread crumb characteristics. Values followed by a different letter are significantly different ($p < 0.05$).

NaCl concentration	Dough samples	Specific bread volume [ml/g]	Number of cells	Porosity (area of cells) [%]	Wall Thickness [mm]	Cell Volume [mm^3]
1.04%	Standard salt control	2.7 ± 0.2^{a}	3620 ± 148^{a}	53.8 ± 0.4^{a}	0.466 ± 0.006^{a}	8.2 ± 0.5^{a}
0.26%	Low-salt control	3.0 ± 0.3^{b}	3564 ± 103^{a}	54.8 ± 0.3^{b}	0.488 ± 0.005^{a}	$9.5 \pm 0.4^{a,b}$
	6% *L. reuteri* FF2hh2	3.36 ± 0.08^{c}	3506 ± 630^{a}	$55.7 \pm 1.3^{b,c}$	0.52 ± 0.03^{a}	$11.7 \pm 2.9^{b,c}$
	12% *L. reuteri* FF2hh2	3.6 ± 0.1^{d}	$3689 \pm 205^{a,b}$	$56.1 \pm 0.8^{c,d}$	0.52 ± 0.02^{a}	11.7 ± 2.1^{c}
	18% *L. reuteri* FF2hh2	3.79 ± 0.09^{e}	$3872 \pm 152^{b,c}$	$56.6 \pm 0.6^{c,d}$	0.52 ± 0.02^{a}	12.0 ± 1.9^{c}
	24% *L. reuteri* FF2hh2	3.37 ± 0.09^{c}	4144 ± 159^{c}	55.3 ± 0.4^{d}	0.49 ± 0.01^{a}	$9.0 \pm 0.5^{a,b}$

Crumb hardness was measured over a storage period of 5 days using a texture profile analyser (TPA). NaCl reduction resulted in a softer crumb after two and five days of storage although no difference could be detected on the baking day. The addition of *L. reuteri* FF2hh2 sourdough softened the bread crumb significantly independent of the addition level and the time of storage with the exception of day 5 where no difference could be detected between the low salt control, 6% and 24% sourdough addition (Figure 15). While the addition of 6% sourdough seemed to be too little to make a difference, the addition of 24% sourdough acidified the bread dough significantly resulting in a reduced loaf volume and harder bread crumb. Increasing amounts of *L. reuteri* FF2hh2 sourdough from 6% up to 18% addition level led to a softer bread crumb. These results correlate with the specific loaf volume as well as with the porosity of the bread crumb samples. Nevertheless, the softening effect is not only based on the higher bread loaf volume but rather due to the positive effect of EPS previously described (Chapter 4) as well as for different gluten free dough system (Galle et al., 2010, Wolter et al., 2014).

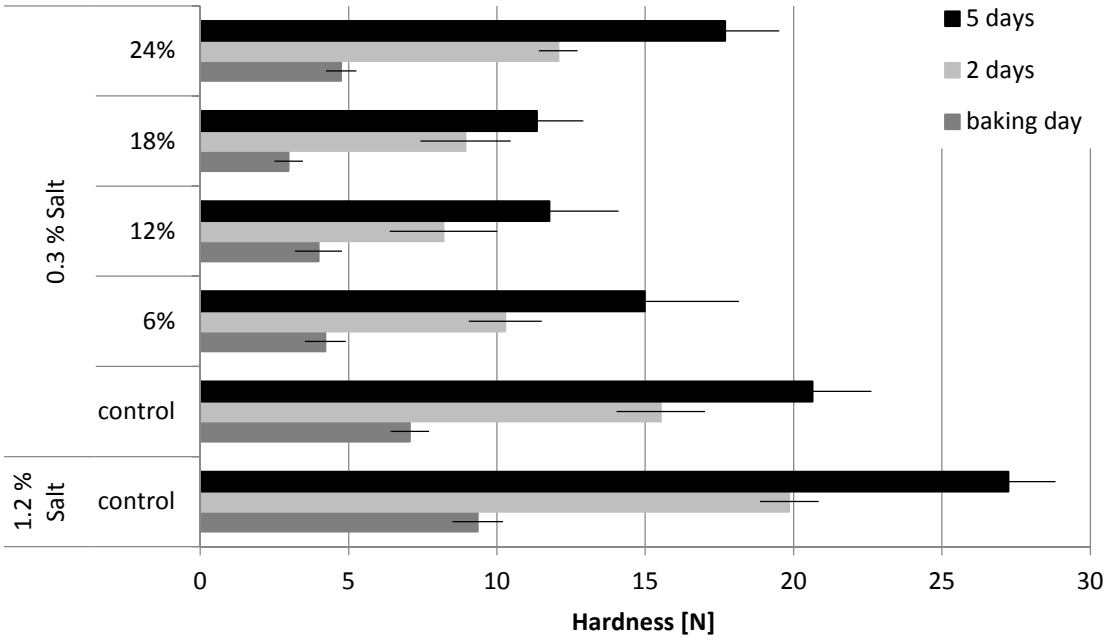


Figure 15: Hardness of bread crumb on day 0, 2 and 5 after baking. Control breads were analysed as well as sourdough breads containing *L. reuteri* FF2hh2 at concentrations of 6%, 12%, 18% and 24%.

The fact that EPS had similar volume improving and crumb softening effects for gluten free breads made out of buckwheat, oat, quinoa, teff and sorghum is evidence that EPS acts independently of the presence and interactions of gluten. Furthermore, the mentioned starch EPS interactions (Di Monaco et al., 2015, Katina et al., 2009) cannot be the main reason for the softening effect of EPS either as the starch characteristics of wheat starch and different gluten free starches differ significantly from each other (Horstmann et al. 2016, Hager et al., 2013, Hager et al., 2012).

5.4.5 Microbial shelf-life and water activity

The microbial shelf life of all bread samples was assessed exposing the different samples of bread slices to the bakery environment for a defined time of 10 min (Figure 16). The reduction of NaCl in bread without sourdough addition from standard salt (1.2%) to low-salt (0.3%) resulted in a reduced shelf life of about two days as previously reported (Belz et al., 2012a, Samapundo et al., 2010).

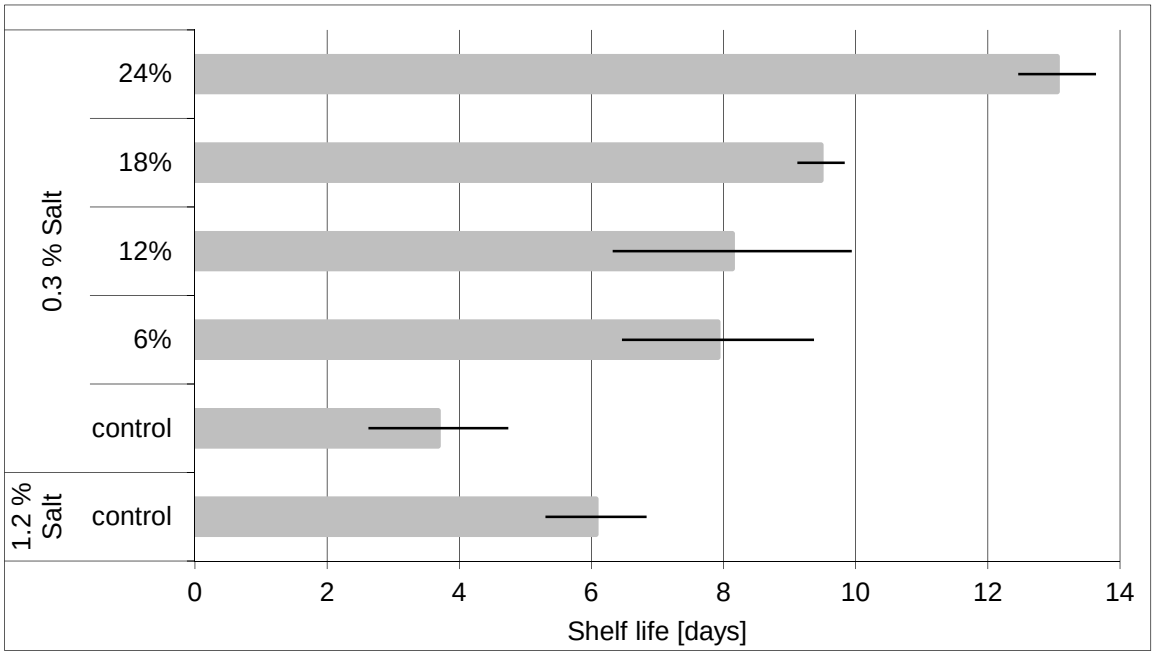


Figure 16: Microbial shelf-life of bread slices exposed to the bakery environment. The following bread samples were considered: standard salt and low-salt control breads without sourdough, sourdough breads at low-salt level with sourdough addition of 6%, 12%, 18% and 24% of sourdough fermented with *L. reuteri* FF2hh2.

The loss of shelf life of two days based on NaCl reduction could be compensated with the addition of 6% - 18% sourdough fermented with *L. reuteri* FF2hh2. Only the highest addition of 24% sourdough prolonged the shelf life significantly to about 13 days. The same shelf life extension was previously reported for the antifungal strain *L. amylovorus* DSM19280 (Belz et al., 2012a).The water activity analyses of the bread crumb samples resulted in a direct correlation to the added amounts of sourdough (Figure 17).

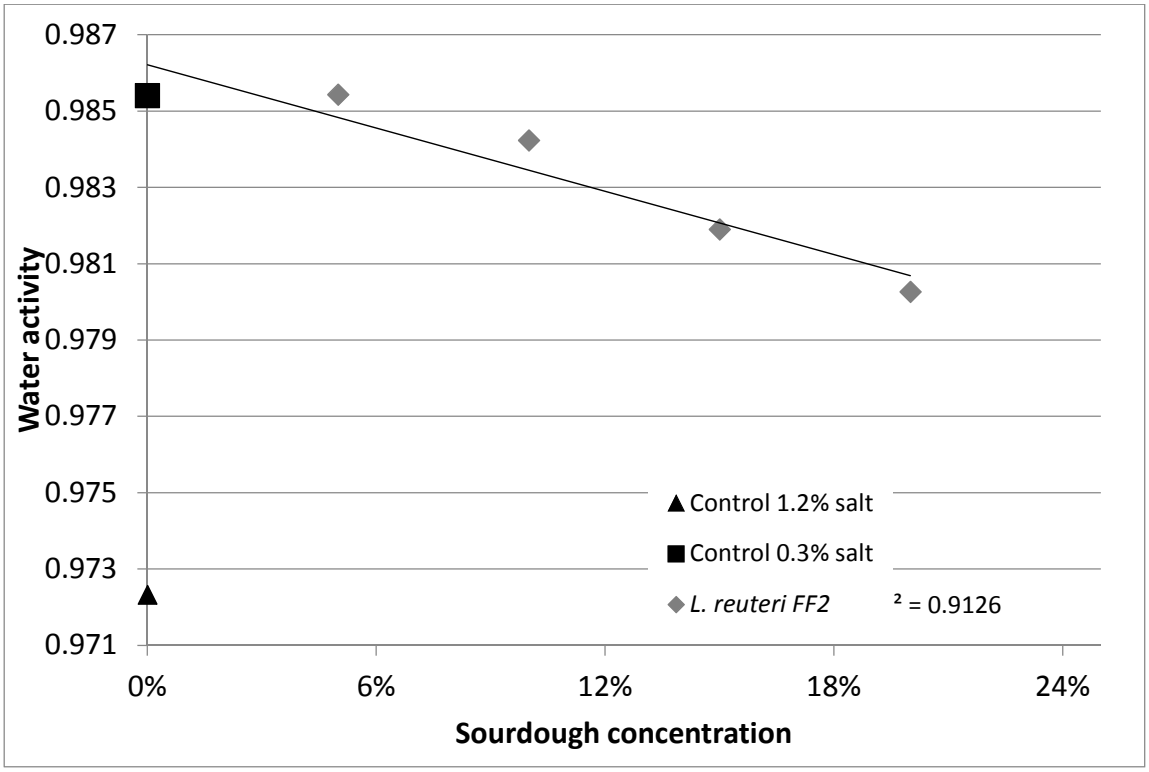


Figure 17: Water activity of control breads at standard salt and low-salt levels and low-salt bread samples with *L. reuteri* FF2hh2 at different concentrations of 6%, 12%, 18% and 24% sourdough

Increasing amounts of sourdough fermented with *L. reuteri* FF2hh2 lead to decreasing a_w values which reached their limit at $a_w = 0.9803 \pm 0.002$ for the addition of 24% sourdough. None of the sourdough breads reached the water activity of the standard salt bread of $a_w = 0.972 \pm 0.002$. All small molecular compounds produced during sourdough fermentation, which have a lowering effect on the water activity are mainly derived from starch and gluten polymers. The polymers are mainly responsible for water binding in dough and bread crumb and due to the fact that they become degraded during fermentation counteracts the lowering influence of the produced compounds. Nonetheless, the antifungal

compounds determined in the sourdough fermented with *L. reuteri* FF2hh2 (Figure 14) were responsible for the increased shelf life of the sourdough bread samples.

