The Impact of Different Fermentations on the Antifungal Activity of Sourdough Bread

by

Zoe Bumanis

A thesis submitted in partial fulfilment of the requirements for the degree of

Master of Science

in

Food Science and Technology

Department of Agricultural, Food, and Nutritional Science

University of Alberta

Abstract

Bread is considered a staple food in North America and Europe, but due to its neutral pH and high water activity it is vulnerable to spoilage by *Penicillium* spp., a problem that costs the commercial baking industry millions each year and contributes significantly to global food waste. Common methods of chemical preservation like calcium propionate (CalPro) and sorbic acid are falling out of favour with ingredient-conscious consumers, leaving a hole in the market for a bread with few or reduced preservatives. Sourdough, a mixture of water and flour fermented by lactobacilli and yeasts, is often used in bread production to improve flavour, texture, and to contribute to leavening. It has also displayed preservative effects against mould which increase the shelf life of the product.

The antifungal impact of 12 strains of lactobacilli incorporated into 10% sourdough breads were compared to one another and revealed to result in equivalent shelf life when inoculated with the highly-resistant spoilage organism *Penicillium roqueforti*. However, altering the time and temperature combinations used in fermentation and proofing protocols resulted in an increase in shelf life which matched that of bread containing 0.3% calcium propionate, even in a 'worst-case' exposure scenario. It was also determined that a combination of 0.1% calcium propionate and sourdough was similarly effective in delaying fungal spoilage. The inclusion of buckwheat in the fermentations and the subsequent impact on shelf life was recorded for the first time, and the benefits of isomerized α-acids sourced from hops were supported by the findings of this study, which identified both additives as effective ways to extend the mould-free shelf life of sourdough bread past what is possible using only wheat flour.

This research posits that a three-pronged approach utilising the products of sourdough fermentation, antifungal adjuncts, and a clean processing area can not only reduce instances of fungal contamination but also lead to improved resistance and extended shelf life when contamination does occur in preservative-free or reduced-preservative bread, providing a benefit that cannot be achieved by any one of these factors alone.

This thesis is dedicated to my incredible parents, Jeff Clarke and Sarah Bumanis. Thank you for helping me grow into the person I am today.

Acknowledgement

I would like to first and foremost thank Dr. Michael Gänzle for the many hours of feedback and advice, and your willingness to share your invaluable knowledge on anything and everything sourdough. Your calm, unwavering sureness in me and my abilities as a researcher lent me confidence that I didn't always have, and working with you gave me a chance to build those skills until I believed in them as well. I would also like to thank Dr. Lynn McMullen, not only for all the advice and feedback provided to me in your time as my committee member, but also for your contagious enthusiasm for food safety and research. I extend my gratitude to both NSERC and Grupo Bimbo for funding my research over the last 2 years.

Thank you as well to the friendly, intelligent, and dedicated members of lab 2-50, all of whom I'm incredibly lucky to have worked with for these past two years. I'm particularly indebted to Vi Pham for her help with the HPLC machine, to Zheng Zhao for always answering my questions about new procedures, and to Maria Hernandez and Menglin Liu for the many conversations about baking and bread recipes. To Mandy Sun, thank you for the hours spent practicing presentations together and the valuable feedback provided therein, and to Justina Zhang, I will always be grateful for how much you taught me as my mentor.

To my family and friends outside of the lab, I will never forget how you supported me on this journey. Thank you to my family who were always there to let me vent, but always encouraged me to keep going. Thank you to Whitney Van Straten for the many dog walks together, to Johanna for the matcha lattes we shared while I was finishing my thesis, and of

course, thank you to Adrian, Alex, and Orion for making me laugh so much and so hard. I will forever appreciate how you all loved me, encouraged me, and helped me relax and enjoy the ride.

Table of Contents

Abstract ii
Acknowledgement v
Table of Contents vii
List of Tables ix
List of Figures x
List of Abbreviations xii
1. Introduction 1
1.1 Background 1
1.2 Fungal spoilage of baked goods 6
1.3 Sourdough as antifungal biopreservative 8
1.3.1 Buckwheat 11
1.3.2 Hops and isomerized α-acids 16
1.4 Artisanal and industrial fermentation protocols for sourdough 17
1.5 Knowledge gap 20
1.6 Thesis Hypotheses 20
1.7 Thesis Objectives 21
2. Material and Methods 22
2.1 Comparison of the antifungal activity of different strains of lactobacilli 22
2.1.1 Bacterial & Fungal Strains 22
2.1.2 Sourdough and Bread Production 23
2.1.3 Experimental design 26
2.1.4 Sample preparation and laboratory analysis 29
2.2 Impact of extended fermentation on antifungal activity of sourdough lactobacilli. 29
2.2.1 Preparation of sourdough and sourdough bread 29
2.2.2 Sample Preparation and Laboratory Analysis 30
2.2.3 Experimental design 30
2.3 Impact of modified fermentation parameters and antifungal ingredients on the mould-free storage life of sourdough bread. 31
2.3.1 Preparation of sourdough, sourdough breads, and controls 31
2.3.2 Experimental design 33
2.3.3 Sample preparation and laboratory analysis 34
2.4 Impact of antifungal additives on the shelf life of sourdough bread 34
2.4.1 Preparation of sourdoughs and controls 34
2.4.2 Experimental design 35
2.4.3 Sample preparation and laboratory analysis 36

2.6 Statistical analysis and calculations **36**
3. Results **36**
3.1 Comparison of the antifungal activity of different strains of lactobacilli **36**
3.1.1 Cell counts and pH values of sourdoughs and sourdough breads 37
3.1.2 Mould free shelf life of sourdough breads fermented with different strains. 38
3.1.3 Concentrations of organic acids in sourdough bread 40
3.1.4 Mould free shelf life of breads stored with ethanol 43
3.2 Impact of extended fermentation on antifungal activity of sourdough lactobacilli **45**
3.2.1 Volume and pH of extended fermentation sourdoughs and breads 45
3.2.2 Concentrations of organic acids in sourdough and breads 46
3.2.3 Mould free shelf life of sourdoughs made with an extended fermentation 49
3.3 Impact of modified fermentations and antifungal ingredients on the mould-free storage life of sourdough bread **51**
3.3.1 Volume, pH and TTA of sourdoughs and sourdough bread 51
3.3.2 Concentrations of organic acids 53
3.3.3 Mould free shelf life of breads made with modified fermentations and buckwheat 55
3.4 Impact of antifungal additives on the shelf life of sourdough bread **58**
3.4.1 Volume, pH, and TTA of sourdough and control breads 58
3.4.2 Concentrations of organic acids 59
3.4.3 Mould free shelf life of breads made using antifungal additives 61
4. Discussion **64**
5. Opportunities for future research **72**
Bibliography **73**
Appendix **96**

List of Tables

Table 1. Overview of some of the major phenolic compounds in buckwheat, *Lactobacilleae* enzymes active on them, and resulting products…….13

Table 2. Unbound phenolic acids (µg/g DM) in buckwheat biscuits prepared from unfermented and fermented flours by *Lp. plantarum* IB in liquid-state fermentation…….……15

Table 3. Bacterial Strains Used……...…23

Table 4. Bread Recipes…….…….....26

Table 5. pH Values of Sourdough and Bread Doughs Formulated for an a_w of 0.92 and 0.96..…..38

Table 6. Parameters of breads made with 48 h fermentations…….…..……..46

Table 7. Parameters of bread made with modified fermentations……..…..……...…53

Table 8. Parameters of sourdough breads prepared with antifungal additives and controls…..…59

List of Figures

Figure 1. Flow chart detailing the production of the sourdough breads……....32

Figure 2. Flow chart detailing the production of the 2 varieties of control breads…….…...33

Figure 3. Shelf life of 0.96aw bread after 10-spore inoculation with *Penicillium roqueforti* 3969, 6130, and *Aspergillus niger* FUA5004……..…….39

Figure 4. Shelf life of 0.92a_w bread after 10-spore inoculation with *Penicillium roqueforti* 3969, 6130, and *Aspergillus niger* FUA5004……..…….40

Figure 5. Concentrations of lactic and acetic acid in breads made with a water activity of 0.96aw, quantified using HPLC……42

Figure 6. Concentrations of lactic and acetic acid in breads made with a water activity of 0.92a_w, quantified using HPLC……43

Figure 7. Shelf life of breads packaged alone or with ethanol after 10-spore inoculation with *Penicillium roqueforti* 3969..……..……44

Figure 8. Concentrations of lactic and acetic acid for breads made with a 10% addition of sourdough fermented for 48 h, quantified by HPLC…….…..……48

Figure 9. Shelf life of breads made with a 10% addition of sourdough fermented for 48 h after 10-spore inoculation with *Penicillium roqueforti* 3969, 6130, and *Aspergillus niger* FUA5004…..…….…50

Figure 10. Concentrations of lactic and acetic acid in sourdough and bread samples, quantified using HPLC…….55

Figure 11. Shelf life of breads made with modified fermentation parameters and antifungal ingredients after 10-spore inoculation with *Penicillium roqueforti* 3969, 6130, and *Aspergillus niger* FUA5004, or after 1h of environmental exposure……..…57

Figure 12. Concentrations of lactic and acetic acid in sourdough samples, quantified using HPLC……..……..……..60

Figure 13. Concentrations of lactic and acetic acid in bread samples, quantified using HPLC…61

Figure 14. Shelf life of controls and breads made with antifungal ingredients after 10-spore

inoculation with *Penicillium roqueforti* 3969, 6130, and *Aspergillus niger* FUA5004, and after 1h of environmental exposure…….63

Figure 15. Schematic representation of bread preservation with sourdough, hygiene intended to prevent contamination with fungal conidiospores, preservatives or antifungal ingredients, or combinations of the three.……...71

Supplemental Figure 1. Propionic acid content of fermented breads and controls…….…….96

List of Abbreviations

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
CalPro	Calcium propionate
CFU	Colony Forming Units
DM	Dry Matter
FODMAP	Fermentable Oligo- Di- Monosaccharides and Polyols
Gcl	γ-Glutamyl cysteine ligase
HEPA	High Efficiency Particulate Air [filter]
HPLC	High Pressure Liquid Chromatography
HUFA	Hydroxy Unsaturated Fatty Acid
LAB	Lactic Acid Bacteria
MIC	Minimum Inhibitory Concentration
mMRS	Modified De Man-Rogosa-Sharpe
TTA/TA	Total Titratable Acidity/Titratable Acidity
TSA/B	Trypticase Soy Agar/Broth

1. Introduction

1.1 Background

Sourdough breads and baked goods have long been a staple in the diets of people living in temperate climates where wheat and rye are grown. While sourdough is often associated with traditional French breads like the classic baguette or Pain de Campagne, its history is far more extensive and elaborate than one might initially suspect.

The first evidence of baking comes from over 14,000 years ago, in a region known as the Fertile Crescent (Arranz-Otaegui et al., 2018). This sickle-shaped area of the world, sometimes called 'the cradle of civilization', encompasses a large section of the Middle East (Lee, n.d.). Egyptian hieroglyphics that depict the production of 14 distinct varieties of bread, including sourdough (Halawa, 2023), lend credibility to the theory that this period saw some of the earliest instances of bread making. As people travelled they carried sourdough with them and each introduced their own characteristics; the Gauls for instance, utilised the foam from a beer-like beverage called *cervoise* instead of water in their bread making due to its ability to leaven the bread and produce a lighter, fluffier texture in the final product (Cappelle et al., 2023). In Europe, sourdough has been mentioned at least as far back as 2,000 years ago by Pliny the Elder in book 18 of his 37-part series titled '*Natural Historis*', written in 77AD, where he described leaven and its application in bread (Bostock, 1855). Due to its simple nature, requiring only flour and water, and the unique positioning of Paris near major areas of grain production, sourdough became heavily entrenched in French culture (Cappelle et al., 2023).

The introduction of sourdough to North America came about with the arrival of European settlers, but of note was the influence of the California and Klondike Gold Rushes in the 19th

century. The promise of wealth drew many, including families who brought sourdough-leavened bread with them into the United States and Canada. Miners adopted the technique because of how reliable it was compared to commercial leavening agents, which could be extremely expensive due to the high demand and cost of delivery (Eschner, 2017). It became such a widely utilised product that the miners themselves, at least those who had seen the Yukon freeze and thaw, were often called 'Sourdoughs' (Coquitlam Heritage, 2022).

The microorganisms responsible for sourdough's unique properties were identified around this time. While it was Schwann who described yeast cells under the microscope and first determined fermentation was not due to oxygen as conventional theory at the time suggested (Smith, 2012), it was Louis Pasteur who proved the production of alcohol by yeasts and provided a definitive explanation of the connections between microbes and leavening in fermented foods (Pasteur, 1857).

The popularity of sourdough was diminished by the mass production of commercial yeast in the 1940's. Isolated yeast leavened bread quickly and consistently and required less time investment (Kimbell, 2015). Wild yeast and lactobacilli were inextricably linked by their presence on cereal substrates where cultivating one would necessitate the presence of the other, but commercial yeast in the 1920's was grown on molasses, allowing the separation of the two organisms (Cappelle et al., 2023). Sourdough baking was further impacted by the French government's restriction of working hours and banning of night work: a significant problem for any time and labour-intensive baked goods (Sowerwine, n.d.). The practice still survived and thrived in the United States, and in countries like Germany and the Soviet Union it persisted in rye bread making, but wheat sourdough remained a miniscule part of European commercial

enterprises until the 20th century.

The resurgence in sourdough came not from any one change, but rather from a growing public demand for higher-quality bread. Interest exploded in the early 2000's (Gänzle et al., 2023); the sensory and chemical changes made to bread dough during fermentation attracted the interest of food scientists and the relative ease of production and many purported health benefits made sourdough more attractive to the average consumer (Paciulli et al., 2021). One of the many ways sourdoughs can improve the quality of bread is by increasing the available mineral content. Phytic acid is an antinutritive compound, which binds to minerals including Ca^{2+}, Mn^{2+}, and Zn^{2+}, preventing their absorption by the intestines (Harvard T.H. Chan School of Public Health, 2022). The phytase enzyme present in grain and produced by yeasts during fermentation of wheat and rye flours can degrade more than half of the phytate present (Hassan et al., 2008) and its performance is increased in the acidic conditions of a sourdough (Buddrick et al., 2014). While the enzyme is most active in wheat at a pH of 5.0-5.5, it can function until the pH becomes as low as 3.5 (Peers, 1953). Phytate becomes most soluble at a pH below 5.0 (Jackman, 1982). Digestion may also be improved for sensitive individuals, as acidification hydrolyses the disulfide bonds in gluten, allowing for increased activity by proteases present in the cereal substrate (Gänzle et al., 2008). The presence of fermentable oligo-, di-, monosaccharides and polyols (FODMAPs); short-chain sugars which are sometimes poorly absorbed and undergo fermentation by bacteria in the large intestine, are also reduced by the long proofing times used in fermented bread, which allows time for the yeast invertase to act (Loponen & Gänzle, 2018). Some lactobacilli will also produce exopolysaccharides from sucrose, which allows for a softer crumb, delayed staling, and increased gas retention (Galle, 2013).

Fermentation is a significant contributor to the taste and smell of sourdough bread. The production of lactic and acetic acid imparts a sourness reflected in the name, and the same proteolysis that improves digestibility also impacts flavour, releasing free amino acids and allowing them to be metabolised by bacteria and aid in the formation of volatiles. For instance, arginine is converted to ornithine, which can be reacted with a dicarbonyl during baking to create 2-acetyl-1-pyrroline; a compound partially responsible for the smell of wheat bread crust (Thiele et al., 2002). Another key flavour compound is glutamate. Glutamate is an umami compound formed by strain-specific catalysis of glutamine by glutaminase (Weingand-Ziadé et al., 2003). The resulting flavour has secondary benefits as the sour taste intensity is enhanced by the presence of glutamate. Zhao et al. (2015) report that when using glutamate-producing strains in sourdough fermentations, a 1% NaCl bread was reported to be as salty as a 1.5% NaCl formulation by a consumer panel. Sourdough breads made with 1 or 2% salt were also judged by a trained panel to have a more sour or umami flavour than a straight dough of the same salt content.

Flavours are further improved in sourdough because of the abundance of kokumi-active peptides. These are γ-glutamyl-dipeptides that have no flavour of their own and instead amplify the basic taste sensations of sweet, salty, and bitter by activating calcium sensing receptors (Ohsu et al., 2010). They are products of sourdough fermentation formed during the proteolysis of free amino acids by lactobacilli which produce one of the 3 varieties of γ-glutamyl cysteine ligase (Gcl), or γ-glutamyl transferase/transpeptidase (Roudot-Algaron et al., 1994; Yan et al., 2018). *Limisilactobacillus* and *Lentilactobacillus* species often contain 2 or more of the Gcl genes, with *Limosilactibacillus reuteri* often containing at least one, but have been observed to have all 3 as is

the case with *Lm. reuteri* LTH2854 (Xie & Gänzle, 2021). All 3 are most active against cysteine, but Gcl1 exhibits greater activity against hydrophobic amino acids isoleucine, leucine, and phenylalanine than Gcl2 or Gcl 3 (Xie, 2023). While both heterofermenters and homofermenters can produce these enzymes, they are less common among homofermentative species (Xie & Gänzle, 2021). The actual sensory impact of these peptides in sourdough is still under investigation; while bacterial fermentation can increase the amount of γ-glutamyl-dipeptides present in sourdough, some are lost during baking. A consumer panel run by Xie & Gänzle (2021) did not report any change in the taste of bread containing γ-glutamyl compounds. Additionally the taste threshold for these peptides is reported to range from 2.5μmol/kg to 5 mmol/kg but no standardised protocol exists to determine that threshold through sensory analysis, especially since the food matrix impacts that threshold (Xie & Gänzle, 2021). However the findings of Zhao and Gänzle (2016) support the status of γ-glutamyl-dipeptides as important components of sourdough flavour. A consumer panel rated sourdough bread fermented with *Lm. reuteri* as more intensely flavourful than both unfermented bread and a *Latilactobacillus sakei* sourdough; both *L. sakei* and *Lm. reuteri* produce identical amounts of acid but *L. sakei* did not produce kokumi-active compounds. In addition, 2% salt sourdoughs fermented with different strains of *Lm. reuteri*, each with different ability to accumulate γ-glutamyl-dipeptides were compared, and ranked differently based on taste intensity (Zhao & Gänzle, 2016)

Sourdough experienced a bump in popularity during the Covid-19 pandemic, with Google searches for the term spiking to an all-time high in April of 2020 as people isolated at home took an interest in baking (Google Trends, 2023). The market value of sourdough bread was 2.9 billion USD in 2021 and projected to grow to 5.3 billion by 2030 (Singh, 2023).

1.2 Fungal spoilage of baked goods

In 2021, the United Nations Environment Programme reported that 30% of all food is wasted between the stages of production and consumption, with 17% of that lost after its purchase. The resources required to produce and transport that food equates to a carbon footprint of roughly 9.3 billion metric tons of CO_2 (Zhu et al., 2023). This value does not include the impact of changing the land to facilitate farming, transport, or any other factors that occur pre-harvest or during waste management. In emergent nations these losses are primarily at the post-harvest and processing stages, but in industrialised countries most food waste takes place at the retail and consumer level (World Food Programme, 2020). A sizable portion of this food loss is bread products; with >100 million tons produced annually, it is estimated that over 900,000 tons of that is wasted (Rejeb et al., 2022). With its high water activity and neutral pH, bread spoils and stales quickly – usually between 3-7 d if no preservation methods are applied (Legan, 1993). While it is difficult to know exactly how much of this food waste is attributable to spoilage, Garcia et al. (2019a) suggest between 1-5% of baked good waste in Europe to be due to mould depending on the year, and based on consumer feedback, up to 11% in tropical countries like Brazil. This is a loss equivalent to more than €200 million each year.

The heat of baking kills mould spores (Knight & Menlove, 1961) but contamination occurs post-baking when the loaves are exposed to open air and handled by machines and/or humans, especially during slicing and packaging (Garcia & Copetti, 2019). Some of the most common spoilage moulds are of the genera *Penicillium* spp. and *Aspergillus* spp., with *Penicillium* usually dominating in colder countries and *Aspergillus* in warmer ones (Legan, 1993). Though air quality varies among locations, samples taken from 4 Edmonton and 1 Calgary

bakeries had found packaging areas to be some of the most contaminated locations, containing 60-210 CFU/m^3 of yeasts and moulds present (Ooraikul et al., 1987). These findings are consistent with more recent research; slicing and packaging areas of a Brazilian bread factory were indicated to pose a high contamination risk, as they had the highest spore counts of the facility at 1.25×10^4 CFU/m^3 (Garcia et al., 2019). Despite efforts by producers, the risk of post processing contamination remains significant unless clean room technology is utilised.

By industrial standards, a 'clean room' is one designed and used in a way that minimises the overall number of airborne contaminants introduced to, generated in, or retained in said room (Holbrook, 2009). Common methods of control are the use of barrier-type filters that can catch dust and microbes, controlled airflow, and ozone or UV radiation. These methods are extremely beneficial for protecting the shelf life of products such as bread without impacting taste or quality. Other significant factors in maintaining a clean room are the education and sanitation of employees, appropriate cleaning of surfaces and equipment, and the layout of the room (Wirtanen et al., 2002). While exact universal cleanroom values and standards required for food processing do not currently exist, Garcia et al. (2019b) proposed that air quality standards for bakeries should be based on fungal presence as CFU/m^3, where 0-100 is considered clean, 101-500 is acceptable (low contamination), 501-1,500 is acceptable (moderately contaminated), and anything above 1,500 is highly contaminated and unacceptable.

To delay the growth of these and other spoilage organisms, most large-scale commercial bakeries make use of chemical preservatives. For baked goods like breads and cakes, weak acids such as calcium propionate/potassium propionate and sorbic acid or potassium sorbate are some of the most used antimicrobials due to their combined effectiveness (Axel et al., 2017). Calcium

propionate is able to significantly delay spoilage by moulds and ropy spoilage bacteria such as *Bacillus subtilis*. Sorbic acid, however, has a high level of inhibitory activity against both moulds and yeasts, and so using the two preservatives in combination with one another allows for excellent antimicrobial coverage (Bensid et al., 2022). For calcium propionate, the method of action is to accumulate in the cell, where it competes with amino acids such as alanine and inhibits enzymes required for metabolism, such as pyruvate dehydrogenase (Kagliwal et al., 2014). Sorbic acid functions by altering cellular membranes, inhibiting key transport systems and enzymes, and increasing the hydrogen ion concentration in the medium surrounding the yeast or mould cell (Sofos et al., 1986). Its activity also increases as the pH drops (Zeece, 2020). In Canada, calcium propionate is allowed in bread up to a limit of 2,000 ppm, and sorbic acid up to 1,000 ppm (Health Canada, 2022), although in the European Union the allowable amount of calcium propionate is increased to 3,000 ppm (Official Journal of the European Union, 2008). For the purposes of this thesis, the EU allowable amount was chosen as the limit. While these preservatives are highly effective in delaying mould growth, if resistant species like *P. roqueforti* spp. can contaminate the product, they will still grow after some time (Debonne et al., 2023).

