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UNIVERSITY COLLEGE CORK

School of Food and Nutritional Sciences

UPCYCLING OF BREWING BY-PRODUCTS USING LACTIC ACID BACTERIA FERMENTATION TECHNOLOGY AND ANALYSIS OF THEIR BEHAVIOUR IN CEREAL-BASED APPLICATIONS

Thesis presented by

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BSc, Food Science

for the degree of

Doctor of Philosophy – PhD in Food Science and Technology

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Emma Neylon

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Abbreviations

5'GMP	5' Guanosine Monophosphate
5'IMP	5' Inosine Monophosphate
5'PDE	5' Phosphodiesterase
a*	Redness – greenness
Aw	Water Activity
b*	Yellowness – blueness
BF	Bakers flour
BL	Bake loss
BR	Barley rootlets
BR-FST1.7	Barley rootlets fermented with *Lactiplantibacillus plantarum* FST1.7
BR-FST2.11	Barley rootlets fermented with *Lactobacillus amylovorus* FST2.11
BR-MG1	Barley rootlets fermented with *Weissella cibaria* MG1
BR-R29	Barley rootlets fermented with *Limosilactobacillus reuteri* R29
BR-TR116	Barley rootlets fermented with *Leuconostoc citreum* TR116
BSG	Brewers spent grain
BU	Brabender units
BV	Breakdown viscosity
CFU	Colony forming units
CL	Cooking loss
CO_2Vol	Volume of CO_2 produced
cP	Centipose
CWE	Cold water extract
DB	Dry basis
DDT	Dough development time
DF	Damping factor
DM	Dry matter
DNS	Dinitrosalicyclic acid
DTT	Dithiothreitol
DWB	Dry weight basis

EF	Excess fructose
FAN	Free amino nitrogen
FBSG	Fermented brewers spent grain
FEMAS	Feed material assurance scheme
FOS	Fructo-oligosaccharides
FU	Farinograph units
FV	Final viscosity
FWA	Farinograph water absorption
GI	Glycaemic index
HACCP	Hazard analysis critical control point
HM	Dough height maximum
HPLC	High performance liquid chromatography
HWE	Hot water extract
IVPD	*In vitro* protein digestibility
L*	Lightness
L. amylovorus FST2.11	*Lactobacillus amylovorus* FST2.11
W. cibaria MG1	*Weissella cibaria* MG1
L. citreum TR116	*Leuconostoc citreum* TR116
L. plantarum FST1.7	*Lactiplantibacillus plantarum* FST1.7
L. plantarum F10	*Lactiplantibacillus plantarum* F10
L. reuteri R29	*Limosilactobacillus reuteri* R29
LAB	Lactic acid bacteria
LOD	Limit of detection
MRS	DeMan Rogosa and Sharpe
MSG	Monosodiumglutamate
N	Newton (force)
NPU	Net protein utilisation
OBC	Oil binding capacity
OCT	Optimal cooking time
pGI	Predicted glycaemic index
PMT	Peak maximum time
PV	Peak viscosity
RH	Relative humidity

RSM	Response surface methodology
RSR	Reducing sugar release
RVA	Rapid visco analyser
SEM	Scanning electron microscopy
SV	Specific volume
TA	Titratable acidity
TM	Torque maximum
WAC	Water absorption capacity
WBC	Water binding capacity
WM	Wholemeal
WMF	Wholemeal flour
ΔE	Differential colour index

Abstract

Barley rootlets (BR) and brewers spent grain (BSG) are by-products of the malting and brewing industries and are primarily used in animal feed and landfill. Due to the beneficial nutritional composition of BR and BSG (high fibre/protein) the current uses underestimate their maximum potential. BR are a relatively under explored material and review of the literature available on BR revealed extensive knowledge on the formation, processing, compositional quality, and potential applications of BR. In regard BSG, literature suggests high prospects for BSG in widely available cereal-based applications. However, further processing of BSG such as using lactic acid bacteria (LAB) fermentation technology are required to improve food product quality and further valorise BSG as a food ingredient. The incorporation of BSG and fermented BSG (FBSG) in a pasta matrix revealed inclusion of BSG and FBSG induced changes in gluten properties which depreciated pasta quality (reduced firmness and tensile strength) compared to semolina pasta. However, both BSG and FBSG pasta formulations performed more favourably than the wholemeal control from a techno-functional perspective. A greater reduction in the predicted glycaemic index with FBSG fortification compared to BSG suggested fermentation further enhances nutritional properties of BSG. A follow up study on the application of BSG and FBSG in a bread revealed similar defects to bread quality. Nonetheless, comparing BSG and FBSG breads, FBSG addition improved bread characteristics resulting in increased specific volume, reduced crumb hardness; restricted microbial growth rate over time; and slowed the release in reducing sugars over time during *in vitro* starch digestion. The success observed in the capability of LAB technology to functionalise BSG sparked interest in the application of LAB fermentation in BR processing. As a result, 5 fermented BR ingredients were developed and produced using *Lactiplantibacillus plantarum* FST 1.7 (BR-FST1.7), *Lactobacillus amylovorus* FST2.11 (BR-FST2.11), *Weissella cibaria* MG1 (BR-MG1), *Leuconostoc citreum* TR116 (BR-TR116) and *Limosilactobacillus reuteri* R29 (BR-R29). The changes in sugar/FODMAP/acid compositions; microbial metabolites and techno-functional properties of the developed BR ingredients identified each LAB fermentation imparted a unique set of characteristics to the BR ingredient, further affirming LAB fermentation technology as a functionalising processing technique. The developed BR ingredients were applied to a bread matrix to explore if characteristics of the LAB fermentation translated to the bread product. Inclusion of the fermented BR ingredients improved bread specific volume/reduced crumb hardness (BR-MG1, BR-TR116); substantially slowed microbial spoilage of breads (BR-R29); and produced breads with improved nutritional characteristics and varied sensory flavour profile (BR-FST2.11, BR-FST1.7). The outcome of this research thesis provides extensive knowledge on the effects of BSG and BR on pasta/bread quality as well as showcasing the potential of LAB fermentation technology as a valorisation technique for BR and BSG processing.