5.4.6 Sensory

For the set of breads selected for the descriptive sensory analysis the two control samples, and the sourdough breads at addition levels of 6, 18 and 24% were chosen. The sample with 12% sourdough was eliminated due to the insignificant differences compared to the addition levels of 6% and/or 18%. In addition, sourdough bread samples without any salt and at 1.2% salt were added for comparison reasons.

Table 14 shows the average panel scores for the sensory characteristics determined by the assessors. Increasing amounts of NaCl lead to a higher bread crumb density independent of the addition level of sourdough. This was found for both appearance and texture density. Based on the inhibition of yeast activity by NaCl the increased density was caused by a reduced gas production by the baker's yeast during proofing (Lynch et al., 2009). For the sourdough addition levels of 6% and 18% no significant difference in density could be determined. With a density score of 18.7 the bread without any NaCl and 24% sourdough had the significantly lowest density as well as the only significant difference for the olfactory and flavour attributes "roasted". The aroma note "cheesy" was significantly more intense for some of the sourdough samples while the NaCl concentration showed no significant influence. The attributes "doughy" and "must/stale" behaved similar being more intense for higher concentrations of NaCl whereas higher addition levels of sourdough reduced the "doughy" and "musty" perception. Salt is known to enhance other flavours (Kare, 2012, Gillette, 1985) resulting in a higher recognition of the doughy compounds originating from the wheat flour in combination with water and yeast. No difference of the perception of salt was recognised between 0.0% and 0.3% NaCl for the control bread samples but significantly different for the standard amount of 1.2%. For the sourdough samples the different NaCl concentrations showed significant differences. The organic acids added with the sourdough together with the present NaCl enhanced the salty perception (Breslin and Beauchamp, 1997, Collier and Snyder, 2014).The PCA, generated from all the attributes (Figure 18), was used to summarize the relationship between the

samples and the attributes in a visualized way (Table 14). PC1 accounted for 69% of the determined variances and it distinguished the samples mainly between a “roasted” flavour and odour on the one side and a “doughy” and musty/stale flavour on the other side. Across PC2, which accounts for 15% variances, the samples were split between a “cheesy, sourdough” like flavour and a “salty” flavour. All samples with a NaCl concentration of 1.2% can be found in the right bottom quadrant. Most samples containing *L. reuteri* FF2hh2 sourdough are located in the upper half showing a cheesy note of this particular sourdough. The attributes “density” did not correlate with the determined specific volumes of the bread loaves which was another indication that the softening effect of the bread crumb occurred due to the addition of a functional sourdough and not due to an increased loaf volume. The increased loaf volume in combination with the more porous crumb with lower density led to a more intense recognition of salt and possibly of other flavours too (Pflaum et al., 2013, Konitzer et al., 2013). All NaCl containing samples with added sourdough were also described as slightly saltier than the respective control samples with 1.2% and 0.3% NaCl independent of the sourdough concentration. In addition, the lactic and acetic acids enhanced the saltiness perception of the bread crumb (Collier and Snyder, 2014, Reddy and Marth, 1991).

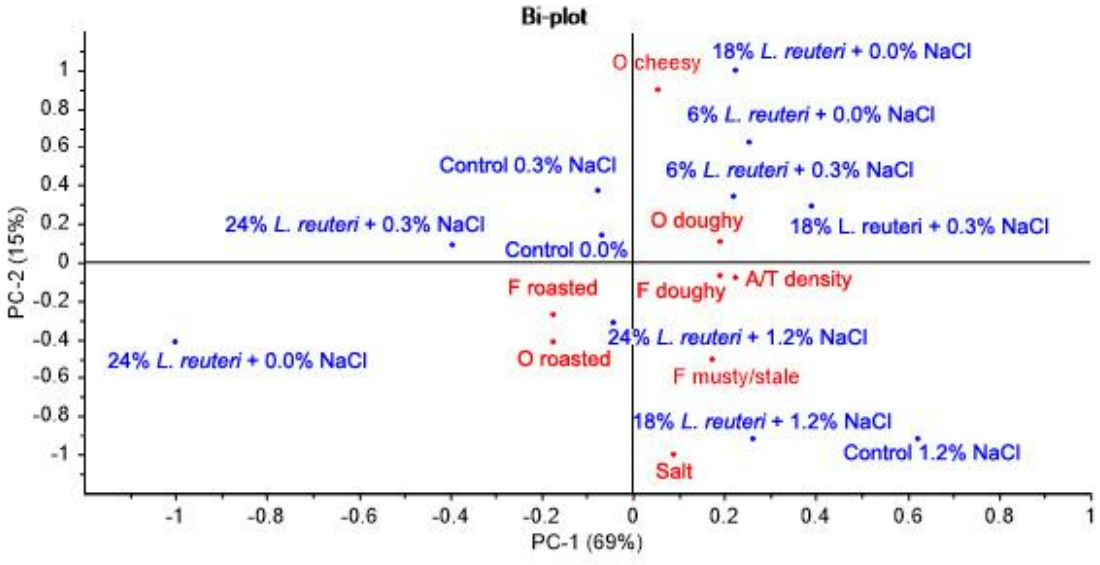


Figure 18: Principle component analysis of sensory attributes of bread samples containing different amounts of NaCl (1.2%; 0.3%; 0.0%) and sourdough fermented with *L. reuteri* FF2hh2. O = odour; F = flavour; A = appearance; T = texture.

Table 14: Mean panel description sensory score for the attributes determined by the descriptive sensory categorised into the four main sensory responses: appearance (A), texture (T), olfactory (O) and flavour (F).

	Appearance	**Texture Response**	**Olfactory Responses**			**Flavour Responses**			
Product	**A Density**	**T Density**	**O Roasted**	**O Cheesy**	**O Doughy**	**F Doughy**	**F Musty/Stale**	**F Roasted**	**Salt**
Control 0.0% NaCl	60.5 ^{b,c}	48.7 ^{b,c}	4.4 ^{a}	0.00 ^{a}	31.3 ^{a,b,c,d}	28.2 ^{a,b}	8.5 ^{a,b,c}	0.1 ^{a}	5.6 ^{a,b}
Control 0.3% NaCl	59.6 ^{b,c}	45.5 ^{b}	4.9 ^{a}	0.75 ^{a}	28.6 ^{a,b,c}	33.1 ^{b,c}	6.6 ^{a,b,c}	0.3 ^{a}	7.6 ^{a,b}
Control 1.2% NaCl	83.8 ^{d}	70.7 ^{d}	2.3 ^{a}	0.04 ^{a}	45.4 ^{d}	44.4 ^{e}	18.2 ^{e}	0.2 ^{a}	58.4 ^{d}
6% *L. reuteri* 0.0% NaCl	70.5 ^{b,c}	54.4 ^{b,c}	2.0 ^{a}	1.8 ^{a,b}	41.3 ^{c,d}	38.5 ^{c,d}	10.1 ^{b,c,d}	0.1 ^{a}	5.1 ^{a,b}
6% *L. reuteri* 0.3% NaCl	70.7 ^{b,c}	51.2 ^{b,c}	2.8 ^{a}	1.1 ^{a}	38.3 ^{b,c,d}	40.5 ^{d,e}	9.5 ^{a,b,c}	0.1 ^{a}	12.2 ^{c,d}
6% *L. reuteri* 1.2% NaCl	73.8 ^{b,c}	51.8 ^{b,c}	3.3 ^{a}	1.0 ^{a}	37.7 ^{b,c,d}	44.9 ^{c}	13.5 ^{c,d,e}	0.2 ^{a}	61.8 ^{d}
18% *L. reuteri* 0% NaCl	63.7 ^{b,c}	51.6 ^{b,c}	2.2 ^{a}	3.4 ^{b}	37.9 ^{b,c,d}	37.6 ^{c,d}	12.0 ^{c,d}	0.3 ^{a}	3.3 ^{a}
18% *L. reuteri* 0.3% NaCl	63.8 ^{b,c}	50.9 ^{b,c}	3.1 ^{a}	2.8 ^{b}	38.8 ^{b,c,d}	37.5 ^{c,d}	12.5 ^{c,d}	0.3 ^{a}	10.9 ^{b,c}
18% *L. reuteri* 1.2% NaCl	65.2 ^{b,c}	52.1 ^{b,c}	5.6 ^{a}	0.0 ^{a}	36.1 ^{b,c,d}	37.8 ^{c,d}	15.5 ^{d,e}	0.2 ^{a}	61.1 ^{d}
24% *L. reuteri* 0% NaCl	18.7 ^{a}	17.1 ^{a}	34.3 ^{b}	0.1 ^{a}	18.4 ^{a}	22.8 ^{a}	4.0 ^{a}	5.9 ^{b}	3.6 ^{a}
24% *L. reuteri* 0.3% NaCl	42.5 ^{a,b}	31.7 ^{a,b}	8.8 ^{a}	0.0 ^{a}	25.7 ^{a,b}	27.5 ^{a}	5.3 ^{a,b}	1.5 ^{a}	8.5 ^{a,b}
24% *L. reuteri* 1.2% NaCl	53.7 ^{b,c}	44.0 ^{b}	13.1 ^{a}	1.8 ^{a,b}	26.8 ^{a,b,c}	33.7 ^{c}	9.3 ^{a,b,c}	0.2 ^{a}	63.2 ^{d}
P Value	0.0244	0.0140	0.0016	0.0139	0.0219	0.0001	0.0025	0.0031	0.0000

5.5 Conclusion

Based on previous research (Chapter 3+4), the use of sourdough for low-salt bread was known as a natural option. Two strains had to be used to achieve the quality benefits in the bread system; EPS to change the volume, crumb structure and salty perception and the antifungal compounds to compensate the lack of shelf life. The presented lactic acid bacteria strain *L. reuteri* FF2hh2 was proven as the first strain to have multifunctional characteristics producing EPS as well as antifungal compounds. Hence, the more efficient option could be provided to compensate the lack of shelf life as well as some of the change in flavour using the multifunctional *L. reuteri* FF2hh2. The antifungal activity was proven *in vitro* on plate assays as well as *in vivo* in the sourdough bread system. For the first time four major antifungal compounds, which have previously been reported to have antifungal activity, could be analysed for the *L. reuteri* FF2hh2 strain,. The shelf life of the low-salt bread could be prolonged by about 2-3 days with the addition of 6% - 18% of sourdough fermented with *L. reuteri* FF2hh2. Significant amounts of EPS could be determined in the sourdough which impacted positive on crumb characteristics as well as bread loaf volume. The bread loaf volume increased significantly for the sourdough addition of 6%-18% and crumb structure got more porous with increasing amounts of sourdough. Only for the highest addition level the crumb was significantly finer and most similar to the standard salt bread. The increased crumb cell surface area and the presence of organic acids enhanced the perception of saltiness of the bread samples containing sourdough compared to the control breads with the same amount of NaCl. The descriptive analyses could clearly distinguish the samples with sourdough addition based on their salty flavour as well as on their crumb density which highlights the correlation between the two attributes. With the novel lactic acid bacteria strain *L. reuteri* FF2hh2, a multifunctional and very interesting strain could be presented. A first application could be demonstrated successfully based on a low-salt bread. It shows a natural way to compensate the lack and changes in product quality of yeasted bread based on salt reduction.