1.3 Sourdough as antifungal biopreservative

While mould remains an ever-present concern for the baking industry, new ideas about how to prevent it from spoiling bread are the basis behind a large body of research. Sourdough has long been studied for such a purpose. This is due in part to its beneficial impact on bread quality as described in Section 1.1, but also because it offers a clean-label alternative to using chemical preservatives. Despite their proven safety and efficacy, consumers are increasingly

concerned with unrecognisable ingredients and additives in their food, and according to the Ingredion 2023 consumer food preference trends report, 33% of consumers were willing to spend 20% or more for foods which advertised themselves as 'all-natural' and 68% would pay more for products with only recognizable ingredients.

Due to their long history of use in food fermentations, lactic acid bacteria, specifically *Lactobacillaceae*, are largely considered food safe organisms. As such, it is relatively simple to use them as starter cultures. While sourdough can confer many benefits to bread, one of particular significance is the increase in mould-free shelf life. Lactobacilli are split into two clades, heterofermenters and homofermenters, the former of which originated from one evolutionary link and therefore all share a common ancestor: *Lactiplantibacillus plantarum* (Gänzle, 2015; Qiao et al., 2022). The two are differentiated by their metabolisms. Homofermenters utilise glycolysis and the pentose-phosphate pathway, preferentially using glucose as fuel (London, 1976) and creating lactic acid as the major product of fermentation, while acetic acid and ethanol are minor products. In contrast, heterofermenters make use of the phosphoketolase pathway (PPT) to metabolise hexoses and pentoses, and preferentially utilise maltose and sucrose. The key metabolites produced are lactate, acetate or ethanol, and carbon dioxide (Gänzle & Gobbetti, 2023). During fermentation a variety of minor compounds can also be produced, some of which have antifungal activity. For instance, hydroxy unsaturated fatty acids (HUFAs) are formed from bacterial conversions of linoleic and oleic acids that are common in cereal products, with the exception of rice (Day, 2016). These conversions by lactobacilli were first proven to take place in

a sourdough matrix by Black et al. (2013), who fractionated samples of antifungal HUFAs produced on media and in sourdough using liquid chromatography coupled with atmospheric pressure photoionization mass spectrometry (LC-APPI-MS). While HUFAs were produced by all strains tested, *Lm. reuteri* was the only strain whose metabolic products had increased activity in response to higher concentrations of linoleic acid. The most abundant HUFA it produced was a $C_{18:1}$ fatty acid that accumulated in sourdough and reached the minimum inhibitory concentration (MIC) for *A. niger*.

Strom et al. (2002) confirmed that some lactic acid bacteria are able to produce cyclic dipeptides when *Lp. plantarum* MiLAB 393 was observed to form cyclo(L-Phe–L-Pro) and cyclo(L-Phe–trans-4-OH-L-Pro) in MRS broth. Antifungal activity was confirmed by plating the strain and overlaying malt extract containing 10^4 spores/ml, then looking for resulting zones of inhibition. However, the amount of cyclic dipeptides produced in sourdough is extremely small; less than 1% of the MIC (Ryan et al., 2011). While the majority of antifungal activity can be attributed to acetic acid, the hurdle effect - a strategic combination of different antimicrobial factors - means that the overall impact of multiple antifungals each present in amounts below their MIC is greater than what any of those components could achieve alone (Jarvis & Paulus, 1982).

Many plant-based additives exhibit antifungal effects but two of particular interest are buckwheat and hops extract. Although the antifungal compounds in buckwheat and the impact of fermentation have been reported on for years, the impact of fermented buckwheat in sourdough

bread during mould experiments has not been assessed. Hops, or specifically the antifungal activity of their isomerized α-acids in sourdough bread remains unexplored with the exception of Gobbetti et al. (2018), who identified it as an effective way to extend the mould-free shelf life of bread.

1.3.1 Buckwheat

While the organic acids produced by lactobacilli have potent antifungal capabilities, other bioactive ingredients have been observed to have similar effects. One of these is buckwheat, a gluten-free pseudocereal which can be hulled and ground to produce flour and is known for its bitter and nutty taste (Starowicz, 2018). It provides significant value to sourdough baked goods through not only its nutritional content, but also its unique interactions with the fermentation process.

Although its lack of gluten makes it unable to retain gas and therefore unable to match the volume of a typical wheat bread on its own (a problem usually remedied by the inclusion of gluten-mimicking additives like hydrocolloids [Culetu et al., 2021]), a combination of the two flours can increase bread volume. Buckwheat in amounts of 10% has been observed to strengthen the gluten network and increase specific volume by up to 20%, though the effect did not increase when a higher percentage of buckwheat was utilised (Moroni et al., 2012). Others report that if the buckwheat content remains below 15%, a reduction in specific bread volume is unlikely (Lin et al., 2009).

Perhaps most useful though, are its potential antifungal properties. Phenolics are the abundant secondary metabolites of plants, and while they come with a variety of characteristics

and functions, many are bitter and exhibit antinutritive effects, usually by binding to vitamins and minerals or inhibiting digestive enzymes (Chung et al., 1998). Three major classes of phenolics are phenolic acids, flavonoids, and polymeric phenols like tannins (González-Sarrías et al., 2020). Phenolics can inhibit fungal growth by diffusing through the cellular membrane of fungi and interfering with protein synthesis or binding to and inhibiting transport proteins (Wink et al., 2012). Hydrophobic phenols can disrupt the cellular membranes of fungi by preventing sterol production, causing ion leakage. Preliminary experiments indicated that 10% and 20% addition of buckwheat to wheat flour sourdough breads can delay fungal spoilage (Moroni et al., 2012). However, the effects of fermentation on antifungal activity of buckwheat flour are not widely studied.

In buckwheat the majority of phenolic acids (the most common phenolic present) are free while flavonoids remain bound to the cell wall, unlike in wheat flour where most phenolic compounds are bound. As such buckwheat has a higher antioxidant activity than wheat (Koval et al., 2020). Even still, a number of phenolics are still found as glycosides and esters (Alvarez-Jubete et al., 2010), but fermentation results in production of strain specific enzymes: esterases, glycosidases, and tannases produced by *Lactobacilleae* which free bound phenols for use by bacteria (Gaur & Gänzle, 2023). The bitter free phenols can then be converted by lactobacilli to aglycones and acids, some of which can undergo further metabolization into more palatable compounds that improve the overall flavour of the product (Koval et al., 2020; Svensson et al., 2010). Below is a list of common phenolic glycosides and esters found in buckwheat, the enzymes which degrade them, and the resulting products (Table 1).

Table 1. Overview of some of the major phenolic compounds in buckwheat, *Lactobacilleae* enzymes active on them, and resulting products

Compound Class	Glycosylated Phenols	Active Microbial Enzymes	Aglycone/Acid	Aglycone/Acid Taste
Hydroxybenzoic acid	2-hydroxy-3-O-β-D-glucopyranosyl-benzoic acid	Esterase (Tannase) Decarboxylase	p-hydroxybenzoic acid	Nutty
Hydroxybenzoic acid	Protocatechuic-4-O-glucoside acid	Esterase (Tannase) Decarboxylase	Protocatechuic acid	Astringent
Hydroxybenzoic acid	Syringic acid-*O*-glucoside	Esterase (Tannase) Decarboxylase	Syringic acid	Astringent
Hydroxycinnamic acid	Caffeic acid hexose	Esterase (Tannase) Decarboxylase Reductase	Caffeic acid	Bitter coffee
Hydroxycinnamic acid	6′-di-feruloyl sucrose	Esterase (Tannase) Decarboxylase Reductase	Ferulic acid	Sour
Hydroxycinnamic acid	3, 6-di-*p*-coumaroyl-1	Esterase (Tannase) Decarboxylase Reductase	*p*-coumaric acid	Bitter
Flavonoid	Rutin	Glycosyl hydrolase	Quercetin	Bitter
Flavonoid	Vitexin	Glycosyl hydrolase	Apigenin	Astringent
Flavonoid	Orientin	Glycosyl hydrolase	Luteolin	Bitter

(Modified from Koval et al., 2020 and Gaur & Gänzle, 2023)

Some free phenols can undergo further conversions. Hydroxycinnamic acids for instance, a subsection of phenolic acids, can be decarboxylated and reduced to form ethyl derivatives which are considered flavour volatiles (Muñoz et al., 2017). Ferulic acid is one example. After being freed from its glycosidic form it can be further metabolised into vanillic acid, and from

there, reduced to vanillin. This turns an astringent compound into one that confers a vanilla scent. Hydroxybenzoic acids can be decarboxylated, but not reduced. The majority of phenolic acid enzymes in lactobacilli were identified in *Lp. plantarum* (Gaur & Gänzle, 2023).

The effect on buckwheat phenolics can be significant. In the comparison of free phenolic acids in unfermented and liquid-state fermented buckwheat crackers, the fermented varieties contained a higher phenolic content as shown in Table 2 (Zieliński et al., 2022). Given that even rigorous extraction is unable to convert all bound phenols from their glycosidic and ester forms (Dey, 2012), the increased amounts of 7 of 8 acids supports the statement that fermentation of buckwheat by lactic acid bacteria can release bound phenols.

Table 2. Unbound phenolic acids (µg/g DM) in buckwheat biscuits prepared from unfermented and fermented flours by *Lp. plantarum* IB in liquid-state fermentation

	Vanillic	Protocatechuic	Syringic	*p*-Coumaric	*t*-Cinnamic	Caffeic	Ferulic
Control biscuits (AF)	187.90 ± 18.83	203.57 ± 6.15[a]	130.65 ± 1.22[a]	26.33 ± 0.14[a]	8.29 ± 0.02[a]	14.64 ± 0.09[a]	8.17 ± 0.21[a]
Fermented biscuits* (AF)	197.89 ± 6.37	327.77 ± 3.6[b]	208.15 ± 6.37[b]	23.60 ± 0.19[b]	46.90 ± 0.26[b]	37.38 ± 0.52[b]	10.48 ± 0.18[b]

(Modified from Zieliński et al., 2022)

Data was repeated in triplicate and expressed as mean ± standard deviation. Means in each column marked by different letters are significantly different based on unpaired t-test ($p < 0.05$).

1.3.2 Hops and isomerized α-acids

Another potential adjunct with antifungal effects is isomerized alpha-acids (iso-α-acids) sourced from hops. Hops are the cones of the plant *Humulus lupulus* (Likens et al., 1978). They are commonly used in beer-making where they add flavour and aroma to the final product; while α-acids themselves have very little taste, the isomerization process causes them to take on moderately sour and intensely bitter flavours (Caballero et al., 2012). Originally though, the main benefit of hops in brewing was a spoilage retardant (Likens et al., 1978). While hops cones contain resins, oils, and a variety of acids, from a food processing perspective the most important compounds are the iso-α-acids. These are formed during the wort boiling stage of beer making and exhibit antimicrobial effects on gram-positive bacteria (Bokulich & Bamforth, 2013) and moulds (Gobbetti et al., 2018). They act as proton ionophores, disrupting the proton gradient of cells and decreasing the cytoplasmic pH. The result is impaired nutrient uptake and eventual cell death (Behr & Vogel, 2009).

The α-acid content of hops can vary widely based on breed, geography and growing conditions; in one examination of 4 varieties of Czech hops over 25 years it was determined that high summer temperatures and increasing age of the plant negatively impacted the α-acid content of cultivars while high rainfall increased the yield of the crop (Donner et al., 2020). The predominant α-acids are humulone, cohumulone, and adhumulone. The average α-acid concentration in hops varies significantly and can be as low as 1.3% (Baker et al., 2008) or as high as 20.5% (Beer Maverick, 2023). The actual values may be somewhat different from what is presented on product labels depending on storage conditions and the hops varieties (Canbas et al.,

2001). The amount isomerized during boiling is usually between 30-50% and reaches maximum conversion after 2h at 100°C (Huang et al., 2013; Jaskula et al., 2008). Isomerization correlates directly to flavour; 1 international bittering unit (IBU) is equivalent to 1 mg/L or 1 ppm of iso-α-acids (Oliver, 2012). While hops also contain β-acids that are antimicrobial in nature (Sleha et al., 2021; Kramer et al., 2015), they have not been observed to exhibit anti-spoilage properties and are oxidised rather than isomerized when boiled (Baker et al., 2008).

While the activity of hops extracts is widely known in beer making, its usefulness in food products is a less-studied endeavour. Kramer et al. (2015) exposed a series of foodborne pathogens to 3 hops extracts (containing α-acids, β-acids, or xanthohumol [a polyphenol]) to determine their antimicrobial impact. The impact of isomerized α-acids was not assessed, but β-acids were found to be effective in inhibiting growth of the pathogens, and acidification with lactic acid lowered the minimum inhibitory concentration (MIC).

While few publications exist on its activity in bread, Gobbetti et al. (2018) found that isomerized hops extract can extend the shelf life of bread when used in conjunction with sourdough. A 25% addition of isomerized hops extract delayed spoilage in bread as effectively as a 0.3% addition of calcium propionate, and the combination of hops and sourdough was more effective than the hops alone. The bread was described as bitter and herbaceous, though still acceptable, by trained panellists.

1.4 Artisanal and industrial fermentation protocols for sourdough

When included in bread sourdough can serve several functions, acting as a leavening agent, an acidifying agent, and a baking improver. The exact benefits it provides are dependent on the fermentation conditions used in its development. In the instance of leavening, the baker is

required to work on the microbe's schedule, 'backslopping' or feeding the sourdough in intervals of 6-24 h (De Vuyst et al., 2023). The exact time depends on the temperature of fermentation; warmer temperatures require more frequent backslopping to keep the microbe's active and in the exponential growth phase. These sourdoughs are often called Type 1 and used in small bakeries rather than large industrial operations (Brandt et al., 2023). Although the exact details of this classification vary among publications, they are generally characterised by frequent backslopping and fermentation temperatures under 30°C (Gänzle & Zheng, 2019). The microbial population of a type 1 sourdough leans towards higher numbers of yeasts, generally dominated by *Kazachstania humilis*, and 1-2 species of lactobacilli, usually insect-associated (Brandt et al., 2023). In a review of the species diversity of 527 sourdoughs, the 3 most common species were (in order of relative abundance) *Fructilactobacillus sanfranciscensis*, *Lp. plantarum*, and *Levilactobacillus brevis* (Van Kerrebroeck et al., 2017).

If the baker wishes to use sourdough as an acidification agent, they must utilise longer fermentations at higher temperatures, usually above 30°C while backslopping less-frequently, often in intervals of 24, 36, or 48 h (De Vuyst et al., 2023). These conditions select for vertebrate-adapted lactobacilli such as *Lactobacillus* and *Limoslactibacillus* and the sourdough will lose its leavening abilities in exchange for a higher total titratable acidity (TTA) (Gänzle & Zheng, 2019), with sourdoughs made under these conditions generally classified as type 2. These tend to be populated by more acid-resistant strains of lactobacilli, meaning that *F. sanfranciscensis* is not a significant contributor to the microbiome. Instead, species such as *Limosilactibacillus reuteri*, *Limosilactibacillus pontis*, and *Limosilactibacillus fermentum* (Gänzle & Zheng, 2019) make up the bulk of the population.

When sourdough is required for a more general purpose, such as that of a baking improver (here considered to be an additive that improves functional features like volume, digestibility, glycemic index score or nutritional quality) (Gobbetti et al., 2014), it is generally agreed upon that a 10% inclusion is enough to confer those benefits, but additions up to 30% are common.

Sponge dough, sometimes called modified pre-ferments, are also frequently used in the pursuit of higher-quality wheat bread. These traditionally involve adding baker's yeast to a 1:1 ratio of flour and water and fermenting for 0.5-24 h; the shorter the fermentation, the larger the amount of yeast added to it. Preferments lasting between 2-3 h for instance would use 1-2% yeast additions; such a process provides the yeast time to acclimate to the osmotic pressure of the sourdough and to improve flavour (Brandt et al., 2023). As time passes, the microbial profile also begins to change. After 8-12 h of fermentation the growth of lactobacilli will exceed the population of the yeasts, eclipsing them and becoming the dominant organisms. As such, it is possible to transition from a sponge dough back to a sourdough (Brandt et al., 2023).

One common fermentation modification includes extending the fermentation of the sourdough breads to overnight while at temperatures at or around 5-10℃. Such a low temperature slows down the metabolism of the lactobacilli and yeasts present in the dough without stopping it entirely. This allows for fermentation with less risk of over-proofing, a defect of bread where overproduction of CO_2 puts too much pressure on the gluten structure and creates small rips, weakening it (Modernist Cuisine, 2023). If this occurs, then the expansion of CO_2 and evaporation of water during baking will no longer result in oven spring (wherein the rising gas is trapped in the gluten matrix and increases the volume of the bread by up to 30%) [Figoni, 2003], but instead a woefully flat and small loaf of bread. Cool proofing is advantageous to the baker

who can comfortably leave their bread to develop overnight, and a functional benefit of such a modification is that the proportion of acetic acid is increased.

Cooler temperatures promote the production of the enzyme invertase by yeast, which lactobacilli are unable to produce (Gänzle et al., 1998). Invertase acts outside the cell, preferentially hydrolyzing the glucose-fructose and fructose-fructose bonds of multi-unit sugars. These include fructans, long chains of fructose that can make up between 1.5-1.7% of the FODMAPs found in cereal grains (Loponen & Gänzle, 2018), and sucrose. Fructans made of 4 sugar units or less can be consumed by lactic acid bacteria and broken down into monosaccharides by intracellular enzymes like β-fructosidase (Gänzle & Follador, 2012). Fructose specifically can be used not just for energy, but also as an electron acceptor by heterofermentative LABs to regenerate NAD+, a cofactor required for cellular function (Loponen & Gänzle, 2018). By taking this pathway they bypass the conversion of acetyl phosphate to ethanol and instead convert it to acetate, producing 1 additional ATP for the cell (Spector, 2009).

1.5 Knowledge gap

Although several publications have compared the impact of different lactobacilli strains on antifungal activity in sourdough bread, the effectiveness of different fermentation parameters on organic acid production and shelf life is not something widely studied. Neither is the impact of buckwheat fermentation on the shelf life of sourdough bread, and the activity of hops as an antifungal adjunct is a subject not-widely explored.

1.6 Thesis Hypotheses

The null hypotheses of this study were that a) The organic acids present in a 10% addition

of sourdough cannot extend the mould-free shelf life of white bread; b) The mould-free shelf life of sourdough bread can be extended by altering the fermentation protocol; c) the inclusion of bioactive adjuncts buckwheat or α-acids in conjunction with sourdough will improve the mould-free shelf life of bread to match or exceed that of chemically-preserved products.

1.7 Thesis Objectives

Based on the null hypotheses above, the objectives of this study were to:

- Assess the effectiveness of a 10% sourdough addition in bread at extending the mould-free shelf life of bread. A secondary objective was to compare sourdough fermentation capabilities among strains of lactobacilli to select a promising starter culture. Twelve strains of lactobacilli were selected as possible starter cultures based on the findings of Liang et al. (2022) who demonstrated their effectiveness in delaying mould spoilage in yoghurt fermentations.
- Alter sourdough fermentation and bread proofing schedules to increase the organic acid content in the final product, thereby delaying mould growth on bread.
- Supplement the effects of sourdough fermentation in bread with addition of anti microbial adjuncts to create a clean-label product with a comparable shelf life to chemically preserved and commercially available bread.

2. Material and Methods

2.1 Comparison of the antifungal activity of different strains of lactobacilli

2.1.1 Bacterial & Fungal Strains

All cultures were stored in screw-cap tubes and maintained at -80℃ glycerol stocks (50% glycerol and 50% mMRS broth). In preparation to produce sourdough the strains were streaked onto mMRS agar plates and incubated anaerobically at 30℃ for 48 h. Individual colonies were selected and added to Nunc™ 15 ml Polypropylene Centrifuge Tubes containing 10 ml of mMRS broth and incubated anaerobically for 24 h at 30°C. All bacterial growth took place in or on mMRS broth and media unless otherwise specified, and all bacterial strains used in the following experiments are listed in Table 3. Species identities were confirmed through 16s rRNA sequencing.

In preparation for mould-resistance experiments strains *Penicillium roqueforti* 3969, *Penicillium roqueforti* 6130 and *Aspergillus niger* FUA5004 were streaked onto trypticase soy agar (TSA) plates and grown aerobically for 5-7 d at 25℃; if not immediately needed, plates were stored at 4℃. These plates were harvested by adding 10 ml of a 0.1% tween 80 solution to the plate and scraping with the tip of a pipette. The dislodged mould was gravity filtered into a 15 ml Nunc™ tube which was centrifuged for 3 min at 5,000 rpm. The supernatant was removed, and the spores were re-suspended in fresh tween 80 solution. Spore counts were confirmed by hemocytometer (Hecht Assistent, Germany). The spore solution was diluted to a concentration of $1x10^3$/ml and stored at -20°C until needed. During the mould experiments, 10 ul was added to each square of bread, equaling to an inoculation of 10 spores. After use the spore stocks were

again stored at -20°C. Spores were re-harvested roughly every 3 months, and stocks of each strain were also made using 50% TSB and 50% glycerol, and stored at -80°C.

Table 3: Bacterial Strains Used

Strain Identifier	Species	Source/Reference
FUA3186	*Lacticaseibacillus paracasei*	Oat drink
TMW1.460	*Lactiplantibacillus plantarum*	Spoiled beer; Ulmer et al., 2000
FUA3073	*Lactiplantibacillus plantarum*	Meat
FUA3183	*Lactiplantibacillus plantarum*	Fermented milk
FUA3428	*Lactiplantibacillus plantarum*	Sourdough
LP38^{T}	*Levilactobacillus hammesii*	Sourdough; Valcheva et al., 2005
FUA3718	*Levilentibacillus parabuchneri*	Sacco S.r.L.
TMW1.154	*Fructilactobacillus sanfranciscensis*	Sourdough; Gänzle and Zheng, 2019
LTH2584	*Limosilactibacillus reuteri*	Gänzle et al., 2000
FUA3583	*Furfurilactobacillus milli*	Mahewu isolate; Pswarayi, 2019
FUA3430	*Furfurilactobacillus milli*	Sourdough; Ripari, 2013
FUA3124	*Furfurilactobacillus milli*	Sourdough

2.1.2 Sourdough and Bread Production

The incubated starter strains (made as described in section 2.1.1) were centrifuged and had the broth discarded. The remaining pellets of bacteria had 10 ml tap water added to the 15 ml centrifuge tube and were vortexed, centrifuged for 5 min at 5,000 RPM, and then had the supernatant removed. This step, referred to as 'washing' the cells, was repeated a second time to

ensure removal of broth. After washing, another 10 ml of tap water was added to the samples which were then vortexed and brought to a food-safe lab where they were each added to their own 50 ml glass beaker and combined with 10g of all-purpose flour, forming sourdoughs. The sourdoughs were covered with plastic wrap to prevent the mixture from drying out. This was the basis for all following sourdough fermentations.