Chapter 1

INTRODUCTION

Introduction

By-product rejuvenation has become an area of interest in food research, in order to align food processing towards the sustainability goals of the future. In 2015, the United Nations set out the 17 sustainable development goals [1] with the intention of merging our world to a more sustainable future. By-product upcycling primarily falls under the 12th sustainable development goal; however, it also aligns with the 2nd and 3rd sustainability goal which emphasises the relevance of the current research.

The brewing industry generates large quantities of by-products annually due to the nature of the processes employed. As the malting industry is inherently associated with the brewing industry, by-products of the malting industry may also fall under the brewing industry by-products. Brewers spent grain (BSG) accounts for approximately 80% of the total by-products produced (approx. 36.4 million tonnes annually [2]). Barley rootlets (BR) on the other hand, are the main by-product of the malting industry which can equate to approx. 5% of the weight of each malt produced. The use of BSG and BR on a commercial scale has not been greatly explored and has primarily been confined to animal feed and/or landfill; consequently underutilising their maximum potential. Their inclusion into the food chain, particularly in cereal-based applications has been a challenging process with quality losses observed in a variety of applications, especially in cereal-based applications. Hence, further processing of BSG and BR is required to improve their acceptability in cereal-based applications. Lactic acid bacteria (LAB) fermentation represents an excellent additional processing technique for BSG and BR, to aid in minimising the techno-functional changes and quality losses observed in cereal-based applications, particularly in bread and pasta. Thus, this work explores the potential of LAB in BSG and BR processing to overcome quality loss and harnessing favourable attributes imparted by the LAB fermentation.

Chapter 2 of this thesis extensively explores the literature available on BR, a rather under explored area of research and focuses on the fundamentals surrounding BR. The chapter details the biological production steps of BR during malting; the formation and processing techniques employed for BR; the nutritional value of BR; and also the potential applications for BR. There is also an additional section (section 2.7) included on the current status of BSG in relation to its application in the cereal-based applications, inspired by the extensive literature review written by Lynch et al. [3].

In **Chapter 3**, the application of a milled, spray-dried BSG (BSG) and milled, spray-dried. fermented BSG (FBSG) (fermented using *Lactiplantibacillus plantarum* F10 under patent number WO 2018/033521 A1) in a pasta system was investigated. BSG and FBSG were supplemented into pasta recipes, partly replacing semolina, at two addition levels "source of fibre" (SF) and **"**high in fibre" (HF) , implying predicted fibre values for the pasta formulations equated to 3 g/100 g (SF) and 6 g/100 g (HF) [4]. The study examines the effect of BSG and FBSG on gluten network development, starch gelatinisation properties, pasta ultrastructure as well as pasta techno-functional characteristics and the predicted glycaemic index of the pasta using an *in vitro* model designed for fibre enriched products. When compared to a wholemeal pasta, BSG and FBSG fortification enhanced techno-functional properties of the pasta, while also reducing the predicted glycaemic index, with FBSG having the most significant effect on this parameter.

As a follow-on study from this, **Chapter 4** investigates the inclusion of BSG and FBSG in a bread system. A similar experimental approach to **chapter 3** was applied with BSG and FBSG replacing bakers' flour (BF) in this case at SF and HF addition levels. The impact of BSG and FBSG on dough quality such as gluten development, starch pasting, yeast fermentation capacity and bread crumb structure was examined. Additionally, final bread characteristics, including techno-functional characteristics, microbial shelf life and *in vitro* starch digestibility were explored. The study reveals inclusion of FBSG rather than BSG increased bread specific volume (SV); reduced bread crumb hardness; restricted mould growth during microbial shelf life and slowed the release of reducing sugars during in-vitro starch hydrolysis, especially at higher levels of inclusion.

BR research is rather limited to date, particularly in relation to their use as food ingredients. **Chapter 5** delves into the fundamentals of BR with a focus on introducing BR as food ingredients. The study examines the effects of additional processing on BR using heat sterilisation and LAB fermentation, utilising 5 different LAB (*Lactiplantibacillus plantarum* FST1.7, *Lactobacillus amylovorus* FST2.11, *Weissella cibaria* MG1, *Leuconostoc citreum* TR116 and *Limosilactobacillus reuteri* R29). The chapter explores the influence of processing on sugar, acid and FODMAP (Fermentable oligosaccharides, di-saccharides, monosaccharides, and polyols) profiles while also examining the microbial metabolites present in BR. In addition, a range of techno-functional properties (structure, water-holding capacity, oil-holding capacity, pH, titratable acidity, and colour) were analysed to provide information on how the BR ingredients may behave in a food system. The changes in sugar, acid, FODMAP and

metabolomic profiles of BR revealed various strain dependent characteristics. Additionally, sterilisation and fermentation processes showed variations in BR enzyme activity, water-binding activity and oil-binding activity indicating further processing alters BR ingredient techno-functional properties.