5.6 Acknowledgement

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Chapter 6

Sodium chloride and its influence on the aroma profile of yeasted bread

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6.1 Abstract

The reduction of the daily sodium intake has been one of the main topics for authorities in the industrialized countries. The excessive intake of sodium was reported to cause hypertension and to increase the risk of serious cardiovascular diseases. Bread was determined as one of the main contributors to the daily sodium intake for a Western diet mainly based on the salt concentration. The reduction of salt in bread was targeted in many countries and the impact on dough processing and bread quality has been investigated in many aspects. While the impact on bread flavour is obvious and well-known, no research has been done to date to investigate the impact of salt reduction on the volatile aroma profile of yeasted bread and the resulting odour characteristics. The volatile bread aroma profile has been reported to consist of a complex mixture of more than 600 compounds where the baker's yeast plays an important role. Baker's yeast as a living metabolism is affected directly by the concentration of sodium chloride which impacts on the yeast activity during the fermentation step of the bread baking process. Besides carbon dioxide and some ethanol, baker's yeast produces many other low molecular weight compounds. The Ehrlich Pathway is an important yeast metabolism which metabolises amino acids into volatile compounds. This work researched for the first time the impact of sodium chloride concentration in bread on the yeast activity in the bread dough and the resulting impact on the volatile aroma profile. Yeast growth was monitored for different salt concentrations from 0.0 – 4.0% on agar plates. The yeast activity in dough was determined for bread samples with different salt and yeast concentrations measuring the gas release with a Chopin rheofermentometer. Aroma compounds were extracted using a solvent-assisted flavour evaporation (SAFE) distillation. The extracts were analysed with a 2-dimensional high-resolution gas chromatograph mass spectrometer (2D-HRGC/MS). In addition, an online analyses of the head space area of bread dough during fermentation was performed using a high sensitive proton-transfer-reaction mass spectrometer. A descriptive sensory as well as discriminating triangle tests evaluated the impact of salt reduction on the volatile aroma profile of the bread crumb samples. The high resolution analytics revealed significant changes for the determined aroma compounds as well as the aroma profile for different salt and yeast concentration. A direct correlation between the metabolites carbon dioxide and phenylethan-2-ol as well as (E)-non-2-enal was found. In contrast, the sensory

panel could not determine any significant differences between samples with the different salt concentrations whereas different yeast levels showed significant impact on the odour profile of some samples. It was proven that salt reduction does influence the aroma profile of yeasted bread below the perception limit of the human olfactory sense and hence, the consumer cannot recognise any changes by the odour of low-salt bread crumb.

6.2 Introduction

Sodium chloride (NaCl), or salt, is a major taste contributor to food. A reduction of salt in food products generally leads to less intense taste and flavour. The impact of salt reduction on taste profiles has been demonstrated for numerous foods, amongst them white yeasted bread. An investigation by Tuorila-Ollikainen and co-workers (1986) on white yeasted, rye and rye-sourdough breads indicated a low consumer preference for the reduced-salt breads. In contrast, however, Wyatt (1983) observed no significant difference in consumer preference for white bread with a 50 % reduction in NaCl compared with the reference bread. The current challenge for food producers is to develop products with a reduced salt content but an unimpaired and consistent taste. This has been investigated with the use of salt replacers such as potassium chloride, magnesium chloride, ammonium chloride, calcium chloride and calcium carbonate (Bassett et al., 2014, Charlton et al., 2007), the use of sourdough (Rizzello et al., 2010), the inclusion of flavour-enhancing acids and other aroma-intensive compounds (Ghawi et al., 2014, Jimenez-Maroto et al., 2013, Breslin, 1996, Hellemann, 1992, Reddy and Marth, 1991) or by changes to the bread crumb texture influencing the saltiness perception (Kuo and Lee, 2014, Pflaum et al., 2013a, Pflaum et al., 2013b, Noort et al., 2012, Noort et al., 2010). In contrast to taste, less is known about the influence of salt reduction on the volatile aroma profile of food and there are no reports of studies investigating the relationship of these properties in bread crumb. Volatile aroma compounds impart flavour to food, and the volatile fraction of bread is highly complex with about 600 volatile compounds reported to be present in bread crumb (Schieberle and Grosch, 1991). In particular, the yeast metabolism plays a key role in the development of a bread's aroma profile and salt, primarily its sodium ions, has a direct impact on yeast activity (Matz, 1992).

In addition to ethanol and carbon dioxide, many low molecular weight flavour compounds such as further alcohols, aldehydes, acids, esters, sulphides and carbonyl compounds are produced by the yeast metabolism. These volatile compounds are essential contributors to the flavour of fermented foods and beverages (Suomalainen and Lehtonen, 1979, Whiting, 1976). The Ehrlich pathway is one of several routes responsible for the generation of aroma compounds by yeast in bread. In particular it leads to the formation of potent compounds such as fusel alcohols and acids. The efficacy of the Ehrlich pathway in converting amino acids into alcoholic odorants was investigated, amongst others, by Czerny and Schieberle

(2006), who used stable isotope dilution assays (SIDA) to demonstrate the conversion of C^{13}(6)-leucine to the metabolite 3-methylbutanol.

The Ehrlich pathway is named after Felix Ehrlich, who in 1904 isolated isoleucine from leaches of molasses sugar extracts and characterised and elucidated the constitution of isoleucine (Ehrlich, 1907a). Ehrlich later described the metabolism of the amino acids to the corresponding alcohols, CO_2 and ammonia that is used by the yeast for the metabolism of protein (Ehrlich, 1906, Ehrlich, 1907b). In 1911, Neubauer and Fromherz outlined the steps in the reaction process of the Ehrlich pathway, which is still valid today. Furthermore, transaminase, decarboxylase and subsequent alcohol dehydrogenase were documented as the key reaction sequence of the pathway (Sentheshanmuganathan, 1960). Numerous studies concluded that all of the fusel alcohols produced by the yeast are derived from amino acid catabolism (Lampitt, 1919, Yamada, 1932, Thorne, 1937, Hazelwood et al., 2008). Figure 19 shows the reactions that take place along the Ehrlich pathway. Transamination and decarboxylation are followed by either an oxidation to the corresponding acid or a reduction to the corresponding alcohol. The cultivation conditions dictate which of the two reactions are favoured. During bread dough fermentation, the reduction reaction dominates over the oxidation reaction (Hazelwood et al., 2008, Sentheshanmuganathan, 1960). The amino acids valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan and methionine are converted into alcohols and acids. A selection of amino acids and their corresponding Ehrlich pathway products is shown in Table 15.

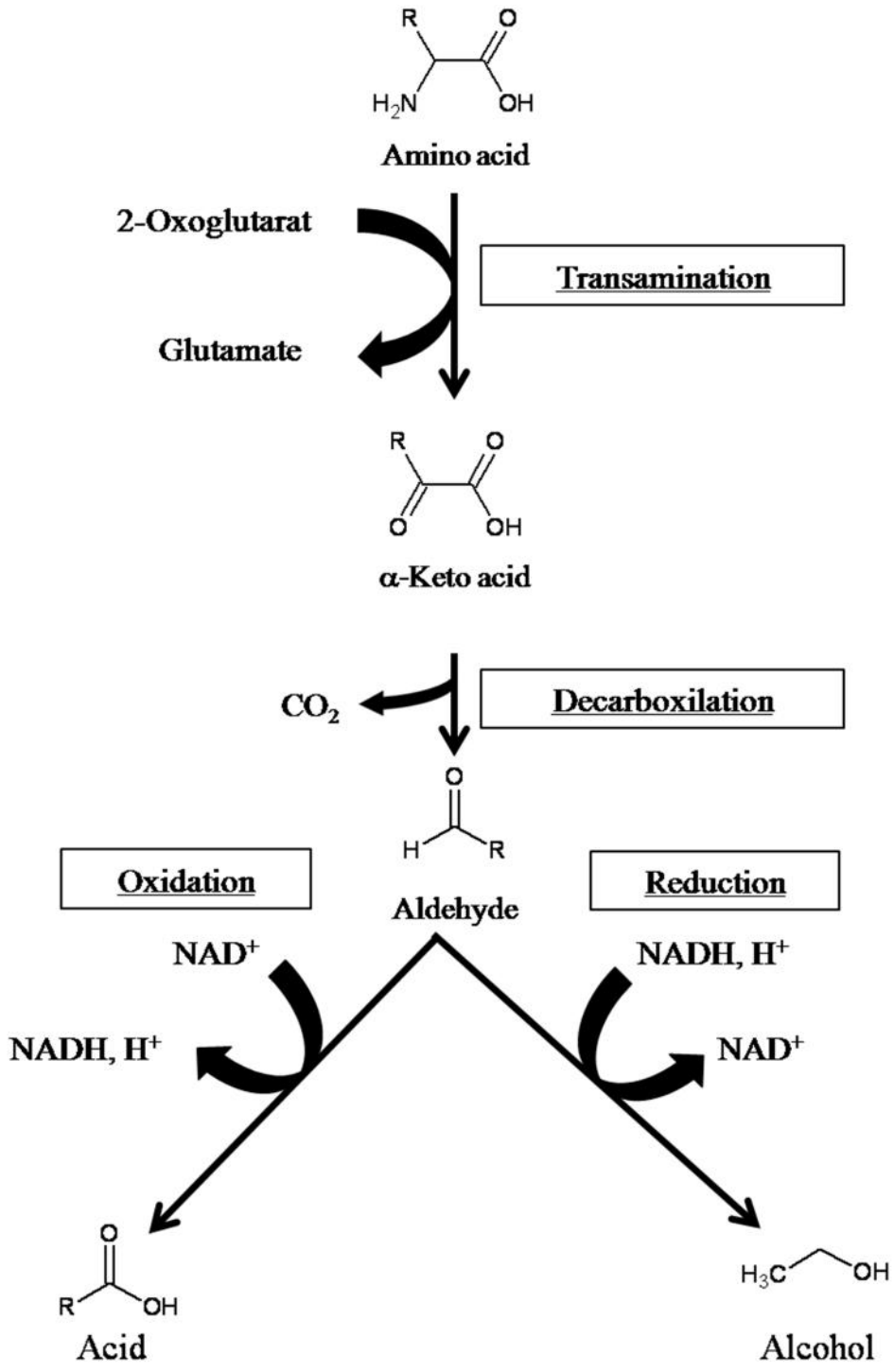
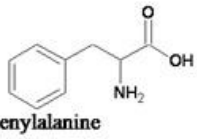
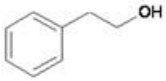
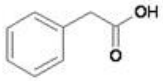
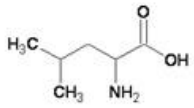
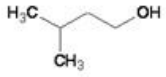
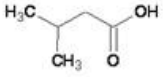
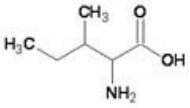
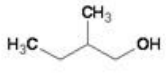
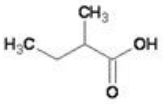


Figure 19: Reactions along the Ehrlich pathway (Taken from Hazelwood et al., 2008)

Table 15: Examples of amino acids and their corresponding alcohols and acids produced along the Ehrlich pathway (Belitz et al., 2008).