The 12 different sourdoughs were incubated at 30°C for 24 h, then backslopped (aka 'refreshed') by the addition of flour and water in a 1: 4.5: 4.5 ratio (36g wheat flour and 36g of room-temperature tap water to 8g of the sourdough) following the University of Alberta sourdough baking protocol (UofA, n.d.). After mixing thoroughly, the refreshed sourdough was covered again with plastic wrap and stored at 30°C for another 24 h, after which it was ready to be added to bread dough.

The chemically acidified 'sourdough' was created by mixing equal parts flour and water one day before it was used in bread dough, and acidifying the mixture with a 1:4 parts solution of acetic and lactic acid. This solution was added by eyedropper. The pH was measured using a pH meter (Fisher Scientific accumet AE150): 5 drops of the acidifying solution would be added to the water/flour mixture, mixed with a sterile tongue depressor, and then measured. This continued until the pH measured between 3.4-3.8. All sourdough production and bread baking took place in a food-safe laboratory. For each sourdough, a series of dilutions up to 10^{-7} was performed using 0.1% peptone water. Dilutions 10^{-5}-10^{-7} were plated on mMRS agar by hand or using a spiral plater, then incubated 2 d at 30°C, after which the colonies were counted. The pH of bread and sourdough samples were taken using the same pH metre previously named. Measurements of pH were repeated in triplicate and represented as mean ± standard deviation. Bread pH was taken

prior to inoculation experiments, and sourdough pH was taken immediately before inclusion in the dough.

Bread baking followed the recipes in Table 4. The only difference between the two formulations was the sugar content, which was used to alter the water activity of the baked breads to 0.96a_w or 0.92a_w. Three replicates of each control and sourdough bread were made at each water activity. For the sourdough bread, sourdough was included in the dough such that 10% of the flour in each loaf had been fermented. For the chemically acidified control loaf, the chemically acidified 'sourdough' was also added in the same amount. The dough was mixed using a dough hook (model KSM3316XCA; KitchenAid, USA) for 1 min on low speed and 5 min on high to ensure incorporation of all ingredients. A clean bowl was used for each dough to reduce the risk of contamination. After mixing, the doughs were proofed for 1h at 30°C after which they were kneaded by hand for approximately one min to develop the gluten. Kneaded doughs were covered and left to rest another hour before baking at 177°C for 25-30 min. The breads were cooled at room temperature and packaged in a 10 L plastic bag, then labelled and stored at -20°C. Three independent replicates were produced for each fermentation.

Samples of each sourdough were reserved for plate counts, pH measurements, and HPLC analysis, but no plate counts were performed for the control because it had not been inoculated or fermented. Samples of each bread were also taken for HPLC analysis. A slice of each bread was assessed with an Aqualab 4TW Water Activity Meter to confirm the water activity.

Table 4. Bread Recipes

Ingredient	$0.96a_w$	$0.92a_w$
Flour	270	270
Water*	150	150
Oil	6	6
Sugar	6	27
Salt	6	6
Yeast	6	6
Starter	60	60

All values presented are represented in grams, except for water, which is represented in ml. All percentages used are bakers' percentages.

*100 ml was used to pre-hydrate the yeast (Fleischmann's traditional active dry yeast) before it was added to the dough

2.1.3 Experimental design

For each sourdough, a series of dilutions up to 10^{-7} was made using 0.1% peptone water. Dilutions 10^{-5}-10^{-7} were plated on mMRS agar by hand or using a spiral plater, then incubated 2 d at 30°C.

In order to compare the antifungal capabilities of each fermentation, the sourdough breads and their controls were inoculated with spores of the moulds mentioned in section 2.1.1 such that each bread was compared to each of the 3 moulds. A 7.0L chamber (ThermoScientific AnaeroPack) held the bread during this experiment. The base of the chamber was filled with roughly 800 ml of salt solution to keep the water activity constant throughout the experiment.

These solutions were made of 5.74g K_2SO_4 and 35g/KNO_3 per 100 ml of distilled water for the experiments at 0.92a_w, and 10.7g of K_2SO_4 per 100 ml of distilled water for experiments at 0.96a_w. Plastic test tube racks were placed at the base of the containers and held the aluminium foil support structure on which the bread samples would be placed. After the chamber was set up, the inoculation proceeded as follows. First, the sourdough and chemically acidified control breads were removed from their plastic bags inside the biosafety cabinet and cut into 7 squares approximately 2.5 cm in length and height and 1.5 cm in width. The squares were then placed side-by-side a few millimetres apart from one another. In the chamber 2 of the 7 squares acted as negative controls and a third was placed in a petri dish and parafilmed; kept separate on the benchtop as an additional negative control. These controls were used to ensure all mould development was the result of inoculation, and not prior contamination of the bread. The remaining 4 squares were inoculated with 10 spores of *P. roqueforti* 3969, 6130, or *A. niger* FUA5004 to simulate a 'worst-case' contamination scenario using 10 ul of the 1,000 spore per ml solution described in section 2.1.1. Once the containers were full they were sealed and placed underneath a webcam (Logitech C922 Pro HD Stream) which took a photo once every 2 h for 9 d. The time limit was chosen based on constraints of the researcher and is supported by literature which determined that to be the maximum shelf life of bread containing the highest allowable amount of propionic and sorbic acid when exposed to *P. roqueforti* (Debonne et al., 2023). The resulting time-lapse video was used to determine the mould-free shelf life for each individual square of bread. Mould development was considered the time when the mould first became visible to the naked eye. This procedure was replicated such that each sourdough and control was exposed to each of the 3 moulds, and 3 independent baking trials were tested.

After each shelf life experiment, the salt solution was filtered and autoclaved and the anaerobic chambers were soaked briefly in a 1:16 Clorox and water solution, then washed with soap and water and allowed to air-dry.

Strains that were used to make breads with the shortest mould-free shelf life were removed from the experiment; only those with performances consistently equivalent to or better than to the controls moved forward to organic acid quantification by HPLC. *F. sanfranciscensis* TMW1.154 (included as a biological control) was also removed.

A set of experiments was also conducted to assess the impact of different concentrations of ethanol on shelf life using the 10% sourdough breads made with *Lp. plantarum* FUA3183 and a water activity of 0.96 described in section 2.1 to determine if the ethanol content impacted the rate of fungal growth. Inoculations were performed as described above, with the following alterations: once cut in the biosafety cabinet, each square of bread was placed into a petri dish containing a polystyrene weighing boat approximately 1.5" in diameter, which was filled with 0, 156, 312, 624, 1560, or 3120 mmol/kg of ethanol. The bread was placed beside the weighing boat and inoculated with 10 spores of *P. roqueforti* 3969 before the petri dishes were sealed with parafilm, labelled, and monitored by camera for 280 h; a chemically acidified bread packaged with no added ethanol acted as a control. Each inoculation was repeated with three independent baking trials and no salt solution to achieve a water activity of 0.96 was utilised due to lack of space in the petri dish. However, the a_w of unmoulded bread was taken after 280 h and determined to be equivalent to the original value.

2.1.4 Sample preparation and laboratory analysis

In preparation for HPLC, organic acids were released from bread samples. Bread and water (5:1 dilution) were stored in 3 lb plastic bags and stomached for 5 min, then incubated overnight at 4°C. After centrifugation 1 ml of supernatant was pushed through a 0.2 mm filter. 750 µL of filtrate was mixed with 7% perchloric acid in a 1:1 dilution and protein precipitation occurred over 24 h at 4°C. Samples were centrifuged for 7 min at 13,000 RPM and 200 ml of the supernatant was pipetted into an autosampler vial, while 1 ml of supernatant was decanted into an Eppendorf tube and kept at -20°C. For sourdough samples all steps were repeated as described above, but protein precipitation took place before filtration to prevent clogging of the pores. Samples were stored at -20°C until analysed by HPLC (Agilent Technologies 1200 series, Germany). An Aminex HPX-87H column maintained at 70°C with 5 mM H_2SO_4 as solvent, run at a rate of 0.4 ml/min, was used. Solvent was mixed and filtered one or more days prior to use, and final concentrations of acetic and lactic acid are reported as mmol/kg of sample.

2.2 Impact of extended fermentation on antifungal activity of sourdough lactobacilli.

Bacterial strains and experimental procedures are identical to those described in section 2.1 except where described below.

2.2.1 Preparation of sourdough and sourdough bread

Sourdoughs were produced using the protocol detailed in section 2.1.1 and 2.1.2 with the following modifications: after backslopping, the sourdough was fermented for 48 h at 25℃ and the 0.96 a_w recipe (Table 4) was removed from the experiment. The pH of sourdough and bread samples were taken as described in section 2.1.2. A one-way ANOVA ($P<0.05$) was used to

compare sourdough pH values and it was determined that all strains had equivalent activity. Results are the mean ± standard deviation of 3 biological replicates (3 independent sourdough fermentations).

2.2.2 Sample Preparation and Laboratory Analysis

Sample preparation followed the protocol outlined in section 2.1.2 with the modification that the specific volume of the bread loaves was measured by the millet displacement method (American Association of Cereal Chemists, 1998). The loaves were weighed accurately (n=3) and a 2L plastic container was filled with millet seeds measured by burette and the volume was recorded. The bread was placed in the container and the remaining space was filled with millet; the initial volume - volume with bread was recorded and divided by the weight of the loaf to obtain ml/g of bread.

2.2.3 Experimental design

The experimental design was identical to that described in section 2.1.3, with the exception that samples for HPLC analyses were taken after the bulk and final proofs in addition to after sourdough fermentation and baking to track the changes in organic acid quantities during bread-making. UV detection during HPLC was also used to confirm the accuracy of the lactic and acetic acid quantifications. Inoculations were performed following the procedure detailed in section 2.1.3.

2.3 Impact of modified fermentation parameters and antifungal ingredients on the mould-free storage life of sourdough bread.

Bacterial strains and experimental procedures are identical to those described in section 2.1 except where described below.

2.3.1 Preparation of sourdough, sourdough breads, and controls

Sourdoughs were produced using the protocol of sections 2.1.1 and 2.1.2 with the following modifications. All sourdough breads were made with a mixed culture of *Lp. plantarum* FUA3183 and *Lv. hammesii* LP38^T. These strains were chosen due to their consistent performances in previous experiments and for the potential benefits of using a combination of homofermentative and heterofermentative organisms. To make the inoculum, 10 ml of an overnight culture of *Lp. plantarum* FUA3183 and 10 ml of an overnight culture of *Lv. hammesii* LP38^T in water were washed as described in section 2.1.2. 5 ml of each were combined and mixed with 10g flour to create the sourdough starter. After backslopping and an additional 24 h fermentation at 30℃, three different sets of fermentations were produced using an altered recipe for the breads with a 0.92a_w (Table 4). The fermentation and proofing temperatures used were based on the 20% sourdough breads prepared by Zhang et al. (2010). For the first of the 3, labelled ‘Fermentation 1’ or ‘F1’, the recipe was not modified. The sourdough was included in bread dough in an amount of 10%, kneaded immediately after mixing, then proofed following the steps outlined in section 2.1.2 prior to baking. The remaining 2 fermentation methods underwent a 3-stage fermentation similar to that described by Xu et al. (2018) but included a 0.1% addition of yeast before the second fermentation. During bread mixing 30% sourdough was included in the

bread. For the second fermentation method (labelled 'Fermentation 2' or 'F2'), the resulting dough was proofed for 4h after kneading. For the final fermentation ('Fermentation 3' or 'F3') the 30% sourdough dough was kneaded and left to ferment at 5°C for roughly 16 h (Figure 1).

Each of the 3 fermentations was produced using wheat flour, wheat flour with 10% buckwheat sourdough, or wheat flour with a 0.1% addition of calcium propionate to produce 9 different fermented breads. The controls for this experiment were a set of straight doughs which received the same proofing schedule used for the F1 breads. Each control was also made with 3% yeast and either wheat flour, a 10% buckwheat addition, or wheat flour with a 0.1% addition of calcium propionate (Figure 2). Unless otherwise specified, every control bread was made with 0.3% calcium propionate.

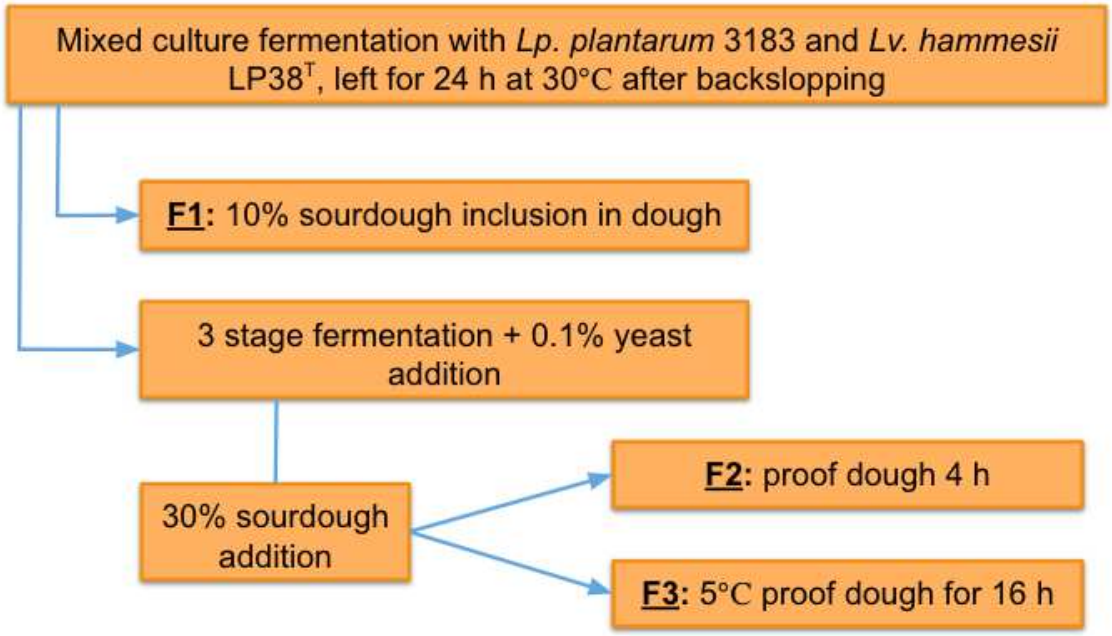

Figure 1. Flow chart detailing the production of the sourdough breads. Each sourdough was produced using wheat flour, wheat flour with a 0.1% addition of calcium propionate, and 10% fermented buckwheat flour.

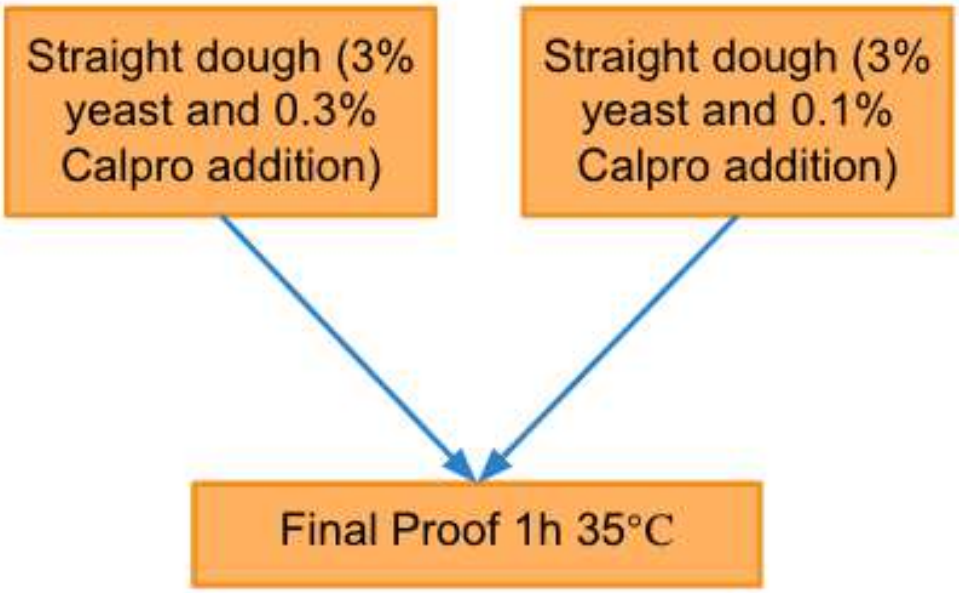

Figure 2. Flow chart detailing the production of the 2 varieties of control breads. The dough containing 0.3% calcium propionate was also produced using wheat flour and 10% buckwheat flour.

2.3.2 Experimental design

An environmental exposure experiment meant to mimic a realistic contamination event was added to the experimental design and performed by preparing bread samples as described in section 2.1.4, but without any inoculation. The containers were sealed and brought to a food-safe laboratory. The lids were removed, exposing the samples inside to air for 1 h before being sealed again and placed under the cameras for a 9 d observation. Humidity during exposure was managed by placing the aluminium foil trays holding the bread (described in section 2.1.3) into containers filled with water, and the salt solution at the bottom of the anaerobic chambers was

able to return the bread to its water activity of ~0.92 after re-sealing.

2.3.3 Sample preparation and laboratory analysis

Sample preparation followed the protocol outlined in section 2.1.4 and 2.2.2 with some modifications: total titratable acidity (TTA) was measured for every loaf of bread by adding 10 g of sample to 90 ml of distilled water and titrating it with 0.1M NaOH until a pH of 8.4 is achieved. The amount of NaOH in ml required is defined as TTA (Hill & Ferrer, 2021). Because it measures the total acids in a sample regardless of if they are dissociated or not, TTA is a more accurate indicator of fermentation than pH, as it continues to increase even after pH has levelled off (Tyl & Sadler, 2017). The impact of different flours on TTA was assessed using a 2-way ANOVA ($P<0.05$). The propionic acid content in the breads was confirmed using HPLC (supplemental Figure A1). Both visual evaluation of the breads after baking and volume measurements confirmed that breads made using the fermentation 3 protocol were undesirably small, dense, and unappetizing. As such the fermentation was removed from the experiment.

2.4 Impact of antifungal additives on the shelf life of sourdough bread

Bacterial strains and experimental procedures are identical to those in section 2.1, except where described below.

2.4.1 Preparation of sourdoughs and controls

The sourdoughs were made with a mixed culture of *L. hammesii* LP38^{T} and *Lp. plantarum* FUA3183 as described in section 2.3.1. Three varieties of bread were produced: one with a 10% buckwheat sourdough (as detailed in section 2.3.1), and two with different concentrations of

iso-α-acids (HopTech, Dublin, CA, USA); 10 mg/L and 30 mg/L. The antifungal activity of iso-a-acids in bread has been reported on by Gobbetti et al. (2018), and was included in these experiments as a comparison against fermented buckwheat. All sourdough breads were made following the F2 protocol detailed in section 2.3.1, and for those breads containing iso-α-acids, they were added by eyedropper to the dough.

The concentration of iso-α-acids needed was determined based on the product's data sheet. 1.23 ml of the extract was diluted with 14.8 ml of distilled water and a pipette was calibrated with 0.62 ml of water (determined to be approximately equal to 17 drops). The resulting concentration was 0.26 IBU per droplet, equivalent to 0.26 mg/L. Following this, 2.26 ml of the diluted solution was added to the 10 mg/L dough and 6.80 ml was added to the 30 mg/L dough to obtain the desired iso-α-acid content.

The controls used were a straight dough with a 0.3% addition of calcium propionate (as described in section 2.3.1), and a straight dough made with 5% yeast.

2.4.2 Experimental design

The experimental design follows the protocol described in section 2.1.4 and includes the environmental mould experiment detailed in section 2.3.2, with the modification that a new control was added. Dempster's white bread was added to the environmental exposure and *P. roqueforti* 6130 inoculation experiments so that comparisons between currently available store bought breads and the experimental sourdoughs could be made. Whole-genome BLAST sequencing was used to identify if *Lp. plantarum* 3183 or *Lv. hammesii* contained the HorA

resistance mechanism after inoculation experiments were performed.

2.4.3 Sample preparation and laboratory analysis

Sample preparation followed the protocol outlined in section 2.1.4, 2.2.2, and 2.3.3 with no modifications.

2.6 Statistical analysis and calculations

Statistics were run using GraphPad Prism (version 9). All data regarding mould experiments are the average of 3 independent baking trials each consisting of 4 technical replicates, and all other data represents the average of 3 independent baking trials.

Measurements of pH were repeated in triplicate and represented as mean ± standard deviation. Bread pH was taken prior to inoculation, and sourdough pH was taken immediately before inclusion in the dough. A one-way ANOVA was performed for each set of pH values to prove uniform fermentation occurred. Quantities of lactic and acetic acid in sourdough samples were compared among fermentations using a one-way ANOVA, the same was done for bread samples. Shelf life of the sourdoughs were contrasted against one another using a one-way ANOVA and compared to that of the control breads by unpaired t-test. Sourdough bread volumes were also compared to the control for each flour treatment group using an unpaired t-test. Significant difference was calculated with a confidence interval of 95%, ($P<0.05$)

3. Results

3.1 Comparison of the antifungal activity of different strains of lactobacilli

These experiments aimed to identify strains of lactobacilli well-suited to sourdough

fermentation so a promising starter culture could be identified and used in future protocols. A secondary goal was to determine if a 10% sourdough inclusion was enough to delay germination of mould spores.

3.1.1 Cell counts and pH values of sourdoughs and sourdough breads

Initially, the activity of 12 strains of lactobacilli already known to exert some level of antifungal activity were compared. In addition to the mould free shelf life, the pH and the organic acid concentration were determined, as they link to antifungal activity. Sourdough and bread pH values were compared within experiments to identify outliers, and between experiments to confirm the equivalency between water activities so comparisons could be made (Table 5). Sourdough and bread pH values were consistent between water activities. Only *Lp. plantarum* FUA3428 varied at the sourdough stage, but the difference was not present post-baking.