Following on from the results observed in **Chapter 5**, **Chapter 6** explores the application of the developed BR ingredients in a bread system investigating if the unique characteristics imparted by the LAB could be of further benefit in a bread application. The study revealed changes in bread SV; crumb hardness; microbial shelf-life; *in vitro* starch hydrolysis; and sensory qualities was unique to the BR ingredient applied, suggesting that the use of fermentation technology on BR may be tailored, depending on the desired characteristic in the bread application.

Figure 1 below details a process flow diagram for guidance of each chapter in this thesis.

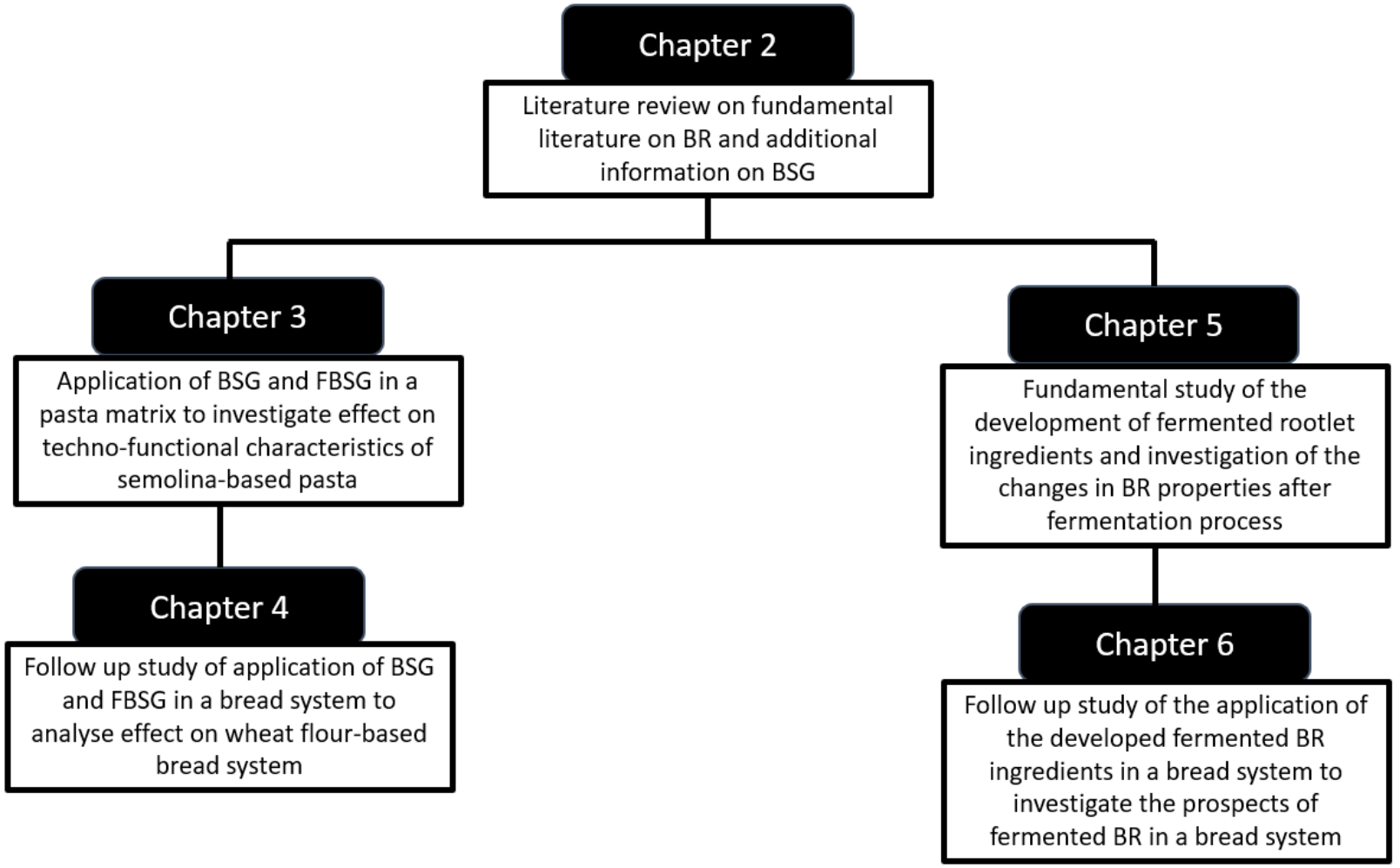

Figure 1. Process flow diagram for thesis.

Chapter 2

BARLEY ROOTLETS AND BREWERS SPENT GRAIN: BREWING BY-PRODUCTS WITH GREAT POTENTIAL

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Additional section (2.7) included on brewers spent grain.

2.1 Abstract

Barley rootlets (BR) are the most abundant by-product from the malting industry which is inherently associated with the brewing and distilling industries. BR are produced during the germination step of malting and are a valuable source of nutrition, with protein and fibre holding a large proportion of their composition. BR are generally pelletised and used as animal fodder; however, their usage may not be limited to this. Efforts have been made to utilise BR as food ingredients, sources of enzymes, antioxidants, raw materials, in fermentations, and also in biochar production. The current BR review focuses on providing information on the formation, production, and processing of BR; while also highlighting the composition, quality, and potential applications of BR. In addition to the literature discussed on BR, there is a minor supplementary section included on brewers spent grain (BSG); which provides an overview on advances made using BSG as a food ingredient and the application of LAB fermentation as a valorisation technique.