Amino acid	Resulting alcohol	Resulting acid
 Phenylalanine	 Phenylethan-2-ol	 Phenylacetic acid
 Leucine	 3-Methylbutan-1-ol	 3-Methylbutyric acid
 Isoleucine	 2-Methylbutan-1-ol	 2-Methylbutyric acid

The reducing activity of yeast during bread dough fermentation has a critical impact on bread aroma, as has been similarly observed during beer wort fermentation (Saison et al., 2010). Unsaturated aldehydes such as (E)-non-2-enal and (E,E)-deca-2,4-dienal derive from the oxidation of linoleic acid (Figure 20) and are well-known for their contribution to fatty odours in wheat bread (Schieberle and Grosch, 1991, Schieberle, 1996). The reduction of these unsaturated volatile compounds by yeast to their corresponding alcohols has an impact on bread aroma; as such, a variation in yeast activity results in an altered aroma of the bread. Notably, it has been observed that *Saccharomyces cerevisiae* fully reduces unsaturated aldehydes to the corresponding alcohols (Vermeulen et al., 2007).

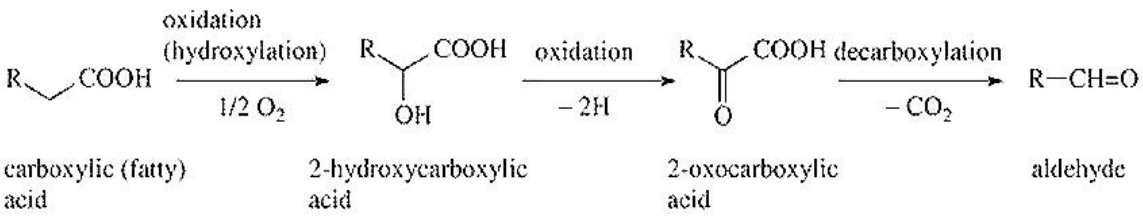


Figure 20: Formation mechanism of unsaturated aldehydes by α–oxidation of fatty acids (after Velisek (2014)).

The present work aimed at determining the impact of NaCl on the yeast metabolism in bread dough during fermentation and its influence on the overall aroma of the bread. More specifically, the unsaturated aldehydes (E)-non-2-enal (*fatty*) and (E,E)-deca-2,4-dienal (*fatty*), and the alcoholic compound phenylethan-2-ol (*rose-like*) were investigated as the key aroma compounds in bread based on preliminary analyses and supported by reports in the literature (Birch et al., 2013, Schuh and Schieberle, 2006, Schieberle, 1996, Gassenmeier and Schieberle, 1995, Frasse et al., 1992, Schieberle and Grosch, 1992, Schieberle and Grosch, 1991). A complementary analytical approach using two-dimensional high-resolution gas chromatography-mass spectrometry (2D-HRGC-MS) and proton-transfer-reaction mass spectrometry (PTR-MS) was used to quantify and monitor the generation of the selected aroma compounds in bread crumb samples with different levels of salt and yeast. Sensory analysis was performed on the bread crumb samples to determine their odour characteristics and assess the impact of salt reduction on bread crumb based on a discrimination triangle test.

6.3 Materials and Methods

6.3.1 Microbiology

Instant active dry yeast consisting of living cells of *Saccharomyces cerevisiae* (Panté; Puratos, Belgium) was diluted in Ringer solution at a concentration of 10^{-5} g mL^{-1}. 10 µL of the yeast solution was grown as a centre colony on yeast-selective potato dextrose agar plates containing different amounts of sodium chloride (NaCl) (0, 0.3, 1.2, 2.0, 3.0, and 4.0% w/w) at 30 °C for 8 days. The growth rate was recorded every day by measuring the diameter of the visible colonies.

6.3.2 Baking procedure and loaf analyses

Wheat bread was prepared by mixing Baker's flour (Odlums, Ireland), dry yeast (Puratos Group, Belgium), NaCl at levels of 0, 0.26, 1.04, 1.73, 2.60 and 3.46% (w/w) and tap water (water levels set to 500 Brabender units (BU) depending on the amount of NaCl using a Brabender farinograph) with a spiral mixer (Kenwood KM020). Considering an average bake loss of 13.5 % the NaCl concentrations in the final bread loaves resulted in 0, 0.3, 1.2, 2.0, 3.0 and 4.0% (Table 16). For dough samples with varying amounts of yeast, the

concentrations of water and NaCl relative to the mass of flour were kept constant at 61.75% and 0.49% w/w, respectively (

Table 17). After bulk fermentation for 15 min at 30 °C and 85% relative humidity, bread loaves (450 ± 1 g) were formed using a moulding machine (Machinefabriek Holtkamp B.V., Almelo, Netherlands). The loaves were then placed into non-stick pans (180 × 120 × 60 mm), fermented for 75 min at 30 °C and 85% relative humidity, and then baked for 35 min at 230 °C (top and bottom heating). The ovens were pre-steamed (0.3 L water) and then steamed when loaded (0.7 L water). After baking the loaves were removed from the pans and left to cool on cooling racks for 120 min at room temperature. Bake loss and specific volume were measured for all of the baked loaves. The bake loss was determined as the difference in mass between the dough and finished baked loaf. The specific volume was determined by a 3D laser scan using a VolScan Profiler 300 (Stable Micro Systems, UK).

6.3.3 Rheofermentometer

A rheofermentometer RheoF3 (Chopin Technologies, Villeneuve-la-Garenne, France) was used to evaluate the gaseous release and dough development of the different dough samples. Three hundred grams of each dough were prepared in the same manner as described below for baking trials. The tests were performed at 30 °C over a period of 90 min. A cylindrical weight of 1,500 g was applied onto the fermentation chamber. The total volume of CO_2, the volume of retention as well as the lost volume of CO_2 and the retention coefficient were determined. Results are presented as the average of three measurements.

Table 16: Ingredient quantities in the breads containing varying amounts of NaCl at constant yeast (1.2 % w/w relative to the mass of dough).

	0 % no NaCl			0.26 % low-NaCl			1.04 % standard level NaCl		
	rel. to flour w/w [%]	abs. [%]	[g]	rel. to flour w/w [%]	abs. [%]	[g]	rel. to flour w/w [%]	abs. [%]	[g]
Wheat flour	100.00	60.63	1818.95	100.00	60.92	1827.60	100.00	60.72	1821.49
Yeast	1.98	1.20	36.02	1.97	1.20	36.00	1.98	1.20	36.07
Tap water (30°)	62.95	38.17	1145.03	61.75	37.62	1128.54	61.00	37.04	1111.11
NaCl	0	0	0	0.43	0.26	7.86	1.72	1.04	31.33
Sum	164.93	100.00	3000.00	164.15	100.00	3000.00	164.70	100.00	3000.00

	1.73 % NaCl			2.60 % NaCl			3.46 % NaCl		
	rel. to flour w/w [%]	abs. [%]	[g]	rel. to flour w/w [%]	abs. [%]	[g]	rel. to flour w/w [%]	abs. [%]	[g]
Wheat flour	100.00	60.33	1809.85	100.00	60.13	1803.75	100.00	59.68	1790.40
Yeast	1.99	1.20	36.02	2.00	1.20	36.08	2.01	1.20	35.99
Tap water (30°)	60.90	36.74	1102.20	60.00	36.08	1082.25	59.75	35.66	1069.77
NaCl	2.87	1.73	51.94	4.32	2.60	77.92	5.80	3.46	103.84
Sum	165.76	100.00	3000.00	166.32	100.00	3000.00	167.56	100.00	3000.00

Table 17: Ingredient quantities in the breads containing varying amounts of yeast at constant NaCl (0.26 % w/w relative to the mass of flour).

Bread type	0.26 % low-NaCl - 1.5 % yeast			0.26 % low-NaCl - 0.9 % yeast			0.26 % low-NaCl - 0.6 % yeast			0.26 % low-NaCl- 0.3 % yeast		
Ingredients	rel. to flour w/w [%]	abs. [%]	[g]	rel. to flour w/w [%]	abs. [%]	[g]	rel. to flour w/w [%]	abs. [%]	[g]	rel. to flour w/w [%]	abs. [%]	[g]
wheat flour	100.00	60.73	1822.05	100.00	61.10	1833.07	100.00	61.29	1838.69	100.00	61.48	1844.34
Yeast	2.47	1.50	45.00	1.48	0.90	27.13	0.98	0.60	18.02	0.49	0.30	8.85
tap water (30°)	61.75	37.50	1125.11	61.75	37.73	1131.92	61.75	37.85	1135.39	61.75	37.96	1138.88
NaCl	0.43	0.26	7.83	0.43	0.26	7.88	0.43	0.26	7.91	0.43	0.26	7.93
Sum	164.65	100.00	3000.00	163.66	100.00	3000.00	163.16	100.00	3000.00	162.66	100.00	3000.00

6.3.4 Extraction of volatile aroma compounds

For the sample preparation the bread crumb was cut into 1 cm^3 cubes, deep frozen in liquid nitrogen and ground using a standard blender. The isotope-labelled standard solutions (Table 18) were added as internal standard to 50 ± 1 g of the ground crumb and the aroma compounds were extracted with 150 mL dichloromethane that was stirred at 120 rpm for 60 min at room temperature and then filtered to remove the suspension. This extraction step was repeated twice for each sample of 50 g crumb and the filtrates were combined. The extracts were purified using solvent-assisted flavour evaporation (SAFE) distillation (Engel et al., 1999). The distillates were concentrated down to a volume of 0.1 mL and these were subsequently stored at -20 °C prior to the analysis.

Table 18: Isotope-labelled standards of selected aroma compounds

	Concentration [µg mL^{-1}]
[$^2H_2$]-(E)-Non-2-enal	0.240
[$^2H_2$]-(E,E)-Deca-2,4-dienal	0.495
[2H4-5]-Phenylethan-2-ol	10.30

6.3.5 Two-Dimensional High-Resolution Gas Chromatography-Mass Spectrometry (2D-HRGC-MS)

Quantification of the selected aroma compounds was made using a two-dimensional high-resolution gas chromatography mass spectrometer (2D-HRGC-MS), with a cryogenic trapping system (CryoTrap; Gerstel, STADT, Germany) connecting the first GC system (Type 3800, Varian, Darmstadt, Germany) with a preparative DB-5 column to the second GC with a DB-FFAP column (each 30 m × 0.32 mm, 0.25 µm film thickness). The helium carrier gas flow was set to 1.5 mL min^{-1}. The initial temperature of the first GC oven was 40 °C and was subsequently heated at a rate of 8 °C min^{-1} to 230 °C. The eluting aroma compounds were transferred at defined retention times onto the cryo-trap, which was cooled to -100 °C. After thermal desorption at 250 °C, the volatiles were flushed onto the column in the second GC oven. The temperature of this second oven was increased from 40 °C to 250 °C at a rate of 6 °C min^{-1} and then held for 5 min at 250 °C. The eluting

compounds were analyzed with a Saturn 2200 mass spectrometer (Varian, Darmstadt, Germany) by chemical ionisation (CI) using methanol as the reagent gas.

6.3.6 Proton-Transfer-Reaction Mass Spectrometry (PTR-MS)

A high sensitivity proton-transfer-reaction mass spectrometer (hs-PTR-MS; IONICON Analytik GmbH, Innsbruck, Austria) was used to analyse the release of selected aroma compounds from the bread during fermentation. The instrument was operated at an electric field to number density ratio (E/N) of 132 Td, which was established with drift tube settings of 600 V, 2.2 mbar and 60 °C. The PTR-MS was set to measure in mass scan mode in the range *m/z* 20-130 at a dwell time of 500 ms per *m/z*. Five individual scans of 51 s duration were made per sampling period, resulting in a complete analysis time of 255 s. A 1 m long, 1/16" OD, 0.04" ID Silcosteel™ (Restek GmbH, Bad Homburg, Germany) sample inlet line, heated to 65 °C and with a flow of 500 mL min^{-1}, was used to transfer the sample gas to the instrument reaction chamber.

Dough samples of 300 g were placed in 1 L perfluoroalkoxy (PFA) containers (AHF Analysentechnik GmbH, Darmstadt, Germany) for the on-line measurement of volatiles in the headspace of the dough during fermentation at 30 °C and 85 % RH over 75 min. Five scan cycles of zero-air – i.e. VOC-free air – in the empty sample container were made at the beginning of each analysis to determine the background noise and the limit of detection of the system. The mean signals from these scans were subtracted from the sample signals to correct for this background.