Table 5. pH Values of Sourdough and Bread Doughs Formulated for an a_w of 0.92 and 0.96

Strain ID	0.92a_w Sourdough pH	0.96a_w Sourdough pH	0.92a_w Bread pH	0.96a_wBread pH
Chemically Acidified	3.71 ± 0.03	3.59 ± 0.05	5.08 ± 0.18	5.02 ± 0.31
Lc. paracasei FUA3186	3.67 ± 0.14	3.70 ± 0.06	5.08 ± 0.08	4.75 ± 0.29
Lp. plantarum TMW1.460	3.50 ± 0.19	3.31 ± 0.11	4.9 ± 0.22	4.94 ± 0.28
Lp. plantarum FUA3073	3.53 ± 0.06	3.38 ± 0.29	5.06 ± 0.14	5.10 ± 0.03
Lp. plantarum FUA3183	3.46 ± 0.04	3.52 ± 0.16	5.04 ± 0.09	4.93 ± 0.17
Lp. plantarum FUA3428	3.66 ± 0.10[a]	3.35 ± 0.13[b]	4.85 ± 0.20	5.06 ± 0.10
Lv. hammesii LP38^T	3.51 ± 0.12	3.57 ± 0.14	4.99 ± 0.09	4.99 ± 0.04
Ln. parabuchneri FUA3718	3.67 ± 0.12	3.67 ± 0.20	5.09 ± 0.09	4.98 ± 0.19
F. sanfranciscensis TMW1.154	3.55 ± 0.20	3.51 ± 0.14	4.91 ± 0.21	4.90 ± 0.14
L. reuteri LTH2584	3.57 ± 0.06	3.54 ± 0.09	4.50 ± 0.14	4.84 ± 0.30
Ff. milii FUA3583	3.42 ± 0.07	3.50 ± 0.24	5.10 ± 0.15	4.95 ± 0.11
Ff. milii FUA3430	3.52 ± 0.18	3.58 ± 0.09	5.12 ± 0.06	4.84 ± 0.21
Ff. milii FUA3124	3.60 ± 0.18	3.42 ± 0.14	4.88 ± 0.22	4.79 ± 0.12

No outliers were identified in the data set.

3.1.2 Mould free shelf life of sourdough breads fermented with different strains.

The shelf life of the sourdough breads was compared among one another and to the control with the goal of identifying strains with significantly better or worse performances than the others, so the bacteria best suited for fermentation could undergo organic acid quantification

and use in future experiments.

When compared, the sourdough breads' shelf life against any of the 3 moulds at a water activity of 0.92 were identical (Figure 3). When compared directly to the control, sourdough breads produced with *Lm. reuteri* LTH2584 and *Ff. milii* FUA3124 developed *A. niger* and *P. roqueforti* 6130 (respectively) faster (Figure 4). In the experiments performed at a water activity of 0.96 no strain was significantly better at delaying mould spoilage than another, but comparing the shelf life of sourdough breads to the controls highlighted that both *Lp. plantarum* FUA3183 and FUA3428 exposed to *P. roqueforti* 6130 had a significantly longer shelf life (Figure 3).

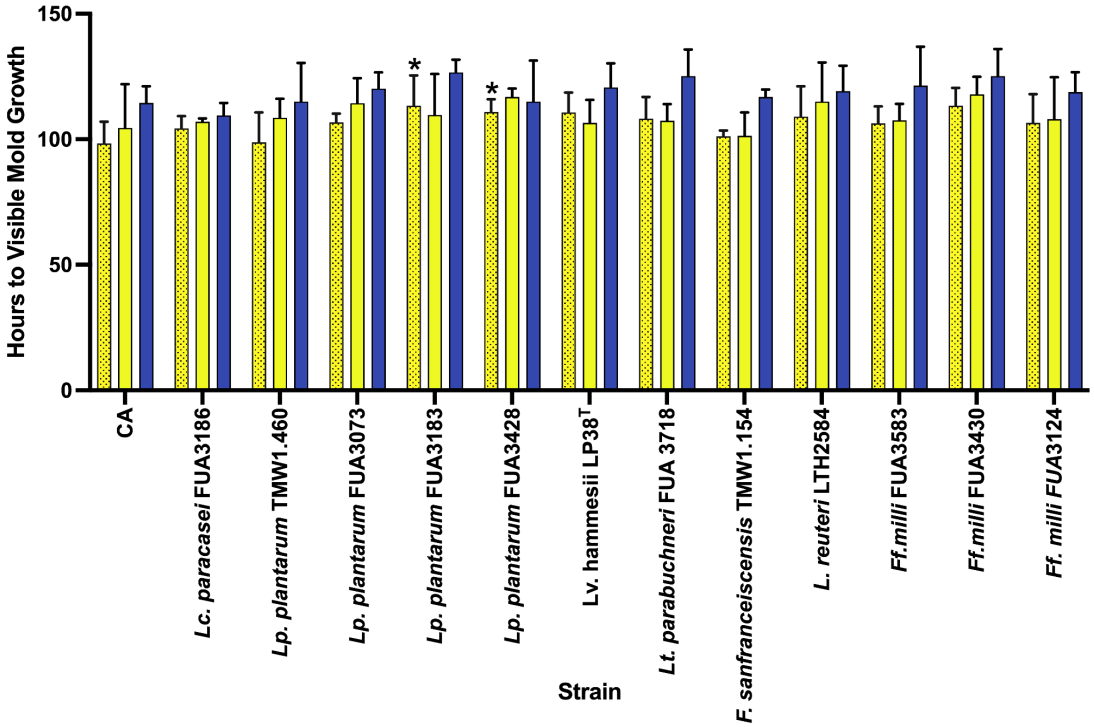

Figure 3. Shelf life of bread with a_W of 0.96 after inoculation with 10 spores of *Penicillium roqueforti* 6130 (▩), 3969 (▭), and *Aspergillus niger* FUA5004 (▭). Shelf life of the sourdoughs were compared to the control where significant difference was marked by an

asterisk. Results are shown as means ± standard deviation of three biological replicates (three independent sourdough fermentation).

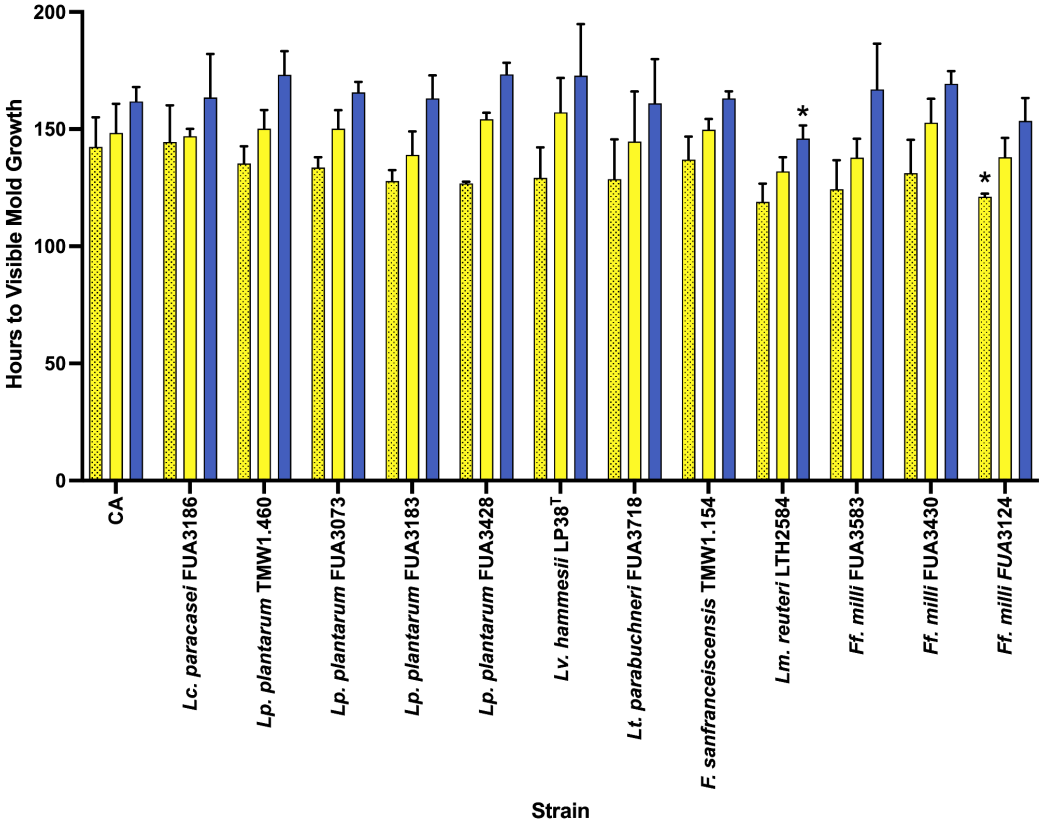

Figure 4. Shelf life of bread with a_W of 0.92 after inoculation with 10 spores of *Penicillium roqueforti* 6130 (▩), 3969 (▭), and *Aspergillus niger* FUA5004 (▭). Results are shown as means ± standard deviation of three biological replicates (three independent sourdough fermentations).

3.1.3 Concentrations of organic acids in sourdough bread

The organic acid concentrations were compared to help add context to the results of the mould experiments and judge the performance of strains. A secondary objective was to confirm

that the metabolic activity of each strain was equivalent across the two water activities. The sourdoughs prepared for bread with a_W of 0.96 contained more lactic acid than the controls, except *Lc. paracasei* FUA3186 and *Ln. parabuchneri* FUA3718. The concentration of acetic acid at this stage was equivalent among all breads. After baking, the organic acid content decreased to the point they were all equivalent (Figure 5).

In the experiments with breads formulated with a water activity of 0.92, 4 strains *(Lc. paracasei* FUA3186, *Ln*. *parabuchneri* FUA3718, *Lp. plantarum* FUA3073 and *Ff. milli* FUA3430) contained equivalent amounts of lactic acid to the control and all samples were equal in acetic acid at the sourdough stage. After baking no differences in the content of lactic acid between the samples was observed, but *Ln. parabuchneri* FUA3718 produced more acetic acid than all other breads except *Lp. plantarum* TMW1.460 *and Lv. hammesii* LP38^T (Figure 6). The organic acid contents found in the breads were equivalent across water activities.

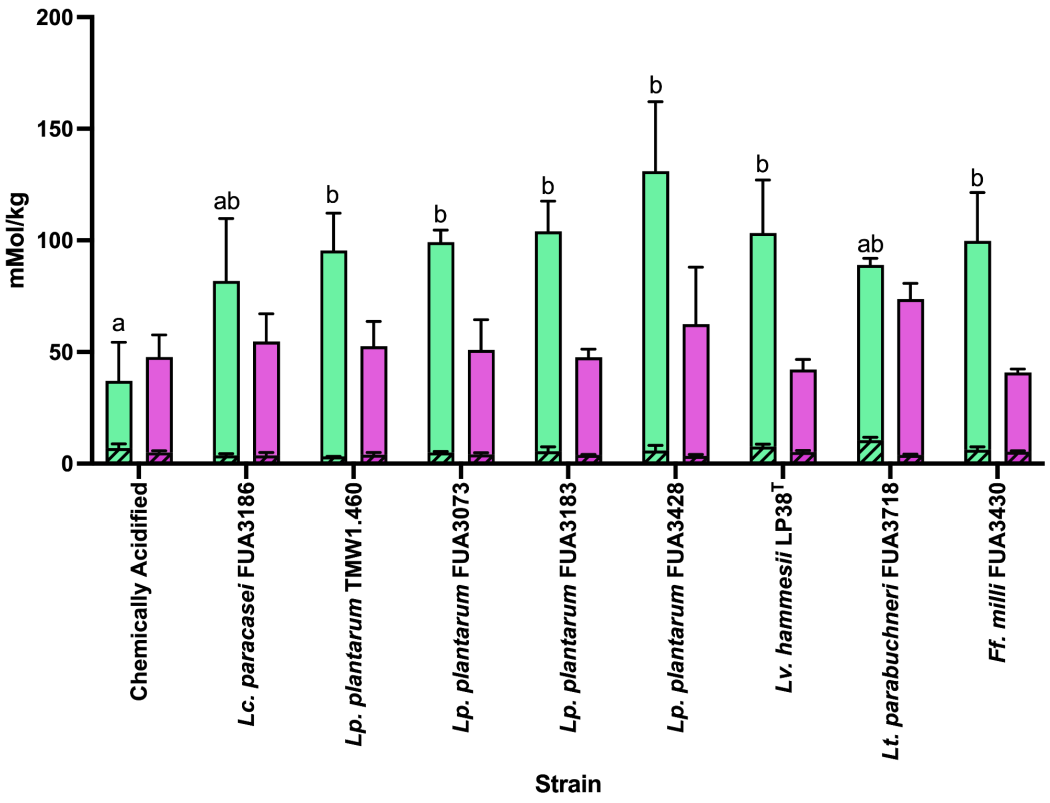

Figure 5. Concentrations of lactic ( ) and acetic ( ) acid in sourdough and lactic and acetic acid in breads (/) made with a water activity of 0.96aw, quantified using HPLC. Differences among all samples as sourdoughs were determined by one-way ANOVA ($P<0.05$) and marked with letters. Results are shown as means ± standard deviation of three biological replicates (three independent sourdough fermentation).

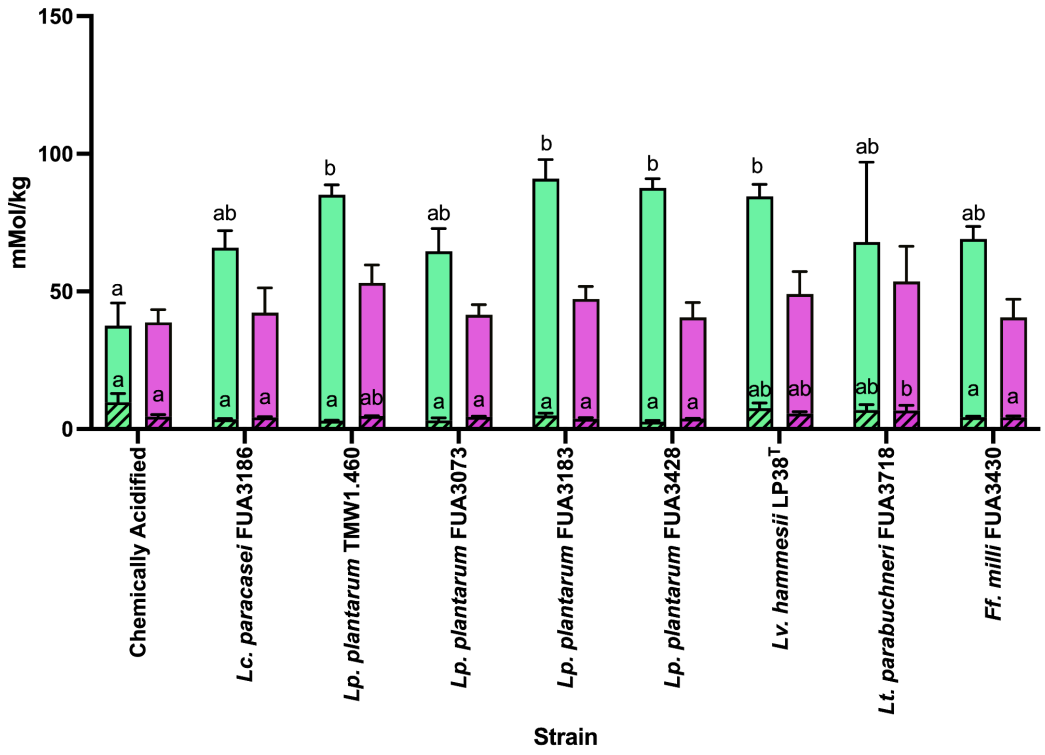

Figure 6. Concentrations of lactic ( ) and acetic ( ) acid in sourdough and lactic and acetic acid in breads (/) made with a water activity of 0.92a_w, quantified using HPLC. Differences among all samples as sourdoughs were determined by one-way ANOVA ($P<0.05$) and marked with lettering. Results are shown as means ± standard deviation of three biological replicates (three independent sourdough fermentations).

3.1.4 Mould free shelf life of breads stored with ethanol

The impact of ethanol as a product of sourdough fermentation by lactobacilli and yeast on shelf life was assessed through a moulding experiment as described in section 2.1.4. The sourdough bread stored without ethanol had a shelf life which was identical to both the control and the sourdough stored with 156 mmol/kg of ethanol. The increase in mould-free shelf life that

312 mmol/kg of ethanol had was equivalent to that displayed by all larger ethanol concentrations tested (Figure 7).

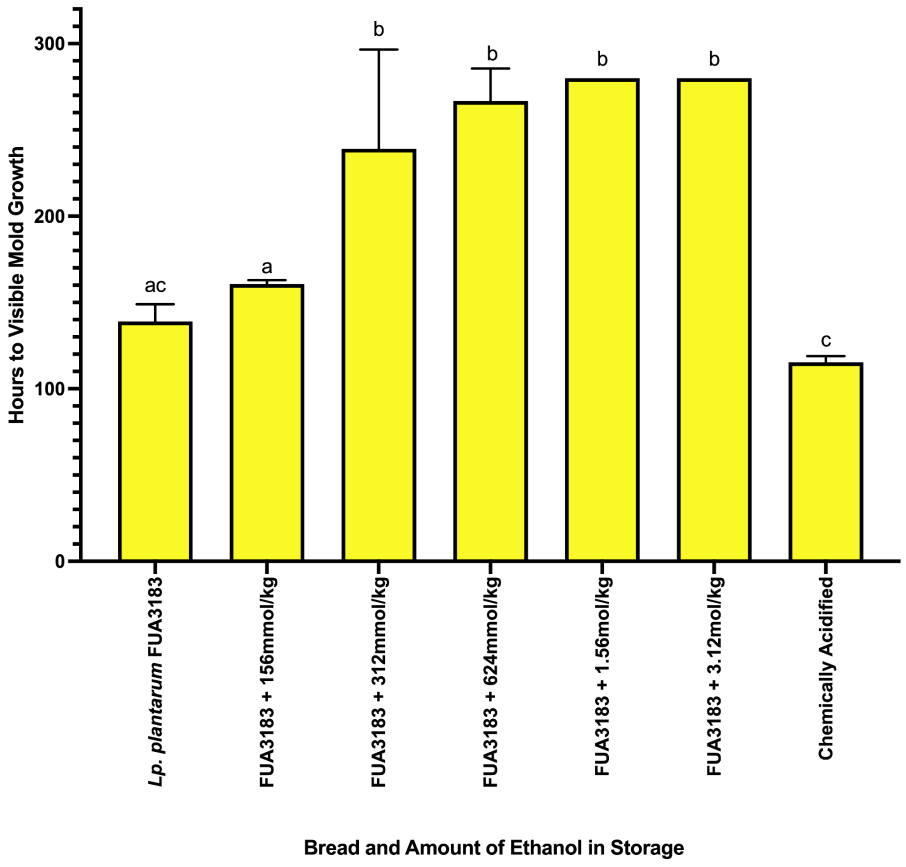

Figure 7. Shelf life of breads packaged alone or with 156, 312, 624, 1560, or 3120 mmol/kg of ethanol after inoculation with 10 spores of *Penicillium roqueforti* 3969 (▭). Shelf life of the sourdoughs were compared by one-way ANOVA ($P<0.05$) and Tukey's test ($P<0.05$) after 280h. The weight of the bread was estimated after the experiment. The ethanol already present in the chemically acidified and *Lp. plantarum* FUA3183 sourdough loaves were not accounted for. All data are the mean ± standard deviation of 3 replicates with 1 inoculated sample of bread.

3.2 Impact of extended fermentation on antifungal activity of sourdough lactobacilli

These experiments were performed to assess the impact of an extended fermentation on organic acid production of previously selected starter cultures and to identify any effects the fermentation conditions may have on bread volume.

3.2.1 Volume and pH of extended fermentation sourdoughs and breads

The pH of all sourdough fermentations were compared to identify outliers indicating different amounts of activity during fermentation. Volume was also assessed to determine if a 10% addition of any of the sourdoughs were able to increase the volume of the fermented bread to a significant degree.

The final pH of the sourdoughs were equivalent. Once the loaves were baked, the majority of sourdough breads (except those made with *Lp. plantarum* FUA3183, FUA3428 and *Lc. paracasei* FUA3186) had a lower pH than the control. Breads made with *Lp. plantarum* FUA3073 was the only sourdough bread with volume greater than the control (Table 6).

Table 6. Parameters of breads made with 48 h fermentations

Strain ID	Sourdough pH	Bread pH	Bread Volume (g/ml)
Control*	-	5.32 ± 0.09	3.29 ± 0.07
Lc. paracasei FUA3186	3.40 ± 0.09	5.03 ± 0.23	2.99 ± 0.40
Lp. plantarum TMW1.460	3.53 ± 0.07	5.13 ± 0.02[a]	3.36 ± 0.04
Lp. plantarum FUA3073	3.50 ± 0.12	5.04 ± 0.03[a]	3.52 ± 0.08[a]
Lp. plantarum FUA3183	3.40 ± 0.09	5.14 ± 0.43	3.08 ± 0.18
Lp. plantarum FUA3428	3.47 ± 0.13	5.05 ± 0.26	3.38 ± 0.12
Lv. hammesii LP38[T]	3.43 ± 0.12	4.91 ± 0.19[a]	3.03 ± 0.53
Ln. parabuchneri FUA3718	3.52 ± 0.05	5.04 ± 0.11[a]	3.29 ± 0.23
Ff. milli FUA3430	3.45 ± 0.09	5.03 ± 0.03[a]	2.98 ± 0.50

Significant difference for pH and volume of bread loaves was determined using an unpaired T-test ($P<0.05$) and is marked by lettering.

3.2.2 Concentrations of organic acids in sourdough and breads

Organic acid concentrations were compared at various stages to see how they changed over the course of bread making, and to determine if the new fermentation protocol resulted in more acetic and lactic acid production. When evaluating the sourdough stage, no difference in the amount of organic acids was found in any of the sourdoughs. Comparison to the control was not possible as it did not have a sourdough stage. The same lack of significance was observed in the samples taken at the bulk proof stage. By the time of the second proof, the metabolic activity of

the lactobacilli had increased enough that some sourdough breads (those made with *Lp. plantarum* TMW1.460, FUA3073, FUA3183, and *L. parabuchneri* FUA3718) contained more lactic acid than the control. Baking caused a reduction in organic acids, with only *Lp. plantarum* FUA3073, FUA3428 and *L. parabuchneri* FUA3718 containing more lactic acid than the control, and *Lp. plantarum* FUA3428, *L. hammesii* LP38^{T} and *Ff. Milii* FUA3430 contained more acetic acid. An observation of importance is that the majority of organic acids were produced between the first and second proof.