Spent barley rootlets

2.2 Introduction

Barley is a cereal grain grass which is a member of the *Poaceae* family and the genus *Hordeum*. The most common form of barley is *Hordeum vulgare* [5]. It is the main raw material used in the production of standard beer, craft beer and malt whiskey [6]. The primary product of brewing is the beverage; however, the process also accumulates various by-products. These include brewers' spent grain, spent hops, and brewers' spent yeast [7,8]. Malt rootlets (sometimes referred to as malt culms/coombes/sprouts) constitute approx. 3–5% by weight of the malt produced [9,10]. However, due to the malting industries inherent association with the brewing industry it can also be considered a brewing by-product [6].

Barley has been considered the most suitable raw material for brewing for centuries. It provides the primary components to produce beer. The starch present in barley, approximately 50–65% of the barley dry weight, is converted to fermentable sugars for the yeast to consume during fermentation [10]. The protein present in barley, representing 11–16% of barley dry weight, makes a considerable contribution to the head retention in beer. Additionally, the husk of the barley grain is used as a filtering aid during the brewing process [10]. However, the starch present in barley, and ultimately the most important material in the grain for beer production, is trapped within the endosperm walls of the barley grain including the aleurone, seed coat, pericarp, and husk layers. Thus, malting is the process used to gain access to this starch.

The malting process induces germination which stimulates rootlet and acrospire production, enzyme formation, and what are collectively known as "modifications" in the barley grain. These "modifications" allow for the starch to be accessed. During the germination stage of malting, rootlets are produced and must be separated from the malted barley after the kilning stage of malting [6].

Currently, the vast majority of the rootlets produced are sold as animal feed and are implemented as a straight feed or a commodity in feed mixtures [6]. The rootlets are pelletised and sold to the animal feed industry. The pellets are often commonly referred to as "malt combings" because they can also include other by-products of the malting industry including malt dust, small and broken barley grains, barley dust, acrospires and parts of the husk. Their

use as animal feed originates from their composition, which is particularly high in protein and fibre.

The composition of BR can vary greatly. They are rich sources of protein, amino acids (both essential and non-essential) and minerals. Fat levels in rootlets are comparable to fat contents found in the barley grain. Starch levels in the rootlets are very low in comparison to the level of starch found in the barley grain. Fibre is a predominant fraction of the rootlet composition, mainly insoluble dietary fibre, while also containing considerable levels of sugars (mono-,di-saccharides) and low levels of maltotriose [11]. Rootlets have also been found to be a source of phenolic compounds [11,12] and enzymes [13,14]. Due to this valuable composition, rootlets have been evaluated as food ingredients, as a source of enzymes such as 5′phosphodiesterase, as a fermentation substrate, and a source of phenolic compounds.

The utilisation of BR has been limited to date; however, this may be due to the limited research available. Increased utilisation of BR will help to reduce the by-products associated with the brewing/malting industry and help merge these processes into a more sustainable future. The purpose of this review is to provide an overview of the information available on BR in relation to their production, composition, and potential uses. It is important to note that in some studies reviewed, it was sometimes difficult to decipher the exact raw material utilised, as a universal term for BR has not been established. Terms such as malt sprouts were often used, particularly for studies involving lactic acid production and growth of lactic acid bacteria. Rootlets have also been referred to as “germs” in literature [15,16]. The authors used the terminology which was outlined in the studies they originated from, however where the term sprouts was used the authors cannot guarantee that this material is the rootlets alone. This term can imply that other parts of the grain are also present, such as the acrospires, barley dust and broken parts of the husk [9,17,18]. However, typically a high percentage of this mixture is comprised of the rootlets and is a good indication of how the rootlets may perform in the analysis. The need for an exact term for rootlets in the future is necessary to avoid confusion.

2.3 Biological steps in grain germination and relationship to malting

The aim of malting for the maltster is to gain access to the desired starch trapped within the endosperm of the barley grain. This is achieved by stimulating germination and exploiting the endogenous enzymes in the barley grain. Malting may potentially be one of the oldest biotechnologies recorded, and is believed to have been practiced in conjunction with brewing for at least 6000 years [9]. Evidence suggests that barley malting has occurred since ancient Egyptian times and was used in beer and bread production [19]. Malting has evolved over time due to the greater understanding of the physiology and biochemistry of the grain. The process is not confined to one type of grain, however historically for numerous reasons, barley malt has proved to be the most suitable malt for beer production [10].