The intensities of the *m/z* relating to the abundance of the selected aroma compounds in the headspace gas of the sample chamber were converted to concentrations (Lindinger et al., 1998). The data were screened for *m/z* 105 specific to phenylethan-2-ol which gets ionised to a cation by dihydroxylation and protonation. For the unsaturated aldehydes the respective *m/z* 141 of (E)-non-2-enal and *m/z* 153 of (E,E)-deca-2,4-dienal were too heavy to be detected.

6.3.7 Sensory analysis

Sensory analyses of the samples were performed via the aroma profile analysis (APA) technique. These descriptive analyses were performed using a trained panel of 15 members, with at least ten assessors participating in each individual sensory session. The panellists

were trained in weekly sessions to recognise the selected aroma compounds orthonasally at different odorant concentrations according to their odour qualities. Training was performed over a period of at least six months prior to participation in the actual sensory experiments and the performance of each panellist was assessed via standard procedures.

Bread loaves were cut into slices of ca. 2 cm thickness and the crust was removed. The yeasted dough and wheat flour bread samples were presented to the sensory panel for orthonasal assessment after storage in a closed glass beaker for 30 min at room temperature. The perceived odour qualities of the bread crumbs were described as being *yeast/dough-like* and *flour-like* based on a comparison to aqueous reference solutions The panel agreed on the characteristic odour attributes of each sample in a group discussion. The pure compounds used for the reference solutions were purchased from Sigma-Aldrich (Taufkirchen, Germany), Acros (Geel, Belgium) and AromaLab (Freising, Germany). Crumb samples were then presented again to the panel in a second sensory session to evaluate the intensities of the aforementioned odour attributes on a scale from 0 (not detectable) over 1 (weak intensity), 2 (medium intensity) to 3 (high intensity). The sensory score of each attribute was calculated as an arithmetic mean. The assessors were trained immediately prior to the analysis with aqueous odorant solutions at defined concentrations (factor 100 above the odour threshold) (Buttery et al., 1976, Czerny et al., 2008, Schuh and Schieberle, 2006).

Sensory triangle tests were additionally performed on selected sample pairs, namely 0.3% and 1.5% yeast, 0.3% and 1.2% NaCl, and 1.2% and 3.0% NaCl. The bread loaves were sliced and punched into uniform round pieces for presentation. The panellists were required to identify which of the three samples differed (forced-choice test). The tests were repeated for all combinations of each sample pair during each of the three independent sessions.

6.3.8 Statistical Analysis

Statistical analyses were performed on the sensory assessment results using Minitab for Windows statistical analysis software package (Systat Software, Inc., Chicago, IL, USA). The data were subjected to one-way analysis of variance (ANOVA). A Fisher's least significant difference (LSD) test was performed for multiple comparisons for cases when an F-test showed significant differences ($p<0.05$). Results are presented as the average of

three separate experiments with three independent samples from each batch, unless otherwise stated.

6.4 Result and Discussion

6.4.1 Microbiology and Rheofermentometer

Initial assessments of the impact of NaCl on activity and proliferation of the yeast – as determined by measuring the diameter of the spot colonies on yeast-selective agar plates with different amounts of NaCl – showed an exponential inhibition of yeast proliferation with increasing amounts of NaCl, as shown in Figure 21. These results reflect previously reported effects of NaCl on yeast. Increased amounts of NaCl in the growth environment reduce the number of viable yeast cells as well as the biomass of the culture, while the length of the log phase is increased (Almagro et al., 2000, Watson, 1970, Wei et al., 1982, Oda and Tonomura, 1993). The most important metabolite of yeast in bread dough is carbon dioxide (CO_2), which leavens the dough and increases the volume of the bread loaf. CO_2 can also be used as a monitor for the yeast activity in dough. The influence of NaCl on yeast performance in wheat dough was measured using a rheofermentometer (RheoF3, Chopin Technologies, Villeneuve-la-Garenne, France). Table 19 lists the parameters relating to the yeast performance in wheat dough during fermentation and indicates that the total volume of CO_2 produced decreased with increasing amounts of NaCl. The higher the amount of NaCl, the higher the osmotic pressure on the yeast cells, which leads to growth inhibition and an inhibitory impact on the yeast metabolism (Almagro et al., 2000, Watson, 1970, Matz, 1992). The non-significant difference between bread dough with 0% and 0.26% NaCl is due to the NaCl threshold concentration required to provide an environment with an inhibitory influence on yeast (Kawai et al., 1999, Oda and Tonomura, 1993). However, the higher the NaCl level, the higher the retention coefficient, which leads to a higher retention of CO_2 by the dough, as previously reported by Lynch et al. (2009). A lower CO_2 production results in a lower pressure on the membranes of the gluten network. In addition, there are several reports that gluten networks are strengthened by NaCl and can thereby retain more gas (Bernardin, 1978, Dal Bello et al., 2007, Lynch et al., 2009, Beck et al., 2012).

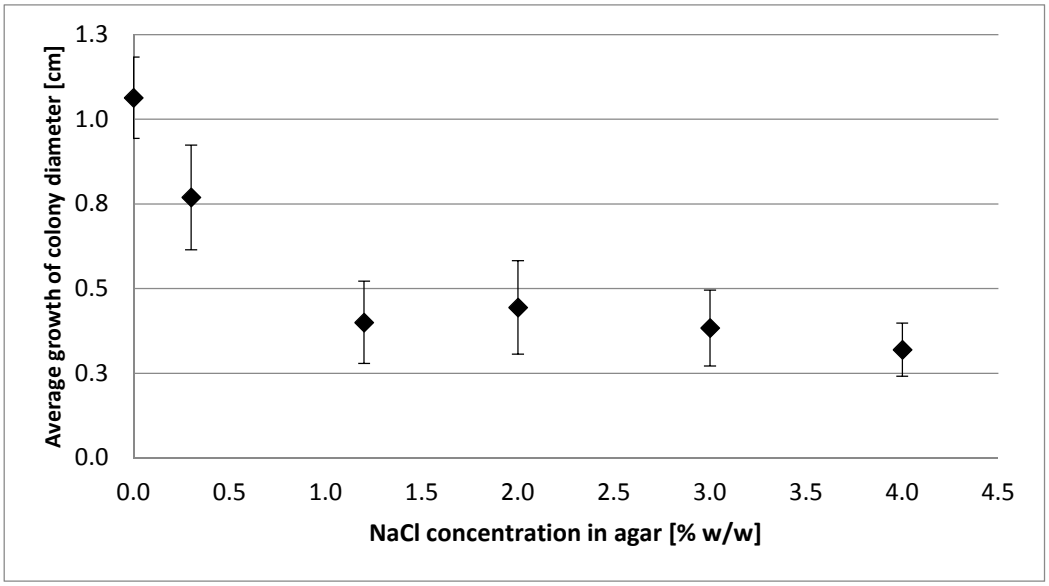


Figure 21: Growth of yeast on yeast-selective agar medium with different NaCl concentrations shown as the difference of the average diameters between growth day 2 and day 8.

Table 19: Yeast performance in wheat dough during fermentation in a rheofermentometer over a period of 3 h. Values followed by a different letter are significantly different ($p < 0.05$).

NaCl level [%]	Total volume [mL]	Volume of retention [mL]	Volume of CO_2 lost [mL]	Retention coefficient
0.00	$2241^{a} \pm 54$	$1435^{b} \pm 23$	$806^{g} \pm 75$	$64.1^{A} \pm 2.4$
0.26	$2108^{a} \pm 40$	$1459^{b} \pm 11$	$649^{d} \pm 49$	$69.2^{B} \pm 1.8$
1.04	$1581^{b} \pm 126$	$1337^{f} \pm 54$	$243^{h} \pm 78$	$84.8^{C} \pm 3.7$
1.73	$982^{c} \pm 28$	$953^{c} \pm 22$	$30^{i} \pm 8$	$97.0^{D} \pm 0.7$
2.60	$573^{d} \pm 15$	$569^{d} \pm 14$	$4.0^{k} \pm 1.7$	$99.4^{E} \pm 0.2$
3.46	$313^{e} \pm 11$	$311^{e} \pm 11$	$2.0^{l} \pm 0.0$	$99.4^{E} \pm 0.1$

*values with the same superscript letter are not significantly different ($p < 0.05$)

6.4.2 Specific bread loaf volume and bake loss

The specific volume and bake loss of the baked bread loaves were determined as a standard quality parameter. The specific loaf volume decreased significantly for increasing amounts of NaCl at a yeast level of 1.2%. The bread containing no NaCl did not show a significantly larger volume than the bread with 0.3% NaCl, despite its water level being the highest at

62.95% (Table 16). Although the dough was mixed to a standard consistency of 500 BU, this observation might be explained by a weaker gluten network in the absence of NaCl (Beck et al., 2012, Lynch et al., 2009, Dal Bello et al., 2007, Bernardin, 1978). The data from the rheofermentometer corroborate this hypothesis; the generation of a non-significant higher amount of CO_2 did not lead to a greater bread volume. The higher the NaCl concentration, the smaller was the bread volume and surface area, and less water could evaporate, which again is supported by the rheofermentometer data. The bake loss expressed as a percentage of the water level for each of the individual bread loaves showed that small adjustments of the water level to achieve a dough consistency of 500 BU did not significantly influence the bake loss (Table 20 and 21).

The breads with yeast levels ranging from 0.3 % to 1.5 % at a constant NaCl level of 0.3 % w/w had the highest specific volume at 0.6 %. At lower yeast concentrations the amount of yeast did not produce sufficient CO_2 to stretch the gluten network to its maximum. At concentrations higher than 0.6 % the specific volume decreased with increasing amounts of yeast. Similarly, increasing amounts of yeast led to excessive fermentation and a resulting expansion of the gluten network, thereby increasing the loss of CO_2 (Oda and Tonomura, 1993, Watson, 1970, Lynch et al., 2009).

Table 20: Specific volume and bake loss of bread loaves with different NaCl concentrations and standard yeast level of 1.2 % w/w. The bake loss is shown as a percentage of the dough mass as well as a percentage of the water level.

NaCl concentration [% w/w]	**Specific volume [mL/g]**	**Bake loss**	
		[% w/w]	**[% of water level]**
0	3.85 ± 0.13^{a}	$14.9 \pm 0.5^{a,b}$	$39.1 \pm 1.2^{a,b,c}$
0.3	3.81 ± 0.08^{a}	15.3 ± 0.3^{a}	40.6 ± 0.8^{a}
1.2	3.49 ± 0.09^{b}	14.4 ± 0.4^{b}	38.6 ± 0.9^{b}
2.0	3.11 ± 0.05^{c}	13.4 ± 0.5^{c}	37.4 ± 0.6^{c}
3.0	2.52 ± 0.02^{d}	12.3 ± 0.4^{d}	32.7 ± 1.0^{d}
4.0	1.93 ± 0.03^{e}	10.6 ± 0.3^{e}	28.6 ± 0.8^{e}

Values labelled with the same superscript letter in the same column are not significantly different ($p < 0.05$)

Table 21: Specific volume and bake loss of bread loaves with different yeast concentrations and standard NaCl concentration of 0.3 % w/w. The bake loss is shown as a percentage of the dough weight as well as a percentage of the water level.