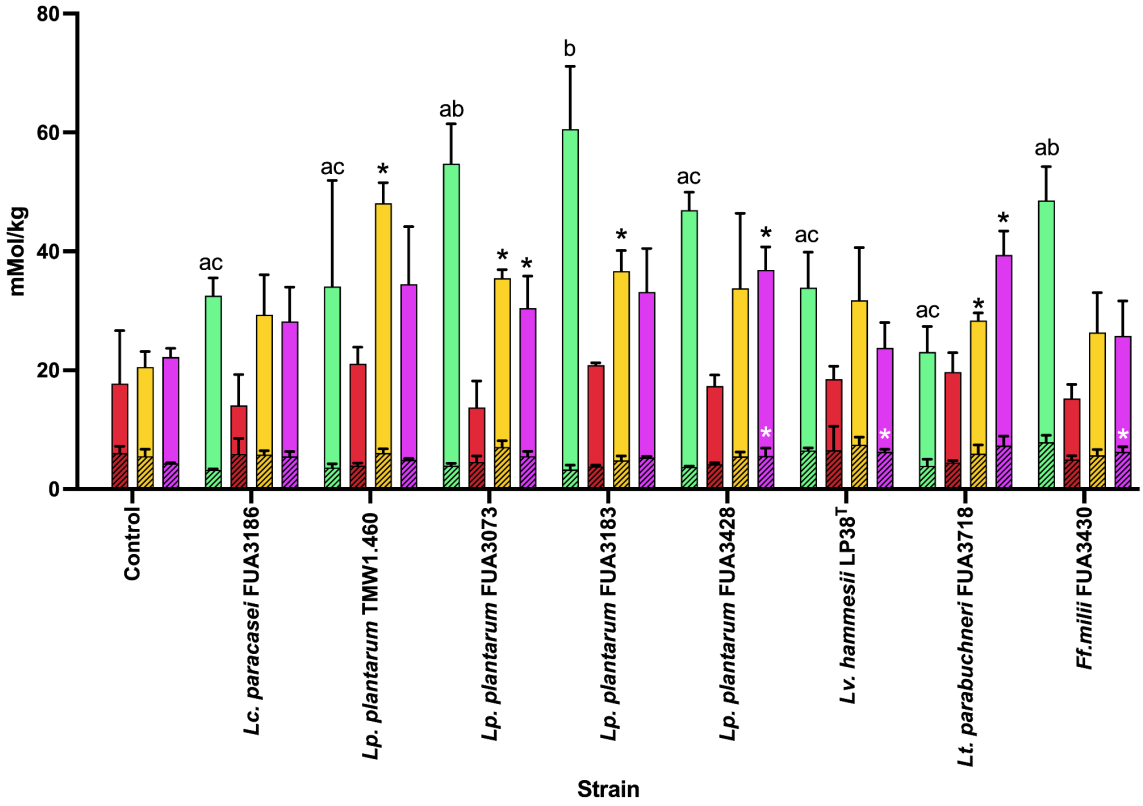

Figure 8. Concentrations of lactic and acetic acid for breads made with a 10% addition of sourdough fermented for 48 h, quantified by HPLC. Differences among strains in the sourdough stage are signified by letters and determined by one-way ANOVA ($P<0.05$). Strains were compared to the control at each of the 4 following stages of bread production: sourdough (/), bulk proof (/), second proof (/), and bread (/) where any differences from the control are represented by an asterisk. Data is mean ± standard deviation of three biological replicates.

3.2.3 Mould free shelf life of sourdoughs made with an extended fermentation

The purpose of the mould experiments was to see if the organic acid concentration in the sourdoughs were enough to translate to a delay in fungal growth. The shelf life of the sourdough breads did not improve over that of the controls in any of the exposure scenarios; in the case of *Lc. paracasei* FUA3186, it was significantly less capable of preventing *A. niger* 5004 growth than the control (Figure 9).

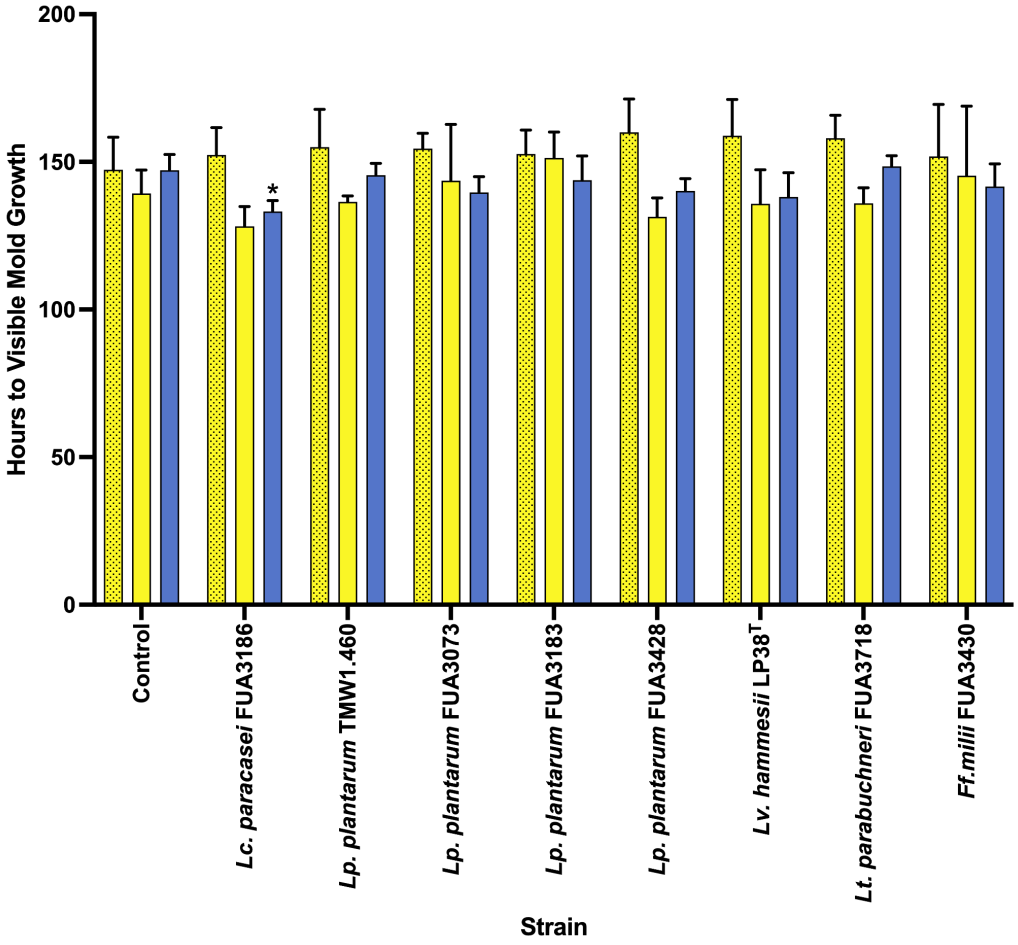

Figure 9. Shelf life of breads made with a 10% addition of sourdough fermented for 48 h after inoculation with 10 spores of after inoculation with 10 spores of *Penicillium roqueforti* 6130 ( ), 3969 ( ), and *Aspergillus niger* FUA5004 ( ). Shelf life of the sourdoughs was compared to that of the control and significant difference was marked by an asterisk. Results are shown as means ± standard deviation of three biological replicates (three independent sourdough fermentations).

3.3 Impact of modified fermentations and antifungal ingredients on the mould-free storage life of sourdough bread

The goal of the proofing modifications and addition of buckwheat was primarily to contrast 3 potential proofs against one another and to select the best one for further experiments. The secondary goals were to compare buckwheat to wheat fermentations and to provide a better idea of what the results of a realistic environmental contamination scenario might look like. All sourdough fermentations were made with a mixed culture of *Lv. hammesii* LP38^{T} and *Lp. plantarum* FUA3183 as described in section 2.3.1.

3.3.1 Volume, pH and TTA of sourdoughs and sourdough bread

As seen in Table 7, the specific volume of breads made using F1 and F2 with buckwheat were greater than the control bread. In the formulations with only wheat flour, F1 had greater specific volume than the control, and in formulations with 0.1% calcium propionate, only F2 had significantly greater volume. In all cases breads made according to the protocol for F3 were comparable to their control, but when contrasted against F1 and F2, were smaller than both in almost all cases. Statistical comparisons supported the researcher's visual evaluation in section 2.3.2.

The pH values of the sourdoughs (excluding F) were compared within their own groups (buckwheat, wheat, and 0.1% calcium propionate) to see if the differences in fermentation resulted in a difference in pH. All F2 sourdoughs were identical to F1, except for the sourdoughs

used in the breads formulated with 0.1% calcium propionate. Among the breads, the controls consistently had a higher pH than the sourdough. F1 with wheat flour and all breads made using F3 were identical to the pH of the control. In comparisons of the same fermentation made using different flours, no treatment resulted in a significant difference, indicating that using 10% buckwheat flour did not significantly impact the pH regardless of the fermentation parameters used.

TTA was also assessed for each bread to determine if any differences present were due to the fermentation or the flour used. The majority of variance (71.53%) was accounted for by the fermentation used, but the impact on variance of the flour at 12.05% was still considered significant according to a 2-way ANOVA ($P<0.05$).

Table 7. Parameters of bread made with modified fermentations

Bread	Sourdough pH	Bread pH	Bread Volume (g/ml)	TTA (ml NaOH)
Control Buckwheat*	-	5.37 ± 0.15^{a}	2.52 ± 0.17^{ab}	3.60 ± 0.17^{b}
F1 Buckwheat	3.44 ± 0.04	5.03 ± 0.11^{b}	3.25 ± 0.36^{a}	4.43 ± 0.51^{b}
F2 Buckwheat	3.58 ± 0.04	4.88 ± 0.01^{b}	3.01 ± 0.44^{a}	5.68 ± 0.39^{a}
F3 Buckwheat	3.99 ± 0.42	5.14 ± 0.13^{a}	1.88 ± 0.58^{b}	-
Control Wheat*	-	5.33 ± 0.16^{a}	2.23 ± 0.16^{ab}	3.10 ± 0.10^{b}
F1 Wheat	3.31 ± 0.05	5.07 ± 0.13^{ab}	2.91 ± 0.34^{ab}	4.25 ± 0.22^{a}
F2 Wheat	3.60 ± 0.08	4.82 ± 0.06^{b}	3.18 ± 0.35^{a}	4.50 ± 0.18^{a}
F3 Wheat	3.83 ± 0.39	5.20 ± 0.14^{ab}	1.93 ± 0.55^{ab}	-
Control 0.1% Calcium propionate*	-	5.42 ± 0.06^{a}	2.51 ± 0.31^{bc}	2.81 ± 0.24^{b}
F1 0.1% Calcium propionate	3.30 ± 0.03^{a}	4.90 ± 0.13^{b}	3.11 ± 0.53^{ab}	4.12 ± 0.34^{a}
F2 0.1% Calcium propionate	3.51 ± 0.04^{b}	4.83 ± 0.20^{b}	3.55 ± 0.41^{a}	4.81 ± 0.71^{a}
F3 0.1% Calcium propionate	3.77 ± 0.13^{c}	5.10 ± 0.04^{a}	1.88 ± 0.26^{c}	-

*No sourdough was used in the control breads. Unless otherwise specified, all control breads contain 0.3% calcium propionate. Significant difference within flour-treatment groups was determined by one-way ANOVA ($P<0.05$) for sourdough and bread pH values, and 'a' or 'b' lettering denotes significant differences. TTA values were compared using a 2-way ANOVA across all experiments ($P<0.05$). F3 breads were removed from the experiment and did not undergo TTA determination. Results are shown as means ± standard deviation..

3.3.2 Concentrations of organic acids

The organic acids concentrations of the sourdoughs were compared among each other to

determine the most suitable flour and fermentation combination (Figure 10). In all cases, the F1 breads contained significantly less lactic acid than the F2 breads, and in only one case, more acetic acid (determined by t-test [$P<0.05$]). When assessing the same fermentation made with different flours, fermentations with buckwheat contained more lactic acid than their counterparts made with wheat flour.

In the case of the bread, comparison of the samples by one-way ANOVA ($P<0.05$) showed that of those made with buckwheat flour, F1 contained more lactic acid than the control and F2 contained even more than F1 breads. Among the breads made with only wheat flour, the two sourdoughs contained more lactic acid than the control, and for those made with 0.1% calcium propionate, F1 and the control both had less lactic acid than F2. In all cases, F1 and F2 contained identical amounts of acetic acid.

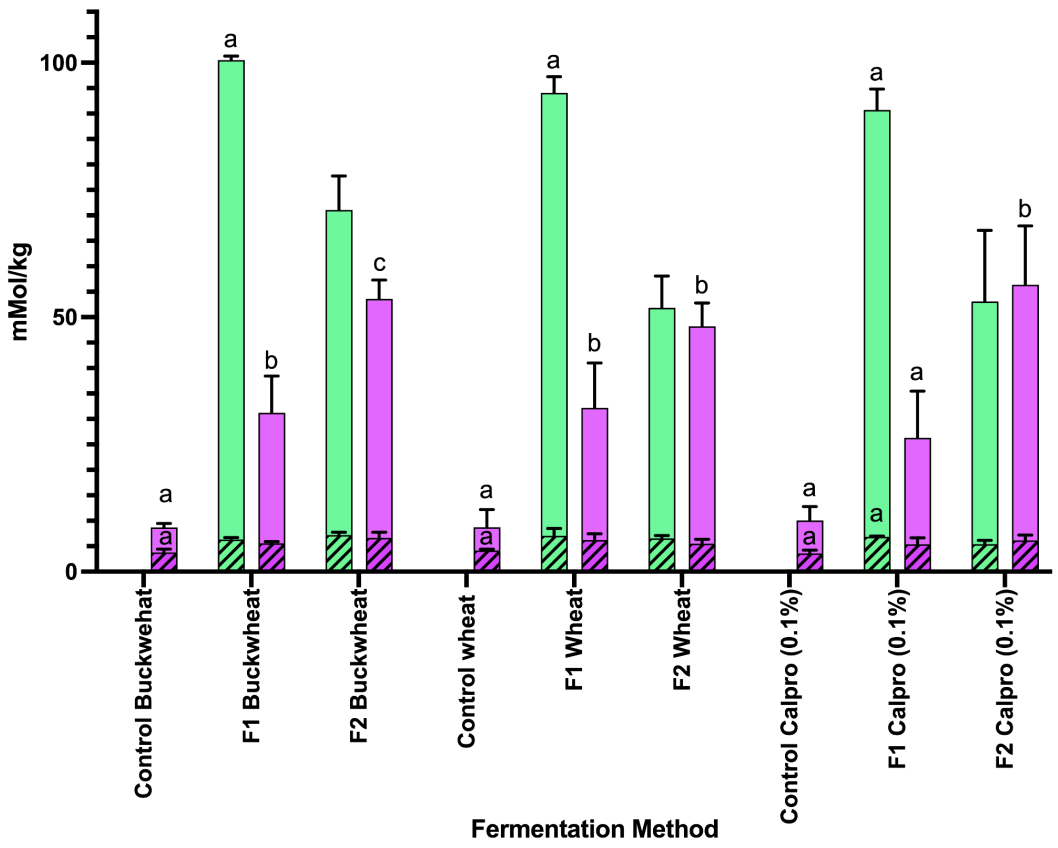

Figure 10. Concentrations of lactic ( ) and acetic ( ) acid in sourdough and lactic ( ) and acetic ( ) acid in bread samples, quantified using HPLC. All F1 breads were made with 10% sourdough and all F2 breads were made with 30% sourdough. Differences between the sourdoughs made with the same flour were determined by t-test ($P<0.05$) and differences among the breads made with the same flour were determined by one-way ANOVA ($P<0.05$). Results are shown as means ± standard deviation of three biological replicates (3 independent sourdough fermentations).

3.3.3 Mould free shelf life of breads made with modified fermentations and buckwheat

In all inoculations F2 breads had an equivalent shelf life to the controls, regardless of the type of flour or amount of calcium propionate. This was also true for the environmental mould

experiments. F1 breads had a more varied performance, developing mould more quickly than F2 breads in multiple scenarios. F1 made with 0.1% calcium propionate was the only F1 bread to meet the shelf life of its respective control in all exposure scenarios, showing how chemical preservatives can improve the shelf life of sourdough bread. Wheat flour fermentations generally had the shortest shelf life (Figure 11).

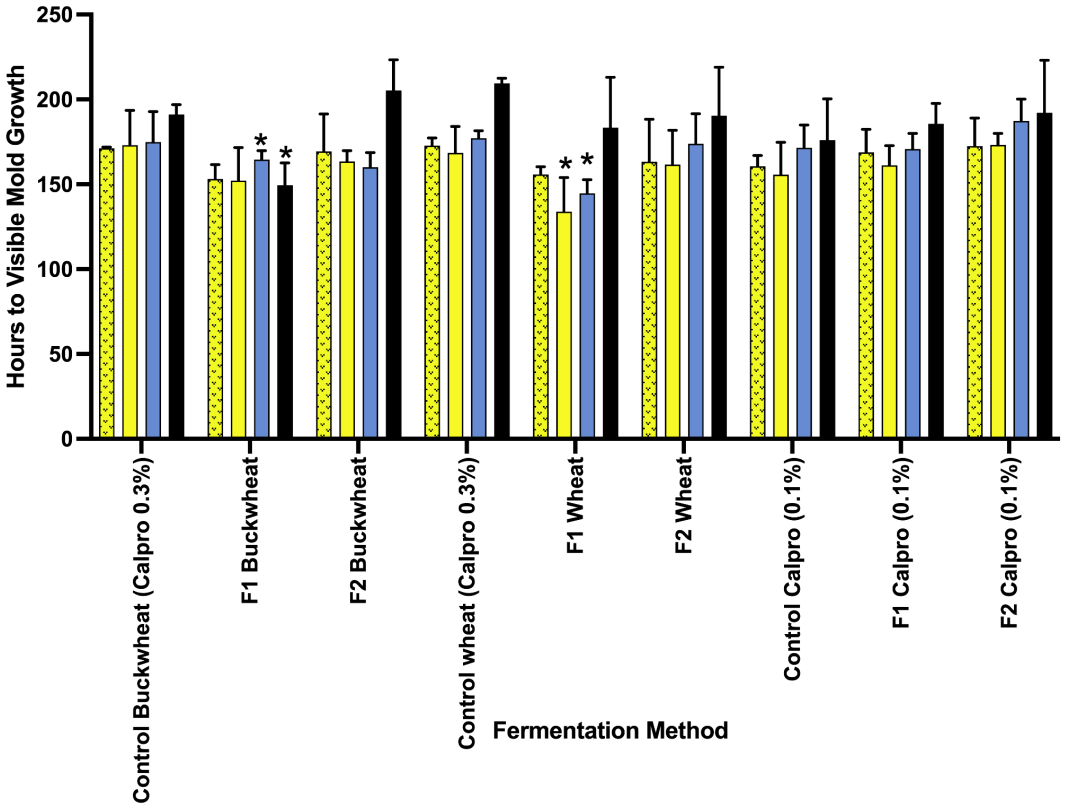

Figure 11. Shelf life of breads made with modified fermentation parameters and antifungal ingredients after 10-spore inoculation with *Penicillium roqueforti* 3969 ( ), 6130 ( ), and *Aspergillus niger* FUA5004 ( ), or after 1 h of environmental exposure ( ). Shelf life of the sourdoughs were compared to that of the control after environmental exposure. After inoculation, all breads made using the same flour were assessed by one-way ANOVA ($P<0.05$). Significant difference from the control in either case was marked by an asterisk. All data are the means ± standard deviation of 3 biological replicates which each had 4 inoculated pieces of bread.

3.4 Impact of antifungal additives on the shelf life of sourdough bread

Antifungal additives sourced from buckwheat and hops cones were included in sourdough bread to determine if they were able to delay mould spoilage, and if so, which treatments had the greatest effect. A comparison to store-brand bread was included to determine if bread free from added preservatives could match the shelf life of commercially available products. All sourdough fermentations were made with a mixed culture of *Lv. hammesii* LP38^{T} and *Lp. plantarum* FUA3183, as in section 2.3.1.

3.4.1 Volume, pH, and TTA of sourdough and control breads

The pH values of the sourdoughs were equivalent, as were the volumes of the final loaves. The pH of the buckwheat bread was the lowest of all samples while the controls were the highest. Buckwheat samples had the highest TTA values while the controls had the lowest. Both breads containing hops were equivalent in pH, volume, and TTA (Table 8). All sourdoughs and volumes of all breads were equivalent among treatments.

Table 8. Parameters of sourdough breads prepared with antifungal additives and controls

Bread	Sourdough pH	Bread pH	Bread volume	TTA (ml NaOH)
10% Buckwheat	3.70 ± 0.09	4.40 ± 0.03[b]	2.60 ± 0.23	7.80 ± 0.087[a]
10 mg/L hops	3.86 ± 0.12	4.88 ± 0.77[ab]	2.20 ± 0.47	6.30 ± 0.3[b]
30 mg/L hops	3.91 ± 0.13	4.51 ± 0.03[ab]	2.77 ± 0.57	6.03 ± 0.20[b]
5% Yeast	-	5.38 ± 0.05[a]	2.64 ± 0.13	3.43 ± 0.38[c]
0.3% Calcium propionate	-	5.42 ± 0.14[a]	2.15 ± 0.32	3.17 ± 0.32[c]

Results are shown as means ± standard deviation of three biological replicates (three independent sourdough fermentations). A one-way ANOVA ($P<0.05$) was performed on bread pH values and total titratable acidity, and significant difference was marked with lettering in the columns.

3.4.2 Concentrations of organic acids

Among the sourdoughs, the fermentation that included buckwheat produced the most lactic acid. The acetic acid values among the formulations were not different (Figure 12).

Comparisons of the organic acid concentrations among the breads in Figure 13 showed that buckwheat contained significantly more lactic acid than the other sourdoughs. The 0.3% calcium propionate and 5% yeast controls contained significantly less lactic acid than any of the sourdough breads. Judging by the reduced organic acid content in the final bread, the α-acids impeded the growth of the lactobacilli during the proofing stages.