Water is key in starting biological processes within the barley grain [20]. Water uptake initiates respiration and allows germination to occur. Respiration rates are highly dependent on water content and increase greatly once moisture content of the barley grain surpasses 15% [10]. As respiration rates increase, so too does the demand for oxygen. This must also be supplied to the barley grain in order to avoid intramolecular respiration and the formation of cell poisons (alkanals, alkanols) which can ultimately kill the barley grain [10]. The grain then draws on its own supply of nutrients to germinate; however, such nutrients are locked within the endosperm and must be accessed. These nutrients become accessible as a consequence of the biological changes occurring in the grain, including enzyme activation, enzyme formation and metabolic changes [21,22]. Rootlets and acrospires are also produced in this process. Enzymes are formed and activated mainly in the aleurone layer as a result of the uptake of water and the release of the complex growth promoting hormone called gibberellic acid. Gibberellic acid is comprised of several classes, and various forms of the hormone are released during grain germination [23]. Alpha-amylase is produced, and beta-amylase is released, which is already present in large amounts in the endosperm. The level

of alpha- and beta-amylase (particularly alpha-amylase) produced strongly correlates with respiration rates and requires oxygen for its formation [10]. A series of other enzymes are also released and activated, some of which include phosphatases, lipases, proteinases and saccharolytic (xylanases, beta-glucanases) enzymes, and are also of huge importance to the grain. These enzymes break down the long chain macromolecules in the grain, supplying energy for the new plant during germination, until the roots are formed [10,24]. These changes to the endosperm are collectively known as grain "modifications" [6,22]. Modifications begin in the starchy endosperm beside the scutellum and continues to the distal end of the grain (Figure 2). The growth element of germination, i.e., the production of rootlets and acrospires from the barley grain, is the visual representation of the extent of germination [25]. To maintain the composition of the barley grain and reap the benefits of the activated enzymes for the brewing process, germination must be stopped. This is achieved by the removal of the water, which previously ignited the life processes to occur [6].

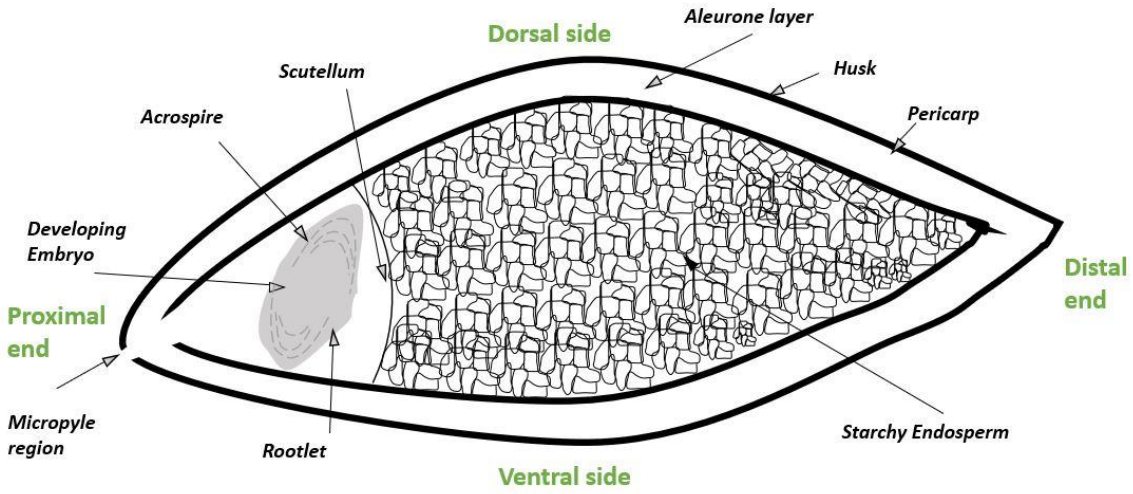


Figure 2. Structure of the barley grain. Adapted from Mosher et al. [26].

Malting can generally be broken into three stages: steeping, germination, and kilning. Although, in reality, a lot more steps are associated with the malting process and the divisions between such stages are not exactly definitive.

Steeping is the first step in malting. The barley grains are immersed in temperature-controlled water in the steeping vessel which promotes the grain to swell, soften and entice living tissues to resume metabolism [9]. As mentioned, the grain requires oxygen for respiration, therefore the barley grain needs to be aerated. In many malting plants, water is drained out and grains are left exposed to the air [9]. The water content of the barley grain needs to reach approx. 45% during steeping [26]. Following this, the grain is transferred to a germination vessel.

The germination process can vary with numerous techniques practiced, however most malting plants carry out germination using circular or rectangular boxes (Saladin boxes) [10]. In short, the basic steps to germination include barley grains spread to a certain depth, held under a controlled temperature, and rotated regularly. This allows for uniform ventilation of the grains, which stimulates even germination as well as preventing the rootlets from entangling [9,20,27]. The temperature and air circulating the barley grains is controlled to manipulate respiration rates [10]. This is done to avoid large compositional losses from the barley grain which would have a negative effect on brewing yield [9]. As germination proceeds, the enzymes are produced/activated, rootlet and acrospire growth progresses due to the increased metabolic activity, and the endosperm "modifications" progress. Figure 3 shows the progression of the rootlets and acrospire growth during germination. Germination generally takes five days to complete and is terminated when conditions of the malt are met. Modifications and malt quality can be assessed by the eye of the maltster along with their crumbly, chalk like texture [10] and through the use of various technological techniques. Some of the technological techniques used include assessment of the malt extract (hot water extract (HWE), cold water extract (CWE), fine and course grind extract), the Kolbach index, friability, viscosity of malt extract/wort, diastatic power, soluble protein, free amino nitrogen (FAN) and β-glucan content [21,28,29].