Yeast concentration [% w/w]	**Specific volume [mL/g]**	**Bake loss**	
		[% w/w]	**[% of water level]**
0.3	3.02 ± 0.02^{a}	13.4 ± 0.2^{a}	36.2 ± 0.5^{a}
0.6	3.70 ± 0.05^{b}	14.7 ± 0.2^{b}	39.8 ± 0.7^{b}
0.9	$3.62 \pm 0.19^{b,c}$	14.7 ± 0.2^{b}	39.7 ± 0.6^{b}
1.2	$3.49 \pm 0.09^{c,d}$	14.4 ± 0.4^{b}	38.6 ± 0.9^{b}
1.5	3.37 ± 0.10^{d}	$13.9 \pm 0.5^{a,b}$	38.2 ± 0.9^{b}

*values labelled with the same superscript letter in the same column are not significantly different ($p < 0.05$)

6.4.3 Analyses of volatile aroma compounds in bread crumb

Three key aroma compounds in wheat bread crumb are the alcoholic compound phenylethan-2-ol (Ehrlich, 1907b, Czerny and Schieberle, 2006, Hazelwood et al., 2008b, Dickinson et al., 1997) and the unsaturated aldehydes (E)-non-2-enal and (E,E)-deca-2,4-dienal (Frasse et al., 1992, Vermeulen et al., 2007). These three compounds were analysed in the aroma extracts of bread crumb by 2D-HRGC-MS.

Phenylethan-2-ol decreased in concentration exponentially with increasing amounts of NaCl, but increased significantly with increased amounts of yeast, albeit not for the highest yeast concentrations (0.9%, 1.2% and 1.5% w/w).

The concentrations of (E)-non-2-enal differed significantly for 3% and 4% NaCl compared to the lowest NaCl concentrations of 0-0.6%. Increasing the yeast content led to a decrease in (E)-non-2-enal with a significance of $p<0.05$ for the samples 0.3% and 0.6% compared to the samples 0.9% and 1.5%. (E,E)-Deca-2,4-dienal did not show any significant change for the different NaCl concentrations, but decreased with increasing amounts of yeast (Table 22). These changes can be explained by the reducing activity of the baker's yeast. Decreasing the yeast concentration or inhibiting the yeast with more salt, lowers the overall yeast activity during the fermentation process, thereby resulting in a lower production of the unsaturated aldehydes. The reducing activity of yeast was reported before (Chwastowski and Koloczek, 2013). Furthermore, Saison et al. (2010) demonstrated a change in beer aroma production based on the reducing activity of (E)-non-2-enal by *S. cerevisiae*, suggesting that the reducing activity metabolism of yeast is not affected by increased concentrations of NaCl in the same manner as the Ehrlich pathway or other parts of the yeast metabolism.

Table 22: Concentration of phenylethan-2-ol, (E)-non-2-enal and (E,E)-deca-2,4-dienal in bread crumb samples as determined by 2D-HRGC-MS. Values followed by a different letter within a column are significantly different ($p < 0.05$).

NaCl concentration [% w/w]	Concentration [µg kg^{-1}]		
	Phenylethan-2-ol	(E)-Non-2-enal	(E,E)-Deca-2,4-dienal
0.0	4441 ± 686^{a}	12.88 ± 1.73^{a,b}	12.70 ± 1.06^{a}
0.3	3055 ± 549^{a}	13.51 ± 1.15^{a,b}	11.07 ± 0.74^{a}
1.2	1582 ± 6^{b}	12.10 ± 0.31^{b}	8.50 ± 0.83^{b}
2.0	1685 ± 88^{c}	15.90 ± 2.55^{a,c}	9.23 ± 1.32^{a,b}
3.0	1189 ± 9^{d}	16.42 ± 1.19^{c}	10.55 ± 1.97^{a,b}
4.0	845 ± 58^{e}	20.75 ± 2.69^{d}	9.25 ± 0.72^{a,b}

	Concentration [µg kg^{-1}]		
Yeast concentration [%]	Phenylethan-2-ol	(E)-Non-2-enal	(E,E)-Deca-2,4-dienal
0.3	1164.00 ± 64.13^{a}	19.67 ± 0.04^{a}	20.16 ± 1.75^{a}
0.6	1773.00 ± 40.00^{b}	16.50 ± 1.87^{b}	17.89 ± 3.05^{a}
0.9	2606.60 ± 165.27^{c}	10.18 ± 2.21^{c,d}	11.38 ± 0.65^{b}
1.2	3054.75 ± 548.90^{c,d}	13.51 ± 1.15^{b,c}	11.07 ± 0.74^{b}
1.5	3287.83 ± 315.12^{d}	7.98 ± 2.31^{d}	8.63 ± 0.14^{c}

6.4.4 PTR-MS analyses of bread dough during fermentation

The development of the aroma profile of the bread dough samples with different salt and yeast concentrations over a 90 min period of fermentation at 3 °C and 85 % RH was monitored on-line by PTR-MS. The PTR-MS data for the sweet, *rose-like* odour compound phenylethan-2-ol at *m/z* 105 in the headspace gas of the dough showed that its concentration increased for increasing amounts of yeast varying from 0.3-1.5 % (Figure 22). The more NaCl was added to the bread samples the less phenylethan-2-ol was present due to the inhibiting effect of salt on yeast (Figure 23).

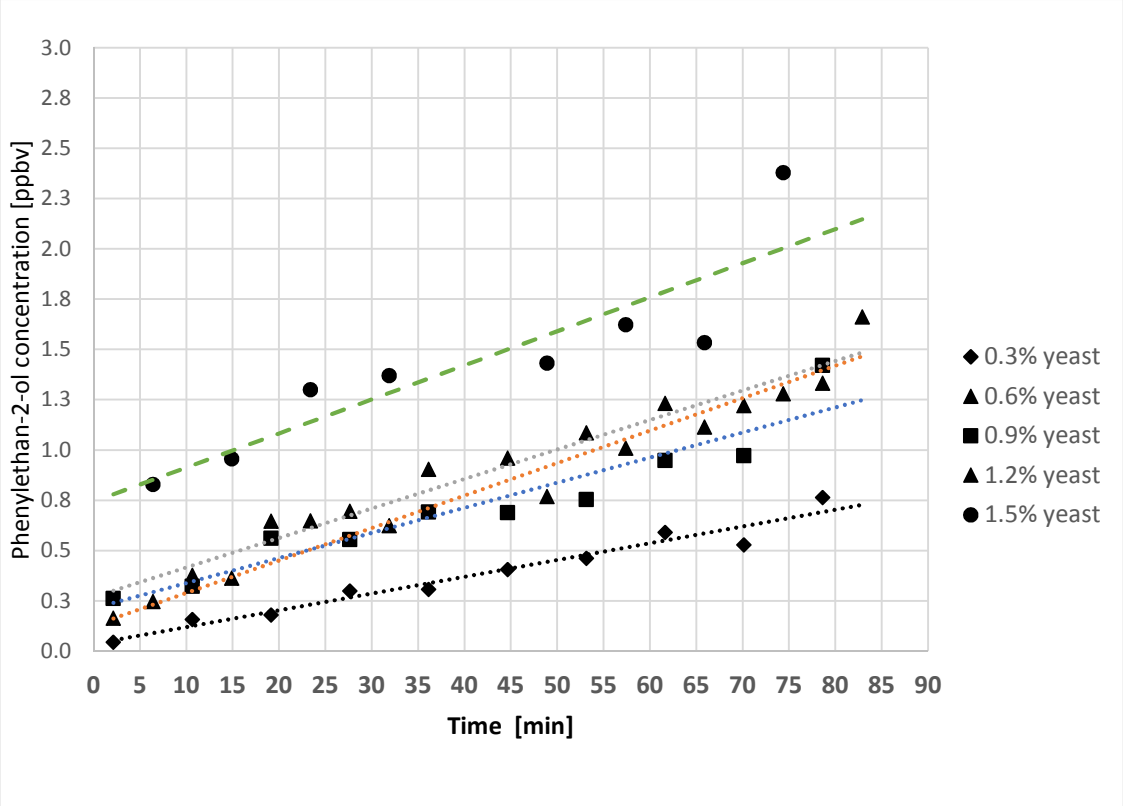


Figure 22: PTR-MS headspace analysis of phenylethan-2-ol of bread dough with different yeast concentrations over 90 min of incubation under fermentation conditions (30 °C, 85 % RH).

The headspace concentrations of phenylethan-2-ol correlate with the concentration of this compound in the respective bread crumb samples, as measured by 2D-HRGC-MS. Correlations with $R^2 \geq 0.75$ were found between the yeast metabolites CO_2 and phenylethan-2-ol or (E)-non-2-enal, indicating that they must arise during yeast metabolism, which is influenced by different amounts of NaCl in bread dough. A negative correlation for the unsaturated aldehyde (E)-non-2-enal shows that the reduction capacity of yeast increased with increasing yeast activity and hence, an increased amount of (E)-non-2-enal was reduced to the corresponding alcohol (Figure 24).

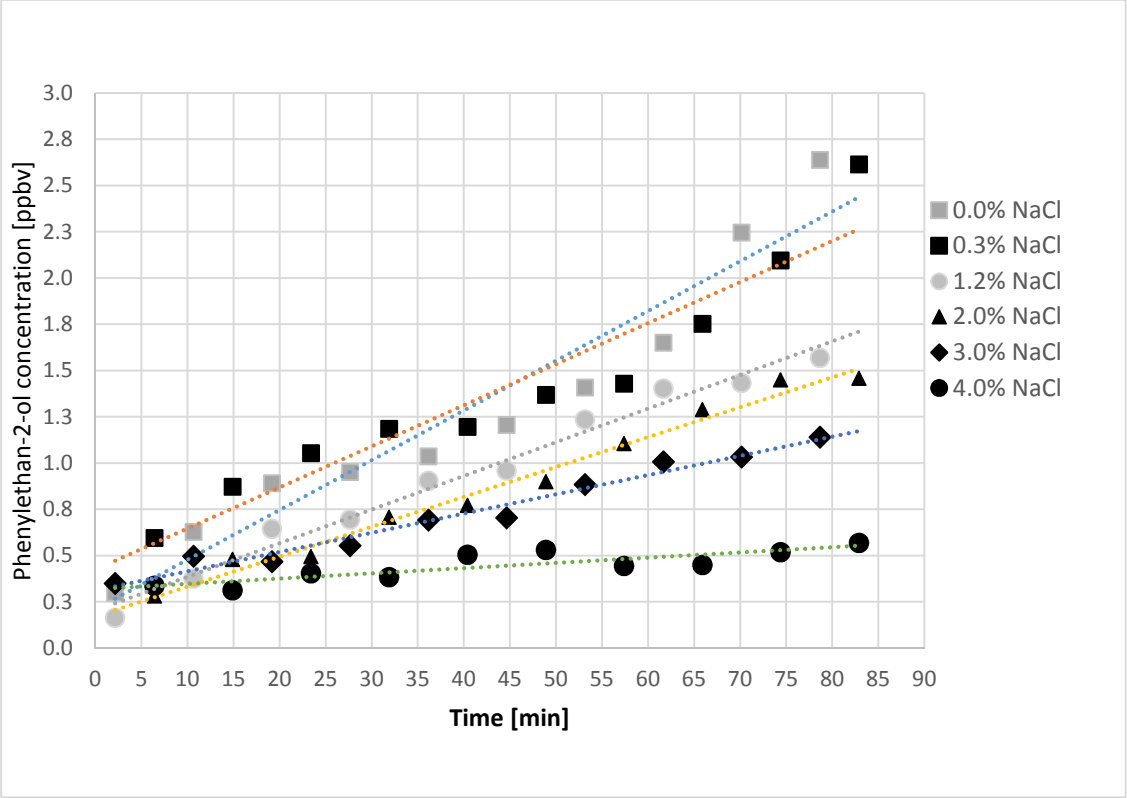
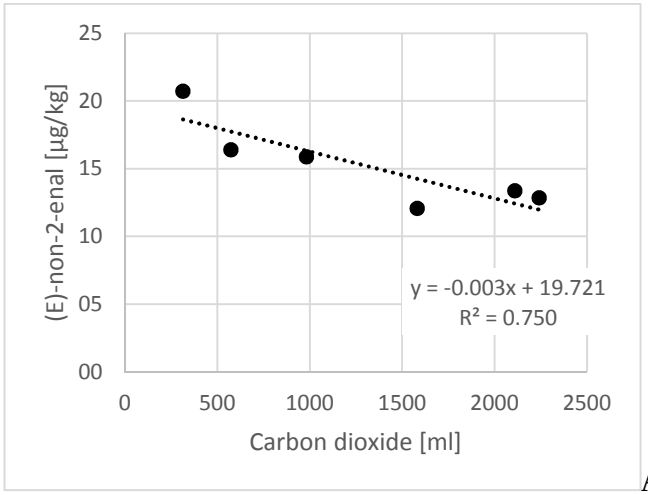
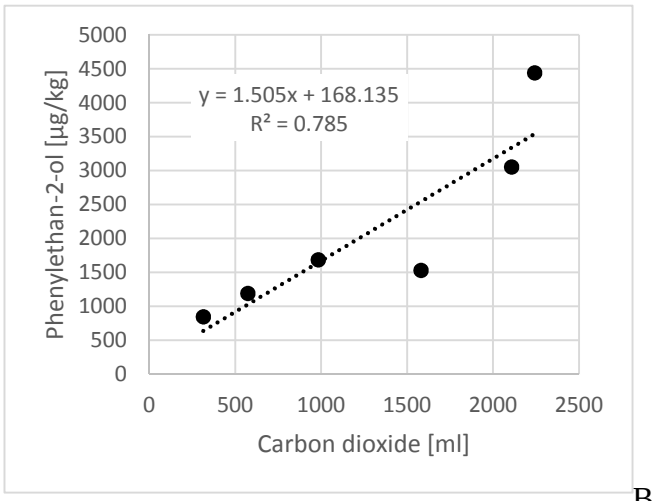


Figure 23: PTR-MS headspace analysis of phenylethan-2-ol of bread dough with different salt concentrations over 90 min of incubation under fermentation conditions (30 °C, 85 % RH).