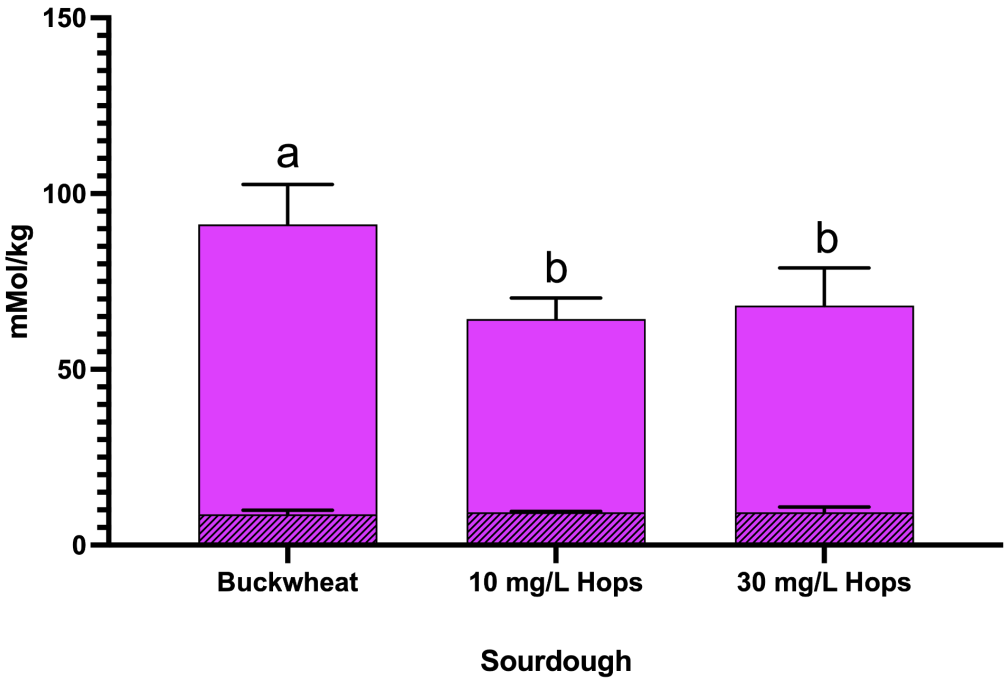

Figure 12. Concentrations of lactic ( ) and acetic ( ) acid in sourdough samples, quantified using HPLC. Buckwheat sourdough and sourdough used to make bread dough containing 10 or 30 mg/L hops were produced with 30% sourdough fermented with *Lp. plantarum* and *Lv. hammesii* (F2 protocol). Differences in lactic and acetic acid across formulations were determined by one-way ANOVA ($P<0.05$) and marked with lettering. Results are shown as means ± standard deviation of three biological replicates (3 independent sourdough fermentations).

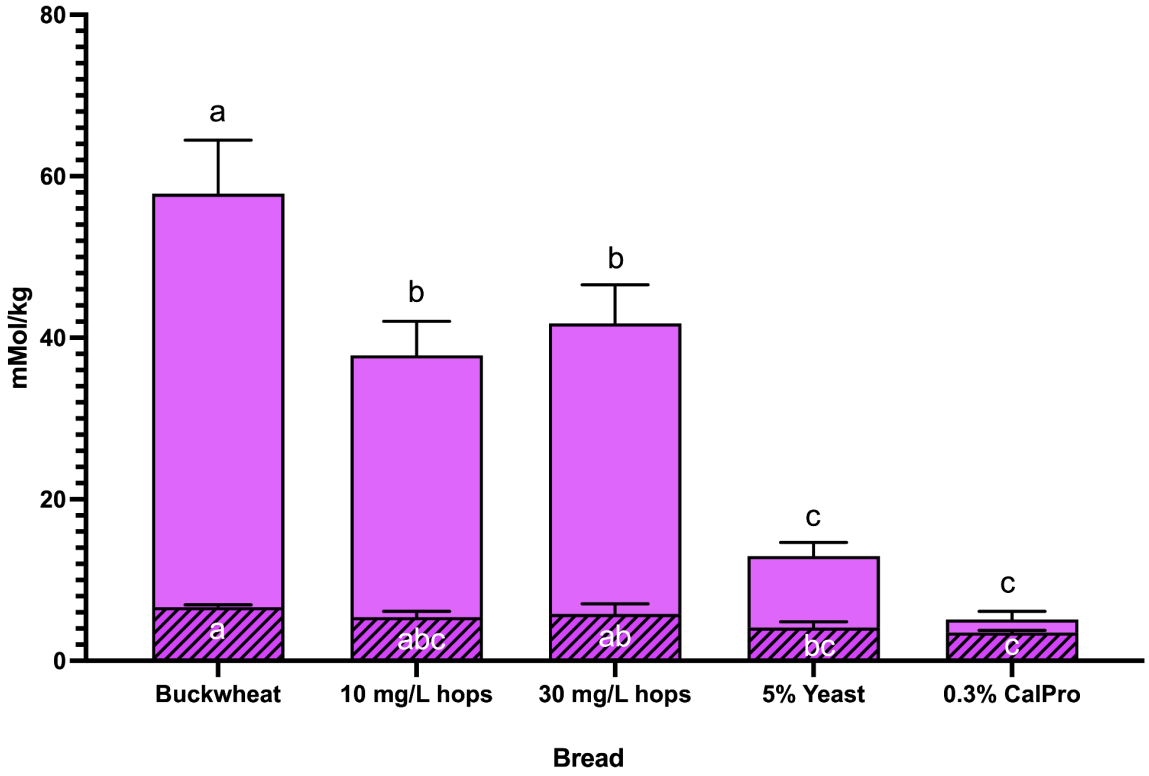

Figure 13. Concentrations of lactic ( ) and acetic ( ) acid in bread samples, quantified using HPLC. The 5% yeast bread and 0.3% calcium propionate bread were produced without sourdough; buckwheat bread and bread with 10 or 30 mg / L hops were produced with 30% sourdough fermented with *Lp. plantarum* and *Lv. hammesii* (F2 protocol). Differences in lactic and acetic acid across formulations were determined by one-way ANOVA ($P<0.05$) and marked with lettering. Results are means ± standard deviation of three biological replicates.

3.4.3 Mould free shelf life of breads made using antifungal additives

In Figure 14 the environmental exposure experiments indicated that all breads had a similar shelf life, but inoculation with *P. roqueforti* 6130 and *A. niger* FUA5004 determined that, when compared to a 0.3% calcium propionate bread, the 5% yeast bread had a significantly

shorter shelf life. All 3 of the fermented bread formulations were able to meet or exceed the shelf life of the control, though none could match that of the Dempsters bread. All breads used in the environmental experiments had a shelf life which exceeded those inoculated with *P. roqueforti* 6130, suggesting the environmental spores were less resistant. Inoculation of bread with *A. niger* FUA5004 did not result in a significantly shorter shelf life than was observed for breads inoculated with *P. roqueforti* 6130. When inoculated with *P. roqueforti* 3969, the buckwheat bread had a longer mould free shelf life than the bread formulated with 0.3% calcium propionate. Conversely the 5% yeast bread lasted significantly less time when inoculated with *A. niger* FUA5004 and *P. roqueforti* 6130.

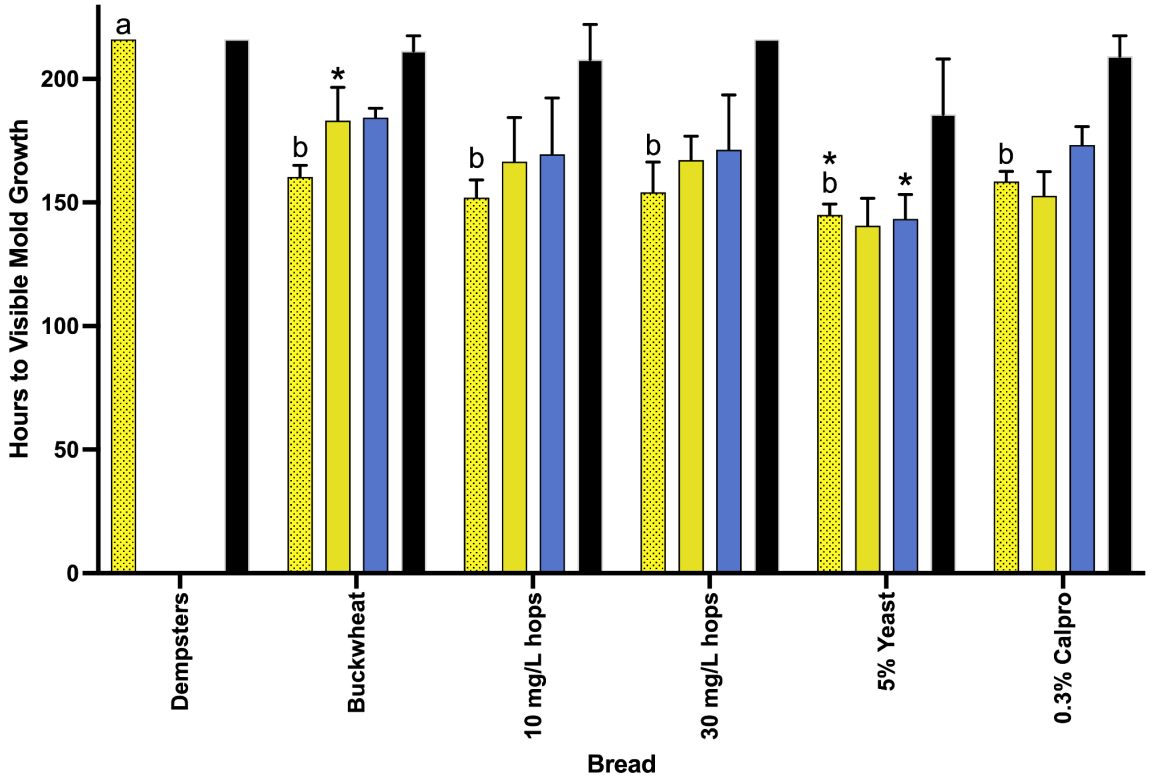

Figure 14. Shelf life of controls and breads made with antifungal ingredients after inoculation with 10 spores of *Penicillium roqueforti* 6130 (), 3969 (), and *Aspergillus niger* FUA5004 (), and after 1h of environmental exposure (). Dempster's, 5% yeast bread and 0.3% CalPro bread were produced without sourdough; buckwheat bread and bread with 10 or 30 mg/L hops were produced with 30% sourdough fermented with *Lp. plantarum* and *Lv. hammesii* (F2). Shelf life of the sourdough breads were compared to the Dempster's brand bread after environmental exposure and inoculation by *P. roqueforti* 6130 (indicated by lettering). A difference in shelf life from the 0.3% calcium propionate control after inoculation with any of the three moulds was marked by an asterisk. Results are shown as means ± standard deviation of three biological replicates (3 independent sourdough fermentations).

4. Discussion

Experiments described in this thesis expand on the current body of knowledge by comparing the ability of several fermentation schemes (all based on common baking practices seen in large- and small-scale bread production) to increase lactic and acetic acid content, and by assessing the impact of antifungal adjuncts on the mould-free shelf life of sourdough bread.

The case was made that a 10% addition of any single-culture sourdough was not enough to contribute to the shelf life in a meaningful capacity, confirming the hypothesis. Additionally, no one strain was consistently and significantly more effective in delaying fungal spoilage than any other. This finding is not in line with most of the literature on this subject. Mota-Gutierrez et al. (2021) compared the antifungal activity of 77 strains of *Lp. plantarum* and *Lactcaseibacillus casei* against 16 fungal cultures *in-vitro* and determined that fungal inhibition was strain specific. Similarly, Garofalo et al. (2012) screened 216 lactobacilli strains against *Aspergillus japonicus*, *Eurotium repens*, and *Penicillium roseopurpureum* and determined *Ff. rossiae* LD108 and *Companilactobacillus paralimentarius* PB127 showed significant activity compared to the others. When incorporated into a panettone sourdough, they delayed fungal spoilage up to 10 d. Unlike those previously-described works, this experiment's methods are unique in that it utilised highly resistant moulds known for their tolerance of chemical preservatives. Most existing research including the 2 examples above use less-resistant species and strains including members of the *Aspergillus, Fusarium* and *Mucor* genus (Mota-Gutierrez et al., 2021; Garofalo et al., 2012). When *Penicillium* strains are utilised, they are almost never *P. roqueforti,* which would represent the worst-case exposure scenario. Rather, species such as *P. nalgiovense, P. citreum,* or *P. chrhysogenum* are often chosen as indicator organisms instead. Fraberger et al. (2020) was one of

the few studies who assessed antifungal activity against *P. roqueforti* and other moulds using *in vitro* experiments and determined it was the most resistant species, with 65% of the 184 lactic acid bacteria isolates unable to impact its growth on MRS agar. *Lp. plantarum* proved to be the most effective of the lactobacilli. However, the authors note that the MRS media contained sodium acetate, a chemical with antifungal effects (Stiles et al., 2002). On APT agar, which is developed for the growth of heterofermentative species and does not include sodium acetate, only 5 strains demonstrated an inhibitory effect of 'weak' or 'intermediate' against *P. roqueforti*, and none were considered to have 'very strong inhibition' (Fraberger et al., 2020). In contrast, Magnusson et al. (2003) found that of 120 isolates screened for antifungal effects, none inhibited *P. roqueforti*. Quattrini et al. (2019) also assessed the impact of sourdough bread on shelf life when exposed to moulds, including *P. roqueforti*, and determined that a combination of 10% sourdough and flaxseed or sourdough and calcium propionate were effective at delaying mould growth, offering preliminary evidence for the benefits of combining sourdough with alternative ingredients or preservatives. Further evidence comes from Ryan et al. (2008), who found that sourdough paired with 0.1% calcium propionate addition was better at preventing *P. roqueforti* growth than 0.3% calcium propionate alone.

The ability of ethanol to prevent microbial growth is widely understood, and in production of bread and baked goods it has been applied as a component of emitters. These are a form of modified atmosphere packaging wherein a combination of ethanol and water, usually mixed into a silicone powder, are packaged in a sachet that allows for the controlled release of vapour (Mexis & Kontominas, 2014). In the preservation of air-packaged rye bread, ethanol emitters were able to more than double the microbial shelf life from 8 to 26 d (Salminen et al., 1996). In sliced wheat

bread a combination of ethanol emitters and packaging with a high barrier material extended shelf life of preservative-free bread from 4 to 24 d before a sensory panel detected off-odours (Latou et al., 2010). The experiments with *Lp. plantarum* FUA3183 detailed in Figure 7 showed that upwards of 312 mmol/kg ethanol has an inhibitory effect on mould development in sourdough, and also that the amount present in the sourdough bread is enough to have an impact on mould growth. In previous research spore germination of 3 different *Penicillium* species in the presence of 190 and 290 mmol/L also revealed that the impact of ethanol was dependent on the water activity of the medium where those spores were produced. Conidia spores produced at an a_w of 0.99 were more sensitive than those at 0.90, 0.93, or 0.95a_w and dry-harvested conidia were not impacted by ethanol (Dao & Dantigny, 2009). The moulding experiment described in sections 2.1.3 and 3.1.4 took place in a petri dish, similar to how bread is packaged in small bags directly after cooling in the commercial bread industry. The smaller headspace would increase the effectiveness of the ethanol, something not accounted for in the other mould experiments that took place in 7L containers where the ethanol vapour had plenty of space to disperse, lowering its concentration. Ethanol concentrations of ≥2% in food is reportedly the limit where the taste becomes noticeable (Seiler & Russel, 1991).

The low fermentation temperature used in the 48 h extended fermentation did not increase the organic acid production (Figure 8) or shelf life of the sourdough bread (Figure 9). Casado et al. (2017) found that when comparing sourdoughs fermented at 25 and 35°C, although the cell counts and overall pH were similar after 24 h, the 25°C sourdough didn't reach its highest titratable acidity (TA) value until 72 h into the fermentation while the 35°C sourdough only needed 48 h to reach peak TA. In this experiment it is likely that the temperature and time

combination chosen for fermentation was less effective than anticipated; while temperatures of 25°C work well for fermentations where the concern is bread quality and leavening (section 1.4), higher temperatures are associated with greater organic acid output at the cost of that leavening (Brandt et al., 2023) The organic acid content of the control (section 3.2.2), which was a straight bread dough, was higher than expected but confirmed by UV detection not to be due to merging of glycerol with lactic acid (section 2.2.3). The majority of organic acids were produced between the first and second proofs, showing that the conditions used for the proofing stages are more important for acid production than the conditions of the sourdough.

The comparison of fermentations highlighted that the multi-stage method (F2) was superior to a 24 h fermentation (F1), as well as a multi-stage and cold proof (F3). The cold proof temperature of 5°C was too low to result in any growth from the lactobacilli in the dough. A growth curve modelling experiment by Gänzle et al. (1998) showed that both the lactobacilli and yeasts assessed were unable to grow at that temperature, with roughly 8-10°C being the threshold for growth.

Though the buckwheat flour inclusion was responsible for less of the difference in shelf life observed, it was still a significant factor. As such, the third hypothesis of section 1.6 can be accepted: altered fermentations with antifungal adjuncts are an effective way of delaying mould spoilage. The organic acid concentrations in Figure 13 suggest that the addition of α-acids to the dough during mixing were enough to impede growth of the lactobacilli during proofing because the buckwheat formulations contained significantly more lactic acid. In previous experiments with the F2 protocol the flour alone was not enough to change the organic acid content of the breads, so the difference is attributable to the iso-α-acids. As lactobacilli are gram-positive, the

inhibitory effects of hop extract (section 1.3.2) also apply to them. While no research exists that documents the resistance of *Lp. plantarum* FUA3183 or *Lv. hammesii* LP38^{T} against hops, some *Lactobacillus* and *Pediococcus* species, including *Lp. plantarum*, have been recognized as spoilage organisms in beer (Priest, 1996). For a lactobacilli to survive in and spoil beer it must possess either the HorA resistance mechanism or the pRH45 plasmid. HorA is a plasmid-encoded multi-drug resistance transporter that protects the cell by mediating ATP-reliant transport of hops bitter compounds. pRH45 contains the HorA genes and allows its transfer from spp. to spp. (Sami et al., 1997; Sami et al., 1998). Plant-associated lactobacilli frequently encounter secondary metabolites with antifungal activity while those found in the intestines of vertebrates can encounter antibiotics, leading them both to develop their own resistance mechanisms (Rao et al., 2018; Rozman et al., 2020). *Lp. plantarum* strains have been identified as being especially resistant to both phenolics such as iso-α-acids and to antibiotics (Pswarayi et al., 2022). The assumption made in this thesis was that most *Lp. plantarum* strains would show some resistance to antimicrobial phenolics.

Lp. plantarum FUA3183 and *Lv. hammesii* LP38^{T} do not contain the HorA gene (section 2.4.2), explaining why their metabolic output is lower than in the buckwheat fermentations of sections 3.3.2 and 3.4.2. Despite this impact, sourdoughs made with 10 or 30 mg/L α-acids had identical shelf life to those made with buckwheat, suggesting that the preservative effect of the hops was enough to make up for the reduced acid output of the lactobacilli. The findings of these mould experiments are consistent with previous research determining that isomerized hops in sourdough could result in a shelf life comparable to 0.3% calcium propionate addition even when tested against *P. roqueforti* (Gobbetti et al., 2018).

Although no experimental breads consistently matched the shelf life of commercially-available Dempster's white bread when inoculated, the environmental exposure experiments showed no difference in the shelf life of the 0.3% calcium propionate controls and the sourdough breads. Dempster's bread contains the preservative sorbic acid, vinegar, ethanol, and the emulsifying agent soybean lecithin (Dempster's White Bread, n.d.), which has also demonstrated an ability to prevent growth of fungi such as *A. niger* (Jolly et al., 2018). In contrast, all sourdoughs using iso-α-acids or buckwheat were able to meet the shelf life of bread containing 0.3% calcium propionate even when inoculated with highly-resistant moulds, a feat that the straight dough was unable to match. In one case, the buckwheat formulation was even able to exceed the shelf life of the 0.3% calcium propionate bread. The preservative effect of the sourdoughs were more impactful in environmental experiments than inoculations, highlighting the importance of clean-room technology. Further supporting this are the results of single spore germinations by Santos et al. (2020) which underscores how effective hurdle technology can be. *Penicillium paneum* germination was delayed by increasing environmental stress. A water activity of 0.93, pH of 5.1 and calcium propionate concentrations of 0.25% took 49% longer to germinate than those exposed to conditions of a water activity of 0.95, pH 5.8 and the same amount of calcium propionate. The Austrian company 'ortner cleanrooms unlimited' offers services specifically tailored to the baking industry, including ortner plus, a plan which guarantees increased product shelf life through filter and UV technologies and offers a lowest bacterial count of <1 CFU (ortner cleanrooms unlimited cleanroom solutions for bread, pastry & bakery industry, n.d.). Air Innovations similarly advertises a variety of options for customizable control units, including HEPA filters that can remove 99.99% of particles ≥0.3µm large (n.d.). Given that *P.*

roqueforti spores are between 3-4.5µm (Punt et al., 2020), these filters have a significant impact on the risk of contamination between baking and packaging, especially in combination with other sanitary controls like positive air pressure, UV surface disinfection and cleanroom-specific clothes for workers. In the case of refrigerated breaded chicken products, use of cleanroom technology in the processing and packaging stages both reduced bacterial contamination and increased the product shelf life. Mould spores on the product packaged in a clean room averaged 1 log CFU/g while those processed normally contained 1.4 log/CFU/g. In all cases, cleanroom-packaged products had lower counts of yeasts, moulds, mesophiles, psychrophiles, and *Enterobacteriaceae* (Barón et al., 2022). The moulding experiments prove that the F2 fermentation method, when used in combination with antifungal adjuncts, can significantly extend the shelf life of sourdough bread in a worst-case exposure scenario. If paired with cleanroom technology to reduce spore contamination, the effect can be even greater.

The issue of mould spoilage and subsequent food waste in any product is not something that can be addressed with a one-step solution, and that remains true in the case of sourdough bread. While a combination of new ingredients and fermentations was able to extend the product shelf life to match that of bread containing 0.3% calcium propionate in worst-case exposure scenarios, Dempster's bread was significantly better at delaying spoilage by even the most resistant moulds. However, this experiment proved that fermented buckwheat can contribute to fungal resistance in bread and also supports previous research by Gobbetti et al., (2018) that identified iso-α-acids in hops as effective in delaying spore germination. In addition, these findings support the novel idea that shelf life can be improved by altering fermentation and proofing schedules to increase organic acid contents in bread. This work posits that a

three-pronged approach including fermentation, antifungal adjuncts, and a clean processing area can not only reduce instances of contamination but lead to improved resistance and extended shelf life when contamination does occur (Figure 15).