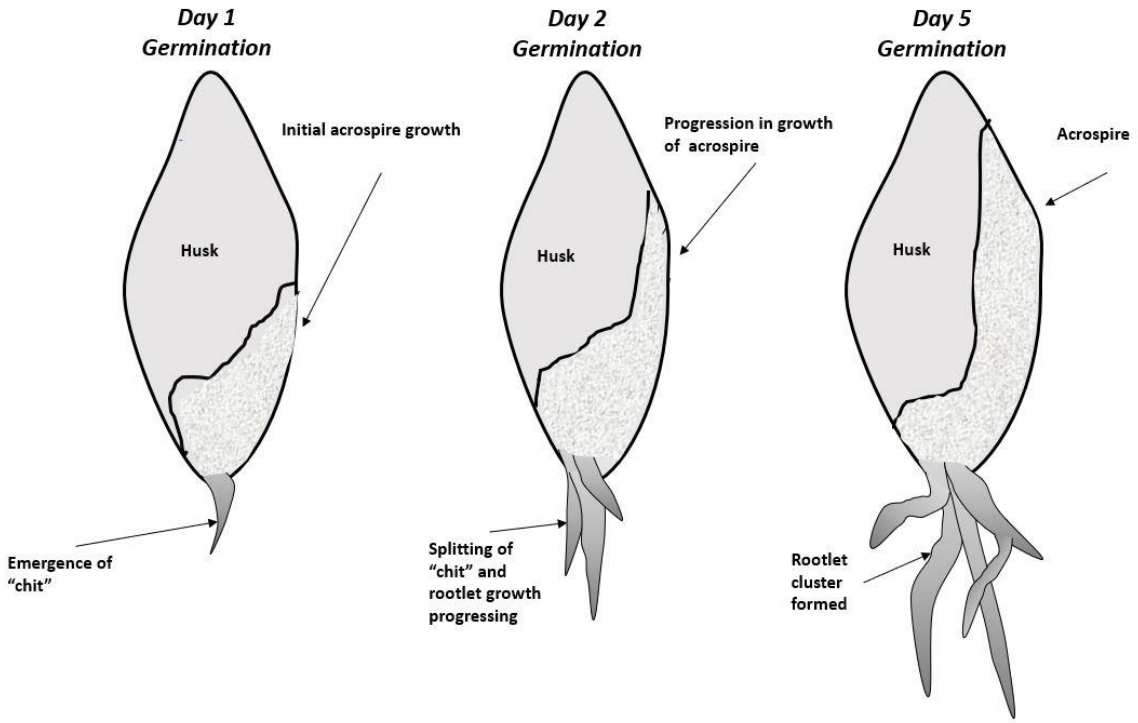


Figure 3. Growth of acrospire and rootlets during germination. Adapted from Kunze et al. [10].

Kilning is the final step of the malting process. During kilning hot air is applied to the germinated barley (known as green malt) [10]. This is done to stop germination, dry the barley grains to approx. 5% moisture, and maintain the enzymatic potential of the grains for further processing [6]. Kilning has an impact on the flavour and colour compounds of the malt. Depending on the brewing process it is destined for, some malts may be roasted in drums, imparting the flavour and colour compounds of the Maillard reaction [9,10]. The green malt is dried using several steps with high temperatures being applied slowly. After pre drying to a moisture content of approx. 12%, the green malt is slowly heated to higher temperatures to continue reducing the moisture of the grain to 4–5% [10]. Kilning regimes cause some loss of enzymatic activity, because high temperatures cause changes to the protein structure [10,25,30]. Kilned malt, unlike green malt, is brittle and fragile and is much more stable in storage due to its lower microbial count [31].

The kilned malt is then cooled and cleaned, removing rootlets and dust. The brittle and fragile nature of the kilned malt makes rootlet and dust removal much easier [9]. Kilned malt is then stored in silos before use for the benefit of the brewing process [10,32].

2.4 Formation and processing of barley rootlets

BR are regarded as the most valuable by-product for the malting industry. Other by-products of malting include malt dust, barley dust and small/broken barley grains, but are of lesser value and quantity than BR. In the U.K., more than 50 tonnes of these malting co-products are produced annually [33]. Rootlets are known as seminal roots because they develop from the embryo of the barley grain, according to terminology outlined by Hackett [34].

The growth of rootlets is initiated by water entering the embryo. Water enters mainly via the micropyle region of the grain (Figure 2) [35]. The embryo swells as a result of the uptake of water, respiration activities start, and formation of new tissues begins [9]. The rootlets are formed by utilising the nitrogenous material (amino acids and peptides) which becomes available in the endosperm and taken up by the embryo [9,24]. The rootlet first emerges as a white "chit", a yellowish coleorhiza or root sheath [9,24,36]. The "chit" breaks through the testa and pericarp layers of the grain and appears between the valves of the husk, extending from the base of the grain. The chit splits as germination progresses, forming rootlets which grow in length and form a cluster (Figure 3). Rootlets can become matted and entangled with each other during germination, causing crowding and non-uniform germination to occur if parameters such as grain turning are not controlled. The rootlets grow to approximately 5–8 mm in length and 0.3–0.4 mm in thickness [9].

Rootlets are separated from the malt because they give bitter flavours to beer [16]. They may also be a source of nitrosamines when stored under unfavourable conditions and are hydroscopic, which can also pose issues during malt storage [4,24]. Rootlets can be sourced from the kiln; removed during the deculming process; in a combined format of rootlets from the kiln and deculming process; or in a pelletised format which also contains other malting by-products. Some of these processes will be discussed further below.