A



B

Figure 24: Correlation between different yeast metabolites. A: CO_2 and (E)-Non-2-enal; B: CO_2 and phenylethan-2-ol

6.4.5 Descriptive Sensory Evaluation

Considering the described impact of salt on the aroma relevant analyts it had to be established whether the measured significant differences are noticeable for the consumer. Hence, aroma profile analyses of bread with different salt and yeast levels were performed describing the impact on the odour characteristics in detail. Therefore, the specific odour attributes were identified by the sensory panel followed by training sessions using solutions of standard aroma compounds. Each set of samples was tested 3 times ranking the attributes on a scale between 0 and 3.

The aroma profile analyses of the bread crumb samples revealed significant differences ($p<0.05$) only for the attributes “cheesy” and “rose-like”. The “cheesy” aroma was significantly different ($p<0.05$) between the samples with the highest three NaCl levels (2, 3, and 4% w/w) and the lowest three levels (0.0, 0.3, and 1.2% w/w NaCl). In the samples with varying yeast levels only the highest (1.5% yeast w/w) and lowest (0.3% yeast w/w) level differed significantly with respect to the “cheesy” odour impression. Butyric acid is well-known as a volatile metabolic compound of yeast with a “cheesy” odour note, and it listed in the Yeast Metabolic Database (YMDB) ID 01392 (Jewison et al., 2012). The results correlate with the yeast activity during fermentation. Increased amounts of yeast as well as decreasing salt levels result in higher yeast activities and hence, in a higher metabolism rate including the metabolite butyric acid, which results in a more intense “cheesy” odour.

The attribute *“rose-like”* determined by the sensory panel correlated directly with the analysed amounts of phenylethan-2-ol and hence, with the yeast activity (Figure 25). Increasing yeast activity during dough fermentation of the bread samples led to higher concentrations of the analyt phenylethan-2-ol. The lower the salt level or the higher the yeast level; the more intensive was the “rose-like” odour recognised by the sensory panel (Figure 26). At 0.0% NaCl w/w the panel did not perceive a “rose-like” aroma. This can be explained with a totally excessive and uncontrolled yeast activity in the complete absence of salt. Odour impressions described as “cheesy” and “buttery” dominated the overall odour characteristic and covered the “rose-like” impression. The same effect is shown for an increased amount of yeast above 0.9% w/w. The “rose-like” compounds, namely phenylethan-2-ol and 2-phenylacetic acid, have higher odour thresholds compared to the other considered aroma components and therefore the “rose-like” aroma fraction can easily be dominated by other volatile aroma compounds or influence the recognition (Czerny et al., 2008).

The “fatty” impression in the samples with varying NaCl content did not vary significantly, which reflects the 2D-HRGC-MS analyses of the unsaturated aldehydes (E)-non-2-enal and (E,E)-deca-2,4-dienal, both of which have characteristic “fatty” odour impressions. By contrast, significantly different concentrations of both aldehydes were found in the samples of varying yeast content, as determined using 2D-HRGC-MS. However, the sensory panel could not differentiate between these samples. This might be due to an increasing yeast

activity, which predominantly produces other volatile aroma compounds such as butyric acid and Ehrlich pathway metabolites which results in the determined increase of buttery aroma (Figure 25 and 26).

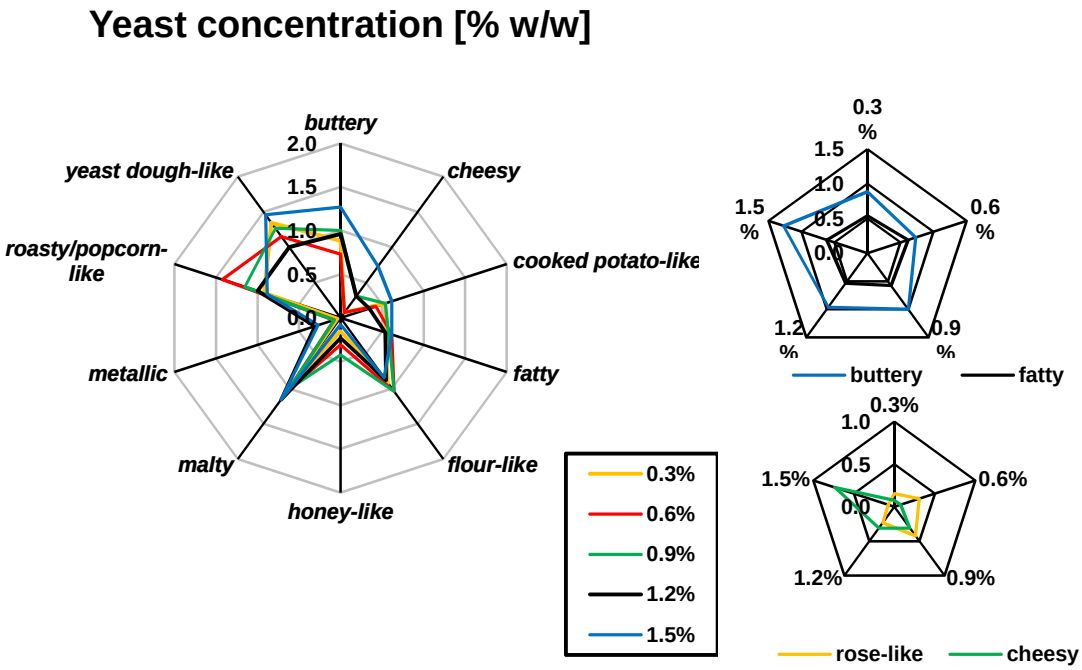


Figure 25: Aroma profile of bread crumbs with different yeast levels.

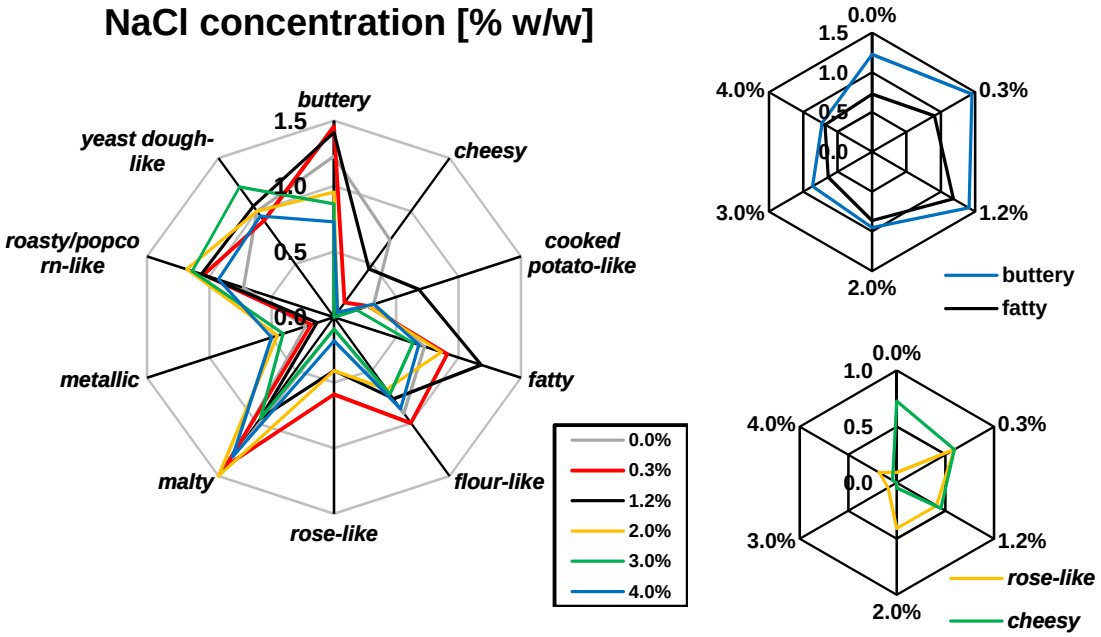


Figure 26: Aroma profile analysis of bread crumbs with different salt levels.

6.4.6 Sensory triangle test

Triangle discrimination tests were performed for the sample pairs 0.3 % and 1.5 % w/w yeast; 0.3 % and 1.2 %, and 1.2 % and 3.0 % w/w NaCl to determine whether the described changes of the volatile aroma fraction can be recognized by consumers or not. The sample pairs were chosen based on a “standard-salt” level of 1.2 % w/w NaCl (Gormley and Morrissey, 1993), a “low-salt” level of 0.3 % w/w NaCl, and an “high-salt” level of 3.0 % w/w NaCl. The sensory panel assessed the bread crumb samples orthonasally in triplicate. Sensory analysis of the two sample pairs with different salt levels indicated that there was no significant difference between the pairs ($p>0.2$). The sample pair with 0.3 % and 1.5 % w/w yeast was significantly distinguishable ($p<0.1$). These findings show that salt reduction has no significant influence on the overall volatile aroma fraction of the bread crumb. The sensory results show several changes for isolated attributes and compounds. But the aroma profile as a whole does not change to an extent where a consumer can recognize a change of odour.

6.5 Conclusion

The influence of yeast activity on the volatile aroma compounds of bread crumb was investigated using sensory assessments in combination with two-dimensional high resolution gas chromatography mass spectrometry (2D-HRGC-MS), and proton-transfer-reaction mass spectrometry (PTR-MS). A correlation between different yeast metabolites was shown. The metabolic pathways in yeast cells seem to correlate with the reducing activity independently of the amount of yeast present or the concentration of sodium chloride. A 5-fold increase in yeast concentration from 0.3 % to 1.5 % w/w was distinguished by the sensory panel. This increase related to an increased yeast activity during the fermentation process and its impact on the bread crumb aroma (Birch et al., 2013). However, a reduction in the sodium chloride concentration and the observed changes in the aroma compounds, as determined by 2D-HRGC-MS and PTR-MS, were proven to be insignificant by the sensory panel. A reduction in sodium chloride from the standard concentration of 1.2 % w/w down to 0.3 % w/w increased the yeast activity but the increase in volatile aroma components could not be detected by the sensory panel. Hence, it can be stated that sodium chloride reduction does not influence the volatile aroma of bread significantly and consumers in general are not able to recognise any changes by

the odour of bread crumb with reduced amounts of salt. While salt reduction in bread impacts on the quality characteristics of taste, shelf-life and texture, the aroma quality remains unchanged.