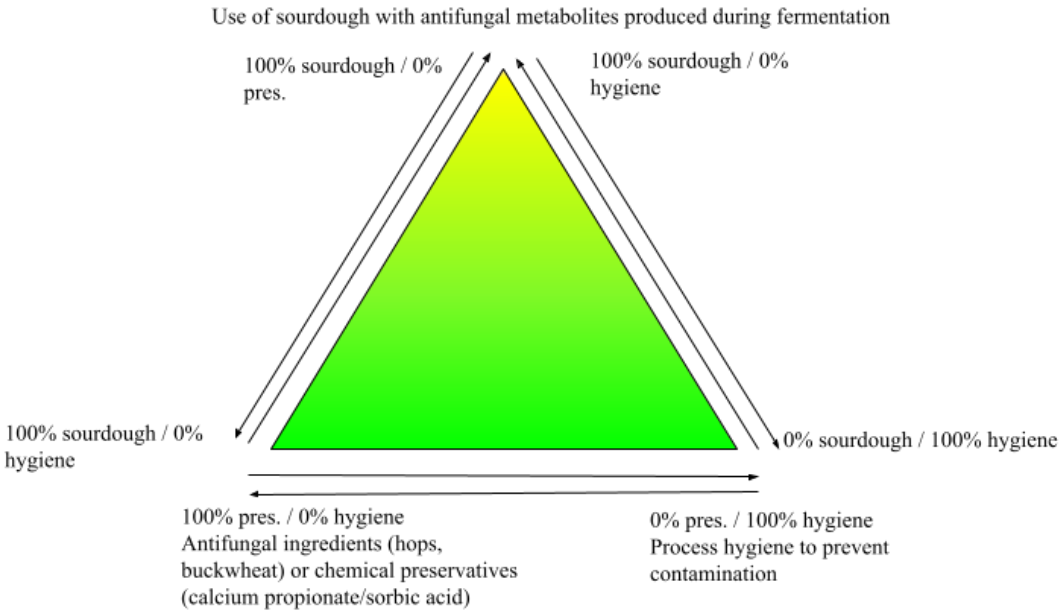

Figure 15. Schematic representation of bread preservation with sourdough, hygiene intended to prevent contamination with fungal conidiospores, preservatives or antifungal ingredients, or combinations of the three. Yellow indicates a low mould-free shelf life while green indicates a long mould-free shelf life.

5. Opportunities for future research

While the shelf life of bread containing iso-a-acids and buckwheat can be compared, no sensory evaluation experiments took place and therefore the acceptability of these sourdough breads to the average consumer cannot be speculated on. Sensory experiments could be a good focus for future research to ensure consumer acceptance before any thought is given to the possible commercialization of the recipes. In this experiment all mixing and baking took place on a very small scale and could not account for the impact of large-scale production or a commercial bread-making recipe. It is possible that the differences in the bread matrix might impact the shelf life of the product. Additionally there is room to expand on the fermentation and proofing schedules assessed by experimenting with different time/temperature combinations and additions of ingredients high in phenols, such as barley or flax seed.

Only lactic and acetic acid were quantified in this study, and no attempts were made to characterise or quantify minor metabolic products of fermentation which might exhibit antifungal effects.

Bibliography

5 facts about food waste and hunger | World Food Programme. (2020, June 2). https://www.wfp.org/stories/5-facts-about-food-waste-and-hunger

2023 consumer food preference trends. (n.d.). Ingredion. https://www.ingredion.com/na/en-us/be-whats-next/2023-consumer-food-preference-trends.html

Alvarez-Jubete, L., Wijngaard, H., Arendt, E. K., & Gallagher, E. (2010). Polyphenol composition and in vitro antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. *Food Chemistry*, *119*(2), 770–778. https://doi.org/10.1016/j.foodchem.2009.07.032

American Association of Cereal Chemists. (1998). *Guidelines for Measurement of Volume by Rapeseed Displacement.* (Method 10-05.01).

Apollo Hops—Substitution, Flavor, Aroma [2023]. (n.d.). Beer Maverick. https://beermaverick.com/hop/apollo/

Axel, C., Zannini, E., & Arendt, E. K. (2017). Mold spoilage of bread and its biopreservation: A review of current strategies for bread shelf life extension. *Critical Reviews in Food Science and Nutrition*, *57*(16), 3528–3542. https://doi.org/10.1080/10408398.2016.1147417

Baker, G. A., Danenhower, T. M., Force, L. J., Petersen, K. J., & Betts, T. A. (2008). HPLC analysis of α- and β-acids in hops. *Journal of Chemical Education*, *85*(7), 954. https://doi.org/10.1021/ed085p954

Barón, M., Ros Chumillas, M., Martínez Hernández, G. B., & López Gómez, A. (2022). Refrigerated clean room processing and packaging increases shelf life of refrigerated map packaged breaded products. *Libro de Actas XI Congreso Ibérico y IX Congreso Iberoamericano de Ciencias y Técnicas Del Frío Cytef: Avances En Ciencias y Técnicas Del Frío - 11,* 322–327. https://dialnet.unirioja.es/servlet/articulo?codigo=8634634

Behr, J., & Vogel, R. F. (2009). Mechanisms of hop inhibition: Hop ionophores. *Journal of Agricultural and Food Chemistry*, *57*(14), 6074–6081. https://doi.org/10.1021/jf900847y

Bensid, A., Abed, N. E., Houicher, A., Regenstein, J. M., & Özogul, F. (2022). Antioxidant and antimicrobial preservatives: Properties, mechanism of action and applications in food – a review. *Critical Reviews in Food Science and Nutrition*. https://doi.org/10.1080/10408398.2020.1862046

Black, B. A.; Sun, C. X.; Zhao, Y. Y.; Gänzle, M. G.; Curtis, J. M. (2013). Antifungal lipids produced by lactobacilli and their structural identification by normal phase LC/atmospheric pressure photoionization-MS/MS. *Journal of Agriculture and Food Chemistry.* 61, 5338-5346

Bokulich, N. A., & Bamforth, C. W. (2013). The Microbiology of malting and brewing. *Microbiology and Molecular Biology Reviews : MMBR*, *77*(2), 157–172. https://doi.org/10.1128/MMBR.00060-12

Braganca, & Quastel. (1957). The phytase of wheat. *Biochem Journal*, *53*(1), 102–110. https://doi.org/10.1042/bj0530102

Brandt, M., Loponene, J., & Cappelle, S. (2023). Technology of sourdough fermentation and sourdough application. In *Handbook on Sourdough Biotechnology*.

Buddrick, O., Jones, O. A. H., Cornell, H. J., & Small, D. M. (2014). The influence of fermentation processes and cereal grains in wholegrain bread on reducing phytate content. *Journal of Cereal Science*, *59*(1), 3–8. https://doi.org/10.1016/j.jcs.2013.11.006

Caballero, I., Blanco, C. A., & Porras, M. (2012). Iso-α-acids, bitterness and loss of beer quality during storage. *Trends in Food Science & Technology*, *26*(1), 21–30. https://doi.org/10.1016/j.tifs.2012.01.001

Canbas, A., Erten, H., & özsahin. (2001). The effects of storage temperature on the chemical composition of hop pellets. *Process Biochemistry*, *36*, 1053–1058.

Cappelle, S., Guylaine, L., & Gänzle, M. (2023). History and social aspects of sourdough. In *Handbook on Sourdough Biotechnology* (2nd ed.).

Caren. (2018, November 20). *How to rescue overproofed dough*. Modernist Cuisine. https://modernistcuisine.com/mc/dough-cpr/

Casado, A., Álvarez, A., González, L., Fernández, D., Marcos, J. L., & Tornadijo, M. E. (2017). Effect of fermentation on microbiological, physicochemical and physical characteristics of sourdough and impact of its use on bread quality. *Czech Journal of Food Sciences*, *35*(6), 496–506. https://doi.org/10.17221/68/2017-CJFS

Chou, K., Yan, C.-T., & Hsiao, H.-I. (2023). Identification of postbaking mold contamination through onsite monitoring of baking factory environment: A case study of bakery company in Taiwan. *Food Control*, *145*, 109495. https://doi.org/10.1016/j.foodcont.2022.109495

Chung, K.-T., Wei, C.-I., & Johnson, M. G. (1998). Are tannins a double-edged sword in biology and health? *Trends in Food Science & Technology*, *9*(4), 168–175. https://doi.org/10.1016/S0924-2244(98)00028-4

Cleanroom Environmental Control and HVAC Systems • Air Innovations. (n.d.). Air Innovations. https://airinnovations.com/environmental-control/cleanroom-ecu-hvac-systems/

Conant, J. B. (2013). CASE 6. Pasteur's study of fermentation. In *CASE 6. Pasteur's Study of Fermentation* (pp. 436–485). Harvard University Press. https://doi.org/10.4159/harvard.9780674598713.c3

Culetu, A., Duta, D. E., Papageorgiou, M., & Varzakas, T. (2021). The role of hydrocolloids in gluten-free bread and pasta; rheology, characteristics, staling and glycemic index. *Foods*, *10*(12), Article 12. https://doi.org/10.3390/foods10123121

Dao, T., & Dantigny, P. (2009). Preparation of fungal conidia impacts their susceptibility to inactivation by ethanol vapours. *International Journal of Food Microbiology*, *135*(3), 268–273. https://doi.org/10.1016/j.ijfoodmicro.2009.08.024

Day, L. (2016). Lipid chemistry. In C. Wrigley, H. Corke, K. Seetharaman, & J. Faubion (Eds.), *Encyclopedia of Food Grains (Second Edition)* (pp. 248–256). Academic Press. https://doi.org/10.1016/B978-0-12-394437-5.00099-1

De Angelis, M., Damiano, N., Rizzello, C. G., Cassone, A., Di Cagno, R., & Gobbetti, M. (2009). Sourdough fermentation as a tool for the manufacture of low-glycemic index white wheat bread enriched in dietary fibre. *European Food Research and Technology*, *229*(4), 593–601. https://doi.org/10.1007/s00217-009-1085-1

de Morais Souto, B., Florentino Barbosa, M., Marinsek Sales, R. M., Conessa Moura, S., de Rezende Bastos Araújo, A., & Ferraz Quirino, B. (2023). The potential of β-glucosidases for aroma and flavor improvement in the food industry. *The Microbe*, *1*, 100004. https://doi.org/10.1016/j.microb.2023.100004

De Vuyst, L., González-Alonso, V., Wardhan, Y., & Pradal, I. (2023). Taxonomy and Species Diversity of Sourdough Lactic Acid Bacteria. In *Handbook on Sourdough Biotechnology* (pp. 97–160).

Debonne, E., Giannotti, G., Verbeke, C., Eeckhout, M., & Devlieghere, F. (2023). Growth/no-growth models of propionic and sorbic acid for bread and cake moulds. *Food Control*, *152*, 109872. https://doi.org/10.1016/j.foodcont.2023.109872

Dempster's® White Bread | Dempster's. (n.d.). https://dempsters.ca/products/white-bread/dempsters-white-bread/675-g

Dey, P. M. (2012). *Methods in Plant Biochemistry* (Vol. 1). Academic Press.

Donner, P., Pokorný, J., Ježek, J., Krofta, K., Patzak, J., & Pulkrábek, J. (2020). Influence of weather conditions, irrigation and plant age on yield and alpha-acids content of Czech hop *(Humulus lupulus L.*) cultivars. *Plant, Soil and Environment*, *66 (2020)*(No. 1), 41–46. https://doi.org/10.17221/627/2019-PSE

Eschner, K. (n.d.). *Gold Miners Kept Their Sourdough Starters Alive By Cuddling Them | Smart News| Smithsonian Magazine*. https://www.smithsonianmag.com/smart-news/gold-miners-kept-their-sourdough-starters-alive-cuddling-them-180962689/

European Union (EU). (n.d.). *Regulation (EC) No 1333/2008 of the European parliament and of the council of 16 December 2008 on food additives*. https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX%3A32008R1333

Figoni, P. (n.d.). *How Baking Works*. https://pdfcoffee.com/how-baking-works-by-paula-figonipdf-pdf-free.html

Fraberger, V., Ammer, C., & Domig, K. (2020). Functional properties and sustainability improvement of sourdough bread by lactic acid bacteria. *Microorganisms*, *8*(12). https://doi.org/10.3390/microorganisms8121895

G. Hassan, E., M. Awad Al, A., & Moniem I., A. (2008). Effect of fermentation and particle size of wheat bran on the antinutritional factors and bread quality. *Pakistan Journal of Nutrition*, *7*(4), 521–526. https://doi.org/10.3923/pjn.2008.521.526

Galle, S. (2013). Sourdough: A tool to improve bread structure. In M. Gobbetti & M. Gänzle (Eds.), *Handbook on Sourdough Biotechnology* (pp. 217–228). Springer US. https://doi.org/10.1007/978-1-4614-5425-0_8

Gänzle, M. G. (2015). Lactic metabolism revisited: Metabolism of lactic acid bacteria in food fermentations and food spoilage. *Current Opinion in Food Science*, *2*, 106–117. https://doi.org/10.1016/j.cofs.2015.03.001

Gänzle, M. G., Ehmann, M., & Hammes, W. P. (1998). Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of sourdough fermentation. *Applied and Environmental Microbiology*, *64*(7), 2616–2623. https://doi.org/10.1128/AEM.64.7.2616-2623.1998

Gänzle, M. G., and Follador, R. (2012). Metabolism of oligosaccharides and starch in lactobacilli: A review. *Frontiers in Microbiology* 3. https://doi.org/10.3389/fmicb.2012.00340

Gänzle, M. G, & Gobbetti, M. (2023). Metabolism of Carbohydrates. In *Handbook on Sourdough Technology*. 215–219.

Gänzle, M., Loponen, J., Gobbetti, M. (2008). Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality. *Trends in Food Science & Technology, 19*(10), 513-521. https://doi.org/10.1016/j.tifs.2008.04.002

Gänzle, M., Qiao, N., & Bechtner, J. (2023). The quest for the perfect loaf of sourdough bread continues: Novel developments for selection of sourdough starter cultures. *International Journal of Food Microbiology*, *407*, 110421. https://doi.org/10.1016/j.ijfoodmicro.2023.110421

Gänzle, M., & Zheng, J. (2019). Lifestyles of sourdough lactobacilli – Do they matter for microbial ecology and bread quality? *International Journal of Food Microbiology*, *302*, 15–23. https://doi.org/10.1016/j.ijfoodmicro.2018.08.019

Garcia, M., & Copetti, M. (2019b). Alternative methods for mold spoilage control in bread and bakery products. *International Food Research Journal*, *26*, 737–749.

Garcia, M. V., Bernardi, A. O., & Copetti, M. V. (2019a). The fungal problem in bread production: Insights of causes, consequences, and control methods. *Current Opinion in Food Science*, *29*, 1–6. https://doi.org/10.1016/j.cofs.2019.06.010

Garcia, M. V., Bernardi, A. O., Parussolo, G., Stefanello, A., Lemos, J. G., & Copetti, M. V. (2019). Spoilage fungi in a bread factory in Brazil: Diversity and incidence through the bread-making process. *Food Research International*, *126*, 108593. https://doi.org/10.1016/j.foodres.2019.108593

Garofalo, C., Zannini, E., Aquilanti, L., Silvestri, G., Fierro, O., Picariello, G., & Clementi, F. (2012). Selection of sourdough lactobacilli with antifungal activity for use as biopreservatives in bakery products. *Journal of Agricultural and Food Chemistry*, *60*(31), 7719–7728. https://doi.org/10.1021/jf301173u

Gaur, G. (2022). *Phenolic acid metabolism in lactic acid bacteria and its ecological relevance*. University of Alberta.

Gaur, G., & Gänzle, M. G. (2023). Conversion of (poly)phenolic compounds in food fermentations by lactic acid bacteria: Novel insights into metabolic pathways and functional metabolites. *Current Research in Food Science*, *6*, 100448. https://doi.org/10.1016/j.crfs.2023.100448

Glycoside | Carbohydrate, Sugar & Structure | Britannica. (2023, December 2). Britannica. https://www.britannica.com/science/glycoside

Gobbetti, M., De Angelis, M., Di Cagno, R., Calasso, M., Archetti, G., & Rizzello, C. G. (2019). Novel insights on the functional/nutritional features of the sourdough fermentation. *International Journal of Food Microbiology*, *302*(2), 103–113. https://doi.org/10.1016/j.ijfoodmicro.2018.05.018

Gobbetti, M., Rizzello, C. G., Di Cagno, R., & De Angelis, M. (2014). How the sourdough may affect the functional features of leavened baked goods. *Food Microbiology*, *37*, 30–40. https://doi.org/10.1016/j.fm.2013.04.012

González-Sarrías, A., Tomás-Barberán, F. A., & García-Villalba, R. (2020). Structural diversity of polyphenols and distribution in doods. In *Dietary Polyphenols*, 1–29. John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119563754.ch1

Google Trends. (n.d.). Google Trends. https://trends.google.com/trends/explore?date=all&q=Sourdough&hl=en

Halawa, A. (2023). Influence of the traditional food culture of Ancient Egypt on the transition of cuisine and food culture of contemporary Egypt. *Journal of Ethnic Foods, 10*(11). https://doi.org/10.1186/s42779-023-00177-4

Harvard T. H. Chan School of Public Health. (2019). *Are anti-nutrients harmful?* The Nutrition Source. https://www.hsph.harvard.edu/nutritionsource/anti-nutrients/

Health Canada. (2006, November 27). *11. List of permitted preservatives (lists of permitted food additives)*. https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/11-preservatives.html

Hill, A., & Ferrer, M. A. (2021). pH and titratable acidity. In *Cheese Making Technology*. https://books.lib.uoguelph.ca/cheesemakingtechnologyebook/chapter/titratable-acidity/

Holbrook, D. (2009). Controlling contamination: The origins of clean room technology. *History and Technology*, *25*, 173–191. https://doi.org/10.1080/07341510903083203

Horvat, D., Šimić, G., Drezner, G., Lalić, A., Ledenčan, T., Tucak, M., Plavšić, H., Andrić, L., & Zdunić, Z. (2020). Phenolic acid profiles and antioxidant activity of major cereal crops. *Antioxidants*, 9(6), 527. https://doi.org/10.3390/antiox9060527

Huang, Y., Tippmann, J., Becker, T. (2013). Kinetic modeling of hop acids during wort boiling. *International Journal of Bioscience, Biochemistry and Bioinformatics*, 3(1). https://doi.org/10.7763/IJBBB.2013.V3.161

Hui, Y. H., Corke, H., Leyn, I. D., Nip, W.-K., & Cross, N. A. (2008). *Bakery Products: Science and Technology*. John Wiley & Sons.

Jackman, R. H., & Black, C. A. (1951). Solubility of iron, aluminum, calcium, and magnesium inositol phosphates at different pH values. *Soil Science*, *72*(3), 179.

Jarvis, B., & Paulus, K. (1982). Food preservation: An example of the application of negative biotechnology. *Journal of Chemical Technology and Biotechnology*, *32*(1), 233–250. https://doi.org/10.1002/jctb.5030320128

Jaskula, B., Kafarski, P., Aerts, G., & De Cooman, L. (2008). A kinetic study on the isomerization of hop α-Acids. *Journal of Agricultural and Food Chemistry*, *56*(15), 6408–6415. https://doi.org/10.1021/jf8004965

Jolly, M., Vidal, R., & Marchand, P. A. (2018). Lecithins: A food additive valuable for antifungal crop protection. *International Journal of Economic Plants*, *5*(3), 104–107. https://doi.org/10.23910/IJEP/2018.5.3.0243

Kagliwal, L. D., Jadhav, S. B., Singhal, R. S., & Kulkarni, P. R. (2014). Preservatives | permitted preservatives – propionic acid. In C. A. Batt & M. L. Tortorello (Eds.), *Encyclopedia of Food Microbiology (Second Edition)* (pp. 99–101). Academic Press. https://doi.org/10.1016/B978-0-12-384730-0.00270-6

Kath. (2020, December 7). What is oven spring? *Veg Patch Kitchen Cookery School*.

https://vegpatchkitchen.co.uk/what-is-oven-spring/

Kim, J., Kim, R. H., & Hwang, K. T. (2023). Flavonoids in different parts of common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*F. tataricum*) during growth. *Journal of Food Composition and Analysis*, *120*, 105362. https://doi.org/10.1016/j.jfca.2023.105362

Kimbell, V. (2015, March 21). The history of sourdough bread. *Nutrition and Digestibility of Bread*. https://www.sourdough.co.uk/the-history-of-sourdough-bread/

Knight, R. A., & Menlove, E. M. (1961). Effect of the bread-baking process on destruction of certain mould spores. *Journal of the Science of Food and Agriculture*, *12*(10), 653–656. https://doi.org/10.1002/jsfa.2740121001

Kosemani, B. S., Ilori, A. T., & Atere, A. O. (2021). Modification and optimization of a baking oven for small scale bread production. *Agricultural Sciences*, *12*(06), 630–644. https://doi.org/10.4236/as.2021.126041

Koval, D., Plocková, M., Kyselka, J., Skřivan, P., Sluková, M., & Horáčková, Š. (2020). Buckwheat secondary metabolites: potential antifungal agents. *Journal of Agricultural and Food Chemistry*, *68*(42), 11631–11643. https://doi.org/10.1021/acs.jafc.0c04538

Kramer, B., Thielmann, J., Hickisch, A., Muranyi, P., Wunderlich, J., & Hauser, C. (2015). Antimicrobial activity of hop extracts against foodborne pathogens for meat applications. *Journal of Applied Microbiology*, *118*(3), 648–657. https://doi.org/10.1111/jam.12717

Kulp, K., Lorenz, K., & Wirtz. (2003). *Handbook of Dough Fermentations*. CRC Press.

Latou, E., Mexis, S. F., Badeka, A. V., & Kontominas, M. G. (2010). Shelf life extension of sliced wheat bread using either an ethanol emitter or an ethanol emitter combined with an oxygen absorber as alternatives to chemical preservatives. *Journal of Cereal Science*, *52*(3), 457–465. https://doi.org/10.1016/j.jcs.2010.07.011

Lee, T. (n.d.). *What is the Fertile Crescent? | University of Chicago News*. UChicago News. https://news.uchicago.edu/explainer/fertile-crescent-explained

Legan, J. D. Mould spoilage of bread: The problem and some solutions. (1993). *International Biodeterioration & Biodegradation* 32(1), 33–53. https://doi.org/10.1016/0964-8305(93)90038-4.