The removal of rootlets from the kilned malt is often referred to as the deculming process [36]. Historically, the deculming process involved crushing the kilned malt by walking on it

while the malt was still on the kiln and shovelling the crushed kilned malt against a sieve/wire screen. The kilned malt was then brushed, and the broken rootlets fell through the screen while the kilned malt slid to the bottom and collected in piles [9]. This highly labour-intensive job was mechanised in the 20th century. In contemporary practice, two different types of machines are used: a malt deculming machine or a deculming screw [9,10,36]. In the deculming screw (Figure 4), the kilned malt moves along angled beaters within a perforated walled, U-shaped trough. The rootlets break off, fall through the perforated walls, and are collected [9]. The deculming machine is a pneumatic device. The kilned malt enters an air stream and is forced into a vertical cylindrical vessel. The impact on entering the vessel breaks off the remaining rootlets. The deculmed malt is heavier and falls through the air stream to the bottom of the cylinder, past a separation cone, and is withdrawn. The rootlets and dust collected from the air stream pass through one or two cyclones and are then collected [9]. Rootlets may also be collected in the kilns because they can fall to the bottom of the heating chamber in the kilning vessel. This occurs most often when very high temperatures are applied during kilning. The rootlets that are collected from the heating chamber in the kiln are of lesser nutritional value due to the exposure to high heat [10]. As a result, these darker rootlets may be kept separate from the less intensely heated rootlets. The kilns must therefore be regularly cleaned to collect the small fine particles which fall from the malt, which may cause fire hazards [9].

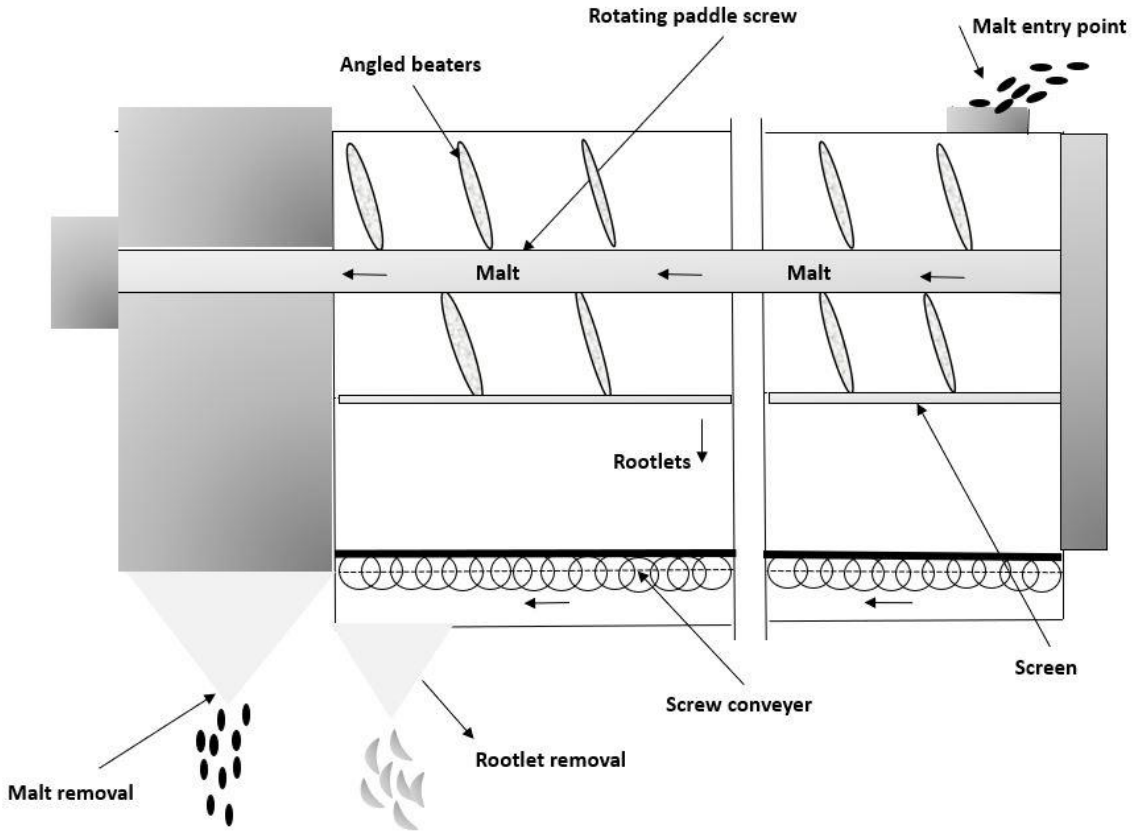


Figure 4. Longitudinal section of deculming screw. Adapted from Kunze et al. [10].

In previous times, the rootlets and dust were bagged separately in moisture-proof bags and sold as animal feed. In some cases, although rarely, they were burned for heat or composted [9]. However, the bulk density of rootlets as a loose material are low, implying that low amounts utilise large volumes of space, so more commonly, the rootlets are pelletised into a blend. The blend includes other malting by-products, such as barley dust, malt dust and floating barley grains from steeping [9,33]. The rootlets are transferred to a pelletising unit pneumatically. The co-product blends are wetted, mixed, and pelletised. The pellets may be produced by compressing and forcing the mixture through a die. The emerging material is then cut to size by rotating blades [9]. Various technologies may be employed for the pelletising process and is subject to the malting plant practice. The composition of the end pellet can vary, however the pellet on dry matter basis is approximately 24.5% crude protein,

2.9% oil, 40% NDF (neutral detergent fibre-mainly insoluble fibre [37]), 5.5% starch, and 13% sugar [38]. Pelletising the rootlets increases their bulk density from 224.3 kg/m^3 to between 561–641 kg/m^3 depending on moisture contents and pellet sizes [9,33]. Converting the rootlets to pellets also makes transport and storage much easier for farmers and the animal feed industry.