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Chapter 7

General Discussion

7.1 General Discussion

The target of a reduced daily salt intake of 6 g is recommended by global institutions (WHO and FAO, 2003, Codex Alimentarius, 1997) as well as by national authorities (European Commission, 2008, FDA, 2005) due to the detrimental impact on human health causing hypertension (Farquhar et al., 2015, du Cailar et al., 2002), which is linked with cardiovascular diseases (O'Donnell et al., 2015, MacGregor and de Wardener, 1998). Since bread has the highest individual contribution to the daily salt intake, any reduction in the salt levels present in bread would equate to a significantly decreased sodium intake in society (Angus, 2007). Reduction of salt in yeasted bread influences many quality characteristics which are important for consumer acceptance and industry suitability. The technological process of bread baking as well as some of the final quality characteristics of bread such as shelf life (Samapundo et al., 2010, Pateras, 2007, Filtenborg et al., 1996), aroma and flavour profile, (Lynch et al., 2009) and crumb structure (Beck et al., 2012) are affected by the reduction of NaCl.

A literature review, as part of the thesis (Chapter 2), revealed that doughs with reduced salt levels possess different rheological behaviour in comparison to regular dough. The reduced dough consistency as well as the decrease of the viscous and elastic moduli and the potential changes of dough stickiness must be considered for industrial processes. Also the shortened dough development time in systems with reduced levels of salt can require adjustments of the mixing process. The earlier start of starch gelatinisation does not significantly influence the final bread quality characteristics. Technologically, the production of bread reduced in salt is feasible, but the sensory characteristics of bread have to be adjusted to meet the consumer’s expectations. The use of sourdough is promising and natural alternative since it has been shown to improve bread quality. In particular, its effect

on flavour and the influence on further sensory characteristics, like crumb texture, can be used to improve the quality of salt reduced bread (Arendt et al., 2007).

NaCl acts as a preservative in bread and any reduction of NaCl decreased the shelf-life of bread significantly by approximately 2 days (Chapter 3). The reduction of shelf-life correlated with the determined values of a_w, pH and TTA. Calcium propionate as well as sourdough fermented with the selected antifungal strain *Lactobacillus amylovorus* (DSM19280) had the potential to compensate the lack of NaCl. For the use of sourdough fermented with *L. amylovorus* DSM19280 the NaCl concentration hardly influenced the preservation effect due to the wide range of antifungal compounds produced by the strain (Ryan et al., 2011, Arendt et al., 2009), which predominated the antifungal effect shown by NaCl. Compared to the respective sourdough breads, the addition of calcium propionate prolonged the shelf-life of breads only when challenged against *P. expansum* (all NaCl concentrations) and against *A. niger* (1.2 % NaCl). Under the environmental contamination conditions of a pilot scale bakery, bread slices were better mould-preserved by the antifungal sourdough than by calcium propionate. Hence, sourdough was the preferred option as it gives also an improvement of flavour and texture (Arendt et al., 2007). Moreover, sourdough is recognised as a natural "green" preservative and is more widely accepted by the consumer. The use of sourdough as a novel approach to prolong the microbial shelf-life of low-salt bread was proven to be a viable option to ensure the microbial integrity of low-salt bread.

The beneficial impact of sourdough addition on low-salt bread was investigated further using two functional lactic acid bacteria strains; the antifungal strain *L. amylovorus* DSM19280 and the EPS producing strain *W. cibaria MG1* (Chapter 4). The use of *L. amylovorus* DSM19280 had previously been shown to prolong the microbial shelf life significantly (Axel et al., 2015, Belz et al., 2012). The present study determined the minimum amount of 6% sourdough addition to compensate the reduced shelf life caused by the salt reduction. The antifungal effect was mainly based on the determined antifungal compounds 3-phenyllactic acid, 4-hydroxyphenyllactic acid, leucic acid and azelaic acid. Each of those detected compounds has been reported previously to have antifungal activity (Sakko et al., 2014, Guo et al., 2012, Dal Bello et al., 2007, Lavermicocca et al., 2003). Due to the presence of the antifungal compounds, the shelf life of a low-salt bread of about

4 days could be prolonged to the same shelf life of a standard salt bread of about 6 days with the addition of 6% of the *L. amylovorus* DSM19280 sourdough. The strain *W. cibaria* MG1 was found to produce a high molecular dextran EPS at an amount of 4.5 g kg^{-1} of sourdough which was previously reported for wheat sourdough by (Galle et al., 2010). The increase of bread volume and bread crumb porosity as well as delaying the bread staling significantly was determined for an optimum addition level of 18%. These findings are in line with previous studies where EPS sourdough increased the bread loaf volume significantly for different flour sourdoughs (Wolter et al., 2014b, Galle et al., 2012, Wolter et al., 2014a). The fundamental rheology analyses showed a significant dough softening with increasing acidification due to sourdough addition. Enhanced enzymatic activity due to a lowered pH during the fermentation process caused partial starch degradation as well as the weakening of the gluten network by increased protease activity (Thiele et al., 2002, Bleukx and Delcour, 2000). Hence, the use of sourdough will change the dough handling, processing may need to be adapted to a softer dough. The descriptive sensory analyses distinguished mainly between a more “roasted” and “yeasty” vs a more “salty” and “dough” perception. The samples at no/lower NaCl levels and the more acidified samples mainly based on the addition of *L. amylovorus* DSM19280 sourdough tended to have a more “yeasty” and “roasted” profile whereas the standard salt levels as well as the control breads and the low acidified samples mainly based on the addition of *W. cibaria* MG1 resulted in a more “salty” and “dough” perception. The combination of both sourdoughs lead to a low-salt bread with improved quality characteristics. The beneficial characteristics of both functional lactic acid bacteria strains could be combined resulting in an improved shelf life, a softer bread crumb, increased bread volume and an improved sensory profile.

Chapter 5 presents the lactic acid bacteria strain *L. reuteri* FF2hh2 which was proven to have multifunctional characteristics producing EPS as well as antifungal compounds. Compared to the approach in Chapter 4, a more efficient option could be provided to compensate the lack of shelf life as well as some changes in flavour, using the multifunctional *L. reuteri* FF2. The antifungal activity was proven *in vitro* on plate assays as well as *in vivo* in the sourdough bread system showing significant antifungal activity against *P. roqueforti* and *F. culmorum*. The screening for antifungal compounds resulted in the presence of leucic acid, hydroxyphenyllactic acid, phenyllactic acid and vanillic acid which have all been found in different fermentation processes and reported to have an

antifungal activity (Axel et al., 2015, Sakko et al., 2014, Ryan et al., 2009, Broberg et al., 2007, Lavermicocca et al., 2003). The shelf life of the low-salt bread could be prolonged by about 2-3 days with the addition of 6% - 18% of sourdough fermented with *L. reuteri* FF2hh2 compensating the reduction of salt. The full potential of this strain could be shown for the highest addition level resulting in a shelf-life of 13 days. Significant amounts of EPS could be determined at an amount of 5.2 g kg^{-1} of sourdough. As reported by Galle et al. (2010) for different *Weisella* strains, the addition of 10% sucrose to the sourdough fermentation favoured the EPS production in combination with the presence of maltose naturally present in the flour. The high level of EPS impacted positive on crumb characteristics as well as bread loaf volume and reduced the staling rate significantly (Di Monaco et al., 2015, Wolter et al., 2014a, Katina et al., 2009). The bread loaf volume increased significantly for the sourdough addition of 6%-18% and crumb structure got more porous with increasing amounts of sourdough. The increased crumb cell surface area and the presence of organic acids significantly influenced the perception of salt of the bread samples containing sourdough compared to the control breads with the same amount of NaCl (Pflaum et al., 2013, Konitzer et al., 2013). The descriptive analyses could clearly distinguish the samples with sourdough addition based on the flavour attribute “salt” as well as on their crumb density which highlights the correlation between the two attributes. With the novel lactic acid bacteria strain *L. reuteri* FF2hh2, a multifunctional and very interesting strain could be presented. A first application could be demonstrated successfully based on a low-salt bread. It shows a natural way to compensate the lack of salt and changes in product quality of yeasted bread based on salt reduction.

The influence of yeast activity on the volatile aroma compounds of bread crumb was investigated in Chapter 6 using sensory assessments in combination with two-dimensional high resolution gas chromatography mass spectrometry (2D-HRGC-MS), and proton-transfer-reaction mass spectrometry (PTR-MS). A correlation between the yeast metabolites carbon dioxide and phenylethan-2-ol as well as (E)-non-2-enal could be shown. The metabolic pathways in yeast cells seem to correlate with the reducing activity of yeast independently of the amount of yeast present or the concentration of sodium chloride. A 5-fold increase in yeast concentration from 0.3 % to 1.5 % w/w could be distinguished by the sensory panel based on the “cheesy” and “fatty” odour attributes. The higher the amount of yeast the more intense the “cheesy” and “fatty” odour perception. A similar trend was

determined for the salt concentration resulting in a more “cheesy” and “fatty” perception the lower the salt concentration was. This increase related to an increased yeast activity during the fermentation process and its impact on the bread crumb aroma (Birch et al., 2013). However, a reduction in the sodium chloride concentration and the observed significant changes in the aroma compounds, as determined by 2D-HRGC-MS and PTR-MS, were proven to be insignificant by the sensory panel. A reduction in sodium chloride from the standard concentration of 1.2% down to 0.3% increased the yeast activity but the increase in volatile aroma components could not be detected by the sensory panel. Hence, it can be stated that sodium chloride reduction does not influence the volatile aroma of bread significantly and consumers in general are not able to recognise any changes by the odour of bread crumb with reduced amounts of salt. While salt reduction in bread impacts on the quality characteristics of taste, shelf-life and texture, the aroma quality remains unchanged.

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Appendix

List of Publications

1 Zannini E., Moroni A., **Belz M. C. E.,** Faltermaier A., Arendt E. K., 2013: Bread, Chapter Sixteen, In: Bread and other baked goods. Bamforth C. (Ed.), Oxford University Press.

2 **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 Micobiology and Biotechnology*, 96 (2):493-501.

3 **Belz M. C. E.**, Ryan L. A. M., Arendt E. K., 2012: The impact of salt reduction on bread: a review. – *Critical Reviews of Food Science and Nutrition*, 52 (6):514-24.

Oral presentations at scientific conferences/seminars

1 **Belz M. C. E.,** Zannini E., Czerny M., Arendt E. K., 2012: Salt reduction in bread - Sourdough as a promising solution – *5th Symposium on Sourdough: Cereal Fermentation of Future Food*, Helsinki, Finland, 2012.

2 **Belz M. C. E.,** Wiegand F., Zannini E., Arendt E. K., 2012: Die Beeinflussung des Hefemetabolismus durch Natriumchlorid während der Brotteigfermentation – *63. Tagung für Getreidechemie (63rd Conference of Cereal Chemisrty)*, Detmold, Germany, 2012.

3 **Belz M. C. E.,** Zannini E., Cashman K. D., Arendt E. K., 2011: Salt reduction in bread – *Baking Industry Workshop*, UCC Cork, Ireland, 15th November 2011.

4 **Belz M. C. E.,** Dal Bello F., Zannini E., Cashman K. D., Arendt E. K., 2011: Salzreduktion in Brot: Die Bedeutung von Sauerteig – 62. *Tagung für Getreidechemie (62nd Conference of Cereal Chemisrty)*, Detmold, Germany, 29th June 2011.

5 **Belz M. C. E.,** Dal Bello F., Zannini E., Cashman K. D., Arendt E. K., 2011: Salzreduktion in Brot – Die Bedeutung von Sauerteig. – *4th Forum Sauerteig, Minden*, Germany, 26th May 2011.

6 **Belz M. C. E.,** Eder D., Zannini E., Arendt E. K., 2011: Fundamental studies on the effect of salt reduction on dough and bread characteristics. – *C&E Spring Meeting*, Munich, Germany, 12th April 2011.

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