Li, Q., & Gänzle, M. (2020). Host-adapted lactobacilli in food fermentations: Impact of metabolic traits of host adapted lactobacilli on food quality and human health. *Current Opinion in Food Science*, *31*. https://doi.org/10.1016/j.cofs.2020.02.002

Liang, N., Zhao, Z., Curtis, J. M., & Gänzle, M. G. (2022). Antifungal cultures and metabolites of lactic acid bacteria for use in dairy fermentations. *International Journal of Food Microbiology*, *383*, 109938. https://doi.org/10.1016/j.ijfoodmicro.2022.109938

Likens, S. T., Nickerson, G. B., Haunold, A., & Zimmermann, C. E. (1978). Relationship between alpha acids, beta acids, and lupulin content of hops. *Crop Science*, *18*(3), https://doi.org/10.2135/cropsci1978.0011183X001800030007x

Lim, Y. J., Lim, B., Kim, H. Y., Kwon, S.-J., & Eom, S. H. (2020). Deglycosylation patterns of isoflavones in soybean extracts inoculated with two enzymatically different strains of lactobacillus species. *Enzyme and Microbial Technology*, *132*, 109394. https://doi.org/10.1016/j.enzmictec.2019.109394

Lin, L.-Y., Liu, H.-M., Yu, Y.-W., Lin, S.-D., & Mau, J.-L. (2009). Quality and antioxidant property of buckwheat enhanced wheat bread. *Food Chemistry*, *112*(4), 987–991. https://doi.org/10.1016/j.foodchem.2008.07.022

London, J. (1976). The Ecology and taxonomic status of the lactobacilli. *Annual Review of Microbiology*, *30*(1), 279–301. https://doi.org/10.1146/annurev.mi.30.100176.001431

Loponen, J., & Gänzle, M. G. (2018). Use of sourdough in low FODMAP baking. *Foods*, *7*(7), Article 7. https://doi.org/10.3390/foods7070096

Magnusson, J., Ström, K., Roos, S., Sjögren, J., & Schnürer, J. (2003). Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiology Letters*, *219*(1), 129–135. https://doi.org/10.1016/S0378-1097(02)01207-7

Mariotti, M., Pagani, M. A., & Lucisano, M. (2013). The role of buckwheat and HPMC on the breadmaking properties of some commercial gluten-free bread mixtures. *Food Hydrocolloids*, *30*(1), 393–400. https://doi.org/10.1016/j.foodhyd.2012.07.005

Mexis, S. F., & Kontominas, M. G. (2014). Packaging | Active Food Packaging. In C. A. Batt & M. L. Tortorello (Eds.), *Encyclopedia of Food Microbiology (Second Edition)* (pp. 999–1005). Academic Press. https://doi.org/10.1016/B978-0-12-384730-0.00434-1

Miners and Sourdough—Coquitlam Heritage at Mackin House. (2022, March 2). Coquitlam Heritage Society. https://www.coquitlamheritage.ca/blog-pages/2022/2/14/miners-and-sourdough

Moroni, A. V., Zannini, E., Sensidoni, G., & Arendt, E. K. (2012). Exploitation of buckwheat sourdough for the production of wheat bread. *European Food Research and Technology*, *235*(4), 659–668. https://doi.org/10.1007/s00217-012-1790-z

Mota‐Gutierrez, J., Franciosa, I., Ruggirello, M., & Dolci, P. (2021). Technological, functional and safety properties of lactobacilli isolates from soft wheat sourdough and their potential use as antimould cultures. *World Journal of Microbiology and Biotechnology*, *37*(9), 146. https://doi.org/10.1007/s11274-021-03114-2

Muñoz, R., B. de las Rivas, F. López de Felipe, I. Reverón, L. Santamaría, M. Esteban-Torres, J. A. Curiel, H. Rodríguez, and J. M. Landete. (2017). Biotransformation of phenolics by *Lactobacillus plantarum* in fermented foods. In *Fermented Foods in Health and Disease Prevention*, edited by Juana Frias, Cristina Martinez-Villaluenga, and Elena Peñas, 63–83. Boston: Academic Press. https://doi.org/10.1016/B978-0-12-802309-9.00004-2.

Nations, U. (n.d.). *Food Loss and Waste Reduction*. United Nations; United Nations. https://www.un.org/en/observances/end-food-waste-day

Nionelli, L., Pontonio, E., Gobbetti, M., & Rizzello, C. G. (2018). Use of hop extract as antifungal ingredient for bread making and selection of autochthonous resistant starters for sourdough fermentation. *International Journal of Food Microbiology*, *266*, 173–182. https://doi.org/10.1016/j.ijfoodmicro.2017.12.002

Ohsu, T., Amino, Y., Nagasaki, H., Yamanaka, T., Takeshita, S., Hatanaka, T., Maruyama, Y., Miyamura, N., & Eto, Y. (2010). Involvement of the calcium-sensing receptor in human taste perception. *The Journal of Biological Chemistry*, *285*(2), 1016–1022. https://doi.org/10.1074/jbc.M109.029165

Oliver, G. (2012). *The Oxford Companion to Beer*. Oxford University Press, USA.

Ooraikul, B., Smith, J. P., & Koersen, W. J. (1987). Air quality in some Alberta bakeries.

Canadian Institute of Food Science and Technology Journal, *20*(5), 387–389. https://doi.org/10.1016/S0315-5463(87)71337-6

Paciulli, M., Littardi, P., Rinaldi, M., & Chiavaro, E. (2021). Wheat bread in the Mediterranean area: from past to the future. In F. Boukid (Ed.), *Cereal-Based Foodstuffs: The Backbone of Mediterranean Cuisine* (pp. 47–88). Springer International Publishing. https://doi.org/10.1007/978-3-030-69228-5_3

Pasteur, L. (1995). Mémoire sur la fermentation appelée lactique (Extrait par l'auteur). *Molecular Medicine*, *1*(6), 599–601. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2229983/

Pliny. (n.d.). *Pliny the Elder, The Natural History, BOOK XVIII. THE NATURAL HISTORY OF GRAIN., CHAP. 26. (11)—THE VARIOUS KINDS OF LEAVEN.* https://www.perseus.tufts.edu/hopper/text?doc=Perseus:text:1999.02.0137:book=18:chapter=26&highlight=leaven

Price, R. K., & Welch, R. W. (2013). Cereal Grains. In B. Caballero (Ed.), *Encyclopedia of Human Nutrition (Third Edition)* (pp. 307–316). Academic Press. https://doi.org/10.1016/B978-0-12-375083-9.00047-7

Priest, F. G. (1996). Gram-positive brewery bacteria. In F. G. Priest & I. Campbell (Eds.), *Brewing Microbiology* (pp. 127–161). Springer US. https://doi.org/10.1007/978-1-4757-4679-2_5

Pswarayi, F., Qiao, N., Gaur, G., & Gänzle, M. (2022). Antimicrobial plant secondary metabolites, MDR transporters and antimicrobial resistance in cereal-associated lactobacilli: Is there a connection? *Food Microbiology*, *102*, 103917. https://doi.org/10.1016/j.fm.2021.103917

Punt, M., van den Brule, T., Teertstra, W. R., Dijksterhuis, J., den Besten, H. M. W., Ohm, R. A., & Wösten, H. A. B. (2020). Impact of maturation and growth temperature on cell-size distribution, heat-resistance, compatible solute composition and transcription profiles of *Penicillium roqueforti* conidia. *Food Research International*, *136*, 109287. https://doi.org/10.1016/j.foodres.2020.109287

Qiao, N., Wittouck, S., Mattarelli, P., Zheng, J., Lebeer, S., Felis, G. E., & Gänzle, M. G. (2022). After the storm—Perspectives on the taxonomy of *Lactobacillaceae*. *JDS Communications*, *3*(3), 222–227. https://doi.org/10.3168/jdsc.2021-0183

Quattrini, M., Liang, N., Fortina, M. G., Xiang, S., Curtis, J. M., Gänzle, M. G. (2019). Exploiting synergies of sourdough and antifungal organic acids to delay fungal spoilage of bread. *International Journal of Food Microbiology*, Special Issue: Seventh International Symposium on Sourdough: Health & Wealth through sourdough innovation, 302, 8–14. https://doi.org/10.1016/j.ijfoodmicro.2018.09.007

Rao, M., Padyana, S., Dipin, Km., Kumar, S., Nayak, B., & Varela, M. (2018). Antimicrobial compounds of plant origin as efflux pump inhibitors: new avenues for controlling multidrug resistant pathogens. *Journal of Antimicrobial Agents*, *4*(01). https://doi.org/10.4172/2472-1212.1000159

Rejeb, B., I., Charfi, I., Baraketi, S., Hached, H., & Gargouri, M. (2022). Bread surplus: A cumulative waste or a staple material for high-value products? *Molecules*, *27*(23), 8410. https://doi.org/10.3390/molecules27238410

Rizzello, C., Coda, R., Mazzacane, F., Minervini, D., & Gobbetti, M. (2012). Micronized by-products from debranned durum wheat and sourdough fermentation enhanced the nutritional, textural and sensory features of bread. *Food Research International*, *46*,

304–313. https://doi.org/10.1016/j.foodres.2011.12.024

Roudot-Algaron, F., Kerhoas, L., Le Bars, D., Einhorn, J., & Gripon, J. C. (1994). Isolation of γ-glutamyl peptides from comté cheese. *Journal of Dairy Science*, *77*(5), 1161–1166. https://doi.org/10.3168/jds.S0022-0302(94)77053-3

Rozman, V., Mohar Lorbeg, P., Accetto, T., & Bogovič Matijašić, B. (2020). Characterization of antimicrobial resistance in lactobacilli and bifidobacteria used as probiotics or starter cultures based on integration of phenotypic and in silico data. *International Journal of Food Microbiology*, *314*, 108388. https://doi.org/10.1016/j.ijfoodmicro.2019.108388

Ortner cleanrooms unlimited. (2022). *Cleanroom solutions for bread, pastry & bakery industry.* Ortner Reinraumtechnik: Bread, Pastry & Bakery Industry. https://ortner-group.com/en/branchen/food/food-processing/brot-gebaeck-backwaren-industrie

Ryan, L. A. M., Dal Bello, F., & Arendt, E. K. (2008). The use of sourdough fermented by antifungal LAB to reduce the amount of calcium propionate in bread. *International Journal of Food Microbiology*, *125*(3), 274–278. https://doi.org/10.1016/j.ijfoodmicro.2008.04.013

Ryan, L., Zannini, E., Dal Bello, F., Pawlowska, A., Koehler, P., and Arendt, E. K. (2011). *Lactobacillus amylovorus* DSM 19280 as a novel food-grade antifungal agent for bakery products. *International Journal of Food Microbiology 146*(3), 276–83. https://doi.org/10.1016/j.ijfoodmicro.2011.02.036.

Salminen, A., Latva-Kala, K., Randell, K., Hurme, E., Linko, P., & Ahvenainen, R. (1996). The effect of ethanol and oxygen absorption on the shelf-life of packed sliced rye bread. *Packaging Technology and Science*, *9*(1), 29–42.

https://doi.org/10.1002/(SICI)1099-1522(199601)9:1<29::AID-PTS351>3.0.CO;2-K

Sami, M., Suzuki, K., Sakamoto, K., Kadokura, H., Kitamoto, K., & Yoda, K. (1998). A plasmid pRH45 of *Lactobacillus brevis* confers hop resistance. *The Journal of General and Applied Microbiology*, *44*(5), 361–363. https://doi.org/10.2323/jgam.44.361

Sami, M., Yamashita, H., Hirono, T., Kadokura, H., Kitamoto, K., Yoda, K., & Yamasaki, M. (1997). Hop-resistant *Lactobacillus brevis* contains a novel plasmid harboring a multidrug resistance-like gene. *Journal of Fermentation and Bioengineering*, *84*(1), 1–6. https://doi.org/10.1016/S0922-338X(97)82778-X

Santos, J. L. P., Chaves, R. D., & Sant'Ana, A. S. (2020). Modeling the impact of water activity, pH, and calcium propionate on the germination of single spores of *Penicillium paneum*. *LWT*, *133*, 110012. https://doi.org/10.1016/j.lwt.2020.110012

Seiler, D. A. L., & Russel, N. J. (1991). Ethanol as a food preservative. In: *Food Preservatives*, 153-171.

Simpson, D. J., Zhang, J. S., D'Amico, V., Llamas-Arriba, M. G., & Gänzle, M. G. (2022). *Furfurilactobacillus milii* sp. Nov., isolated from fermented cereal foods. *International Journal of Systematic and Evolutionary Microbiology*, *72*(5). https://doi.org/10.1099/ijsem.0.005386

Singh, S. (2023, January). *Sourdough market trends, size, share, and industry analysis by 2030*. Market Research Future. https://www.marketresearchfuture.com/reports/sourdough-market-1823

Siwatch, M., Yadav, R. B., & Yadav, B. S. (2019). Influence of processing treatments on nutritional and physicochemical characteristics of buckwheat (*Fagopyrum esculentum*).

Current Nutrition & Food Science, *15*(4), 408–414. https://doi.org/10.2174/1573401313666170405142747

Sleha, R., Radochova, V., Malis, J., Mikyska, A., Houska, M., Krofta, K., Bogdanova, K., Janovska, S., Pejchal, J., Kolar, M., Cermak, P., & Bostik, P. (2021). Strong antimicrobial and healing effects of beta-acids from hops in methicillin-resistant *Staphylococcus aureus*-infected external wounds *in vivo*. *Antibiotics*, *10*(6), 708. https://doi.org/10.3390/antibiotics10060708

Smith, K. (2012). Louis Pasteur, the father of immunology? *Frontiers in Immunology*, *3*. https://doi.org/10.3389/fimmu.2012.00068

Sofos, J. N., Pierson, M. D., Blocher, J. C., & Busta, F. F. (1986). Mode of action of sorbic acid on bacterial cells and spores. *International Journal of Food Microbiology*, *3*(1), 1–17. https://doi.org/10.1016/0168-1605(86)90036-X

Sowerwine, C. (n.d.). *HIST 276—Lecture 2—The Paris Commune and Its Legacy*. Open Yale Courses. https://oyc.yale.edu/history/hist-276/lecture-2

Spector, M. P. (2009). Metabolism, Central (Intermediary). In M. Schaechter (Ed.), *Encyclopedia of Microbiology (Third Edition)* (pp. 242–264). Academic Press. https://doi.org/10.1016/B978-012373944-5.00078-X

Starowicz, M., Koutsidis, G., & Zieliński, H. (2018). Sensory analysis and aroma compounds of buckwheat containing products—A review. *Critical Reviews in Food Science and Nutrition*, *58*(11), 1767–1779. https://doi.org/10.1080/10408398.2017.1284742

Stiles, J., Penkar, S., Plocková, M., Chumchalová, J., & Bullerman, L. B. (2002). antifungal activity of sodium acetate and *Lactobacillus rhamnosus*. *Journal of Food Protection*,

65(7), 1188–1191. https://doi.org/10.4315/0362-028X-65.7.1188

Ström, K., Sjögren, J., Broberg, A., & Schnürer, J. (2002). *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Applied and Environmental Microbiology*, *68*(9), 4322–4327. https://doi.org/10.1128/AEM.68.9.4322-4327.2002

Svensson, L., Sekwati-Monang, B., Lutz, D. L., Schieber, A., & Gänzle, M. G. (2010). Phenolic acids and flavonoids in nonfermented and fermented red sorghum. *Journal of Agricultural and Food Chemistry*, *58*(16), 9214–9220. https://doi.org/10.1021/jf101504v

Thiele, C., Gänzle, M. G., & Vogel, R. F. (2002). Contribution of sourdough lactobacilli, yeast, and cereal enzymes to the generation of amino acids in dough relevant for bread flavor. *Cereal Chemistry*, *79*(1), 45–51. https://doi.org/10.1094/CCHEM.2002.79.1.45

Torrieri, E., Pepe, O., Ventorino, V., Masi, P., & Cavella, S. (2014). Effect of sourdough at different concentrations on quality and shelf life of bread. *LWT - Food Science and Technology*, *56*(2), 508–516. https://doi.org/10.1016/j.lwt.2013.12.005

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients*, *2*(12), 1231–1246. https://doi.org/10.3390/nu2121231

Tyl, C., & Sadler, G. D. (2017). pH and titratable acidity. In S. S. Nielsen (Ed.), *Food Analysis* (pp. 389–406). Springer International Publishing. https://doi.org/10.1007/978-3-319-45776-5_22

Ulmer, H. M., Gänzle, M. G., & Vogel, R. F. (2000). Effects of high pressure on survival and metabolic activity of *Lactobacillus plantarum* TMW1.460. *Applied and Environmental*

Microbiology, *66*(9), 3966–3973. https://doi.org/10.1128/AEM.66.9.3966-3973.2000

United Nations. (2021, April 3). *UNEP Food Waste Index Report 2021*. UNEP - UN Environment Programme. http://www.unep.org/resources/report/unep-food-waste-index-report-2021

Van Kerrebroeck, S., Maes, D., & De Vuyst, L. (2017). Sourdoughs as a function of their species diversity and process conditions, a meta-analysis. *Trends in Food Science and Technology*, 68. https://doi.org/10.1016/j.tifs.2017.08.016

Vrancken, G., Rimaux, T., Weckx, S., Leroy, F., & De Vuyst, L. (2011). Influence of temperature and backslopping time on the microbiota of a type I propagated laboratory wheat sourdough fermentation. *Applied and Environmental Microbiology*, *77*(8), 2716–2726. https://doi.org/10.1128/AEM.02470-10

Weingand-Ziadé, A., Gerber-Décombaz, C., & Affolter, M. (2003). Functional characterization of a salt- and thermotolerant glutaminase from *Lactobacillus rhamnosus*. *Enzyme and Microbial Technology*, *32*(7), 862–867. https://doi.org/10.1016/S0141-0229(03)00059-0

Wijngaard, H. H., & Arendt, E. K. (2006). Buckwheat. *Cereal Chemistry*, *83*(4), 391–401. https://doi.org/10.1094/CC-83-0391

Wink, M., Ashour, M., & Zaki El-Readi, M. (2012). Secondary metabolites from plants inhibiting ABC transporters and reversing resistance of cancer cells and microbes to cytotoxic and antimicrobial agents. *Frontiers in Microbiology*, *3*. https://doi.org/10.3389/fmicb.2012.00130

Wirtanen, G., Miettinen, H., Pahkala, S., Enbom, S., Vanne, L. (Ed.). (2002). Clean air

solutions in food processing. *VTT publications*.

Xie, J. (2023). *Formation of taste-active metabolites in sourdough fermentation and the fermentation of plant cheese analogous* [University of Alberta]. https://drive.google.com/drive/search?q=owner:jxie8%40ualberta.ca

Xie, J., & Gänzle, M. G. (2021). Characterization of γ-glutamyl cysteine ligases from *Limosilactobacillus reuteri* producing kokumi-active γ-glutamyl dipeptides. *Applied Microbiology and Biotechnology*, *105*(13), 5503–5515. https://doi.org/10.1007/s00253-021-11429-1

Xu, D., Tang, K., Hu, Y., Xu, X., and Gänzle, M. G. (2018). Effect of glutathione dehydrogenase of *Lactobacillus Sanfranciscensis* on gluten properties and bread volume in type I wheat sourdough bread. *Journal of Agricultural and Food Chemistry* 66(37), 9770–76. https://doi.org/10.1021/acs.jafc.8b03298.

Yan, B., Chen, Y. Y., Wang, W., Zhao, J., Chen, W., & Gänzle, M. (2018). γ-Glutamyl cysteine ligase of *Lactobacillus reuteri* synthesizes γ-glutamyl dipeptides in sourdough. *Journal of Agricultural and Food Chemistry*, *66*(46), 12368–12375. https://doi.org/10.1021/acs.jafc.8b05056

Yoder, G. (2021). *How to Get Better Oven Spring On Your Sourdough Bread*. Grant Bakes. https://grantbakes.com/how-to-get-better-oven-spring-on-your-sourdough-bread/

Zeece, M. (2020). Chapter Seven—Food additives. In (Ed.), *Introduction to the Chemistry of Food* (pp. 251–311). Academic Press. https://doi.org/10.1016/B978-0-12-809434-1.00007-4

Zhang, C., Brandt, J., Schwab, C., and Gänzle, M. G. (2010). Propionic acid production by

cofermentation of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough. *Food Microbiology* 27(3), 390–95. https://doi.org/10.1016/j.fm.2009.11.019

Zhao, C. J., & Gänzle, M. G. (2016). Synthesis of taste-active γ-glutamyl sipeptides during sourdough fermentation by *Lactobacillus reuteri*. *Journal of Agricultural and Food Chemistry*, *64*(40), 7561–7568. https://doi.org/10.1021/acs.jafc.6b02298

Zhao, C. J., Kinner, M., Wismer, W., & Gänzle, M. G. (2015). Effect of glutamate accumulation during sourdough fermentation with *Lactobacillus reuteri* on the taste of bread and sodium-reduced bread. *Cereal Chemistry*, *92*(2), 224–230. https://doi.org/10.1094/CCHEM-07-14-0149-R

Zhou, Y., She, X., Zhu, S., & Zhou, X. (2022). The study of microbial diversity and volatile compounds in tartary buckwheat sourdoughs. *Food Chemistry: X*, *14*, 100353. https://doi.org/10.1016/j.fochx.2022.100353

Zhu, J., Luo, Z., Sun, T., Li, W., Zhou, W., Wang, X., Fei, X., Tong, H., & Yin, K. (2023). Cradle-to-grave emissions from food loss and waste represent half of total greenhouse gas emissions from food systems. *Nature Food*, *4*(3), Article 3. https://doi.org/10.1038/s43016-023-00710-3

Zieliński, H., Wiczkowski, W., Topolska, J., Piskuła, M. K., & Wronkowska, M. (2022). Bioaccessibility of phenolic acids and flavonoids from buckwheat biscuits prepared from flours fermented by lactic acid bacteria. *Molecules*, *27*(19), Article 19. https://doi.org/10.3390/molecules27196628

Appendix

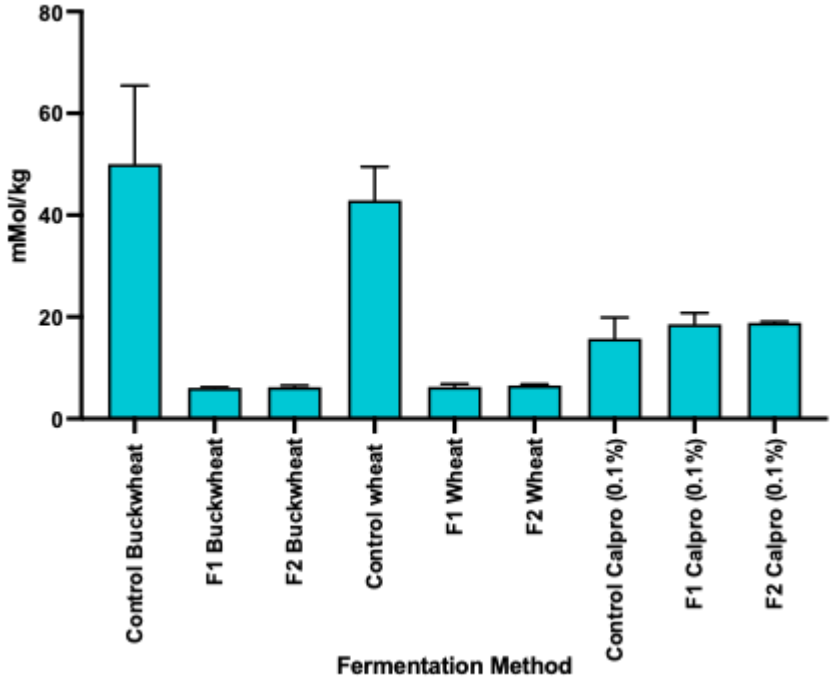

Figure A1. Propionic acid content of fermented breads and controls

Concentrations of propionic acid in bread samples, quantified using HPLC. All F1 breads were made with 10% sourdough and all F2 breads were made with 30% sourdough. Control breads contain a 0.3% calcium propionate addition and F1 and F2 breads contain no calcium propionate unless otherwise specified in the figure legends. Results are shown as means ± standard deviation of three biological replicates.