These pellets are a valuable source of nutrition for animals; however, one may question if there is potential for these pellets to be consumed by humans. Malting plants are certified to FEMAS (Feed Materials Assurance Scheme) which is based on the HACCP principles, and by-products are produced with Good Manufacturing Practice [33]. However, there is the potential to have higher levels of mycotoxins in these pellets than the parent malt due to the higher level of husk and outer layers incorporated into the by-product pellets. Mycotoxins are toxic substances that are produced as secondary metabolites of certain moulds (fungi) [39]. These layers have the highest risk of mycotoxin contamination. This is already of concern for some animals such as weaner piglets, which are as sensitive to mycotoxin exposure as humans [33]. Therefore, these pellets may have limited usage in human nutrition currently; however, if the pellets were produced containing only malt rootlets and quality assured, they may have potential as food ingredients in the future.

2.5 Rootlet composition and Quality

The composition and quality of BR is important when considering their nutritive value and potential applications. The composition of the BR (Table 1) can vary, and often depends on barley variety and malting practices (e.g., germination time, kilning temperatures) [9,17].

Protein constitutes 22.6–38.7% of the composition (Table 1), which makes BR an excellent source of protein. The protein content in BR exceeds the level of protein found in the barley grain itself and malted barley [11], as well as other cereals such as wheat which has an approx. protein content of 11–14% [11,40]. The proteins in rootlets are primarily glutelins, followed by globulins, albumins and prolamins [41]. Analysis of the barley rootlet proteome reveals that the proteins in BR are more diverse and enriched due to its anatomical complexity and the various processes which occur there for growth [12]. Table 2 illustrates the quantity of amino acids present in BR reported across various studies. Glutamic acid and aspartic acid are present in the highest amounts. The essential amino acids isoleucine, phenylalanine, lysine, and leucine also quantify a significant proportion of the amino acids. The presence of lysine in rootlets is of interest because it is widely known that lysine is a limiting amino acid in many plant-based cereals. Salama et al. [18] reported that 5.29 g/16 g N of lysine was present in BR, which was slightly lower than the barley malt sprouts (7.12 g/16 g N), acrospires (6.14 g/16 g N) and husks (7.58 g/16 g N). However, with a content of 5.29 g/16 g N, lysine would not be considered a limiting amino acid in barley rootlet protein according to WHO recommendations for lysine intake in adult diets [42]. It was noted by Salama et al. [18] that the limiting amino acid in rootlets were the sulphur-containing amino acids, methionine and cysteine. The protein profile of rootlets could potentially complement the amino acid profiles of proteins from cereal-based staple foods, such as wheat bread, which contains low levels of lysine but sufficient amounts of sulphur-containing amino acids [43]. The rootlets also have an IVPD (in vitro protein digestibility) value of approx. 81–83.29% [17,18], similar to the IVPD of acrospires and malt sprout mixture [18] and an NPU (net protein utilisation) which supports normal growth in rats [44]. Such parameters combined

provide an indication of the quality of the protein in BR, highlighting its potential in human nutrition.

Fibre levels are difficult to quantify because various analytical methods exist to measure fibre and are used across a variety of different studies [37]. Table 1 refers to both crude fibre and total fibre. Crude fibre determination, developed by Einhoff in 1806, is one of the oldest methods to determine fibre based on acid and alkali digestion [37], hence older studies report crude fibre values. Crude fibre values are still used in the animal feed industry today, however their usage in human nutrition is limited because the measurement obtains a lower fibre value [45]. This is because they do not measure all the polysaccharides present in the plant cell walls that are indigestible for humans and only measure a percentage of the levels of cellulose, hemicellulose, and lignin [37,46]. Total fibre values take into account the insoluble and soluble fibre present [47] which gives a better representation of the fibre content. Therefore, crude fibre and total fibre values are not comparable; however, the values still give an indication of the amount of fibre present. Table 1 shows that fibre represents a substantial amount of the composition of rootlets. Fibre in BR is higher than the fibre in both the barley grain and malted barley [11]. Waters et al. [11] report the most detailed and accurate representation of the level of fibre in BR. Insoluble fibre comprises 91.19% of the total fibre composition. Arabinoxylans, composed of a xylose backbone with arabinose substitutions with ferulic acid esterified to arabinose [3], comprise about one third of the fibre present in BR [11]. Arabinoxylans have the potential to cross-link via di-ferulic acid bridges, which can pose a challenge in food applications, particularly cereal-based applications [48]. Other fibres which have been identified in BR include cellulose [41,49] and lignin [50]. High fibre foods are becoming increasingly popular due to the health benefits associated with them [51], and the high fibre content of BR makes them a potential ingredient as a fibre fortifier in the future.

Starch contents have been reported in the range of 2.6–26.5% (Table 1). The amount of starch found in BR is much lower than the level of starch found in the barley grain [11]. Sugar levels (monosaccharides, disaccharides, reducing and non-reducing) have been reported in the

range of 3.4–13.6% [11,18,41,44]. Glucose and fructose are the main monosaccharides present in BR, with minor levels of sucrose, maltose and maltotriose also present [11]. The rootlets also have much higher levels of glucose and fructose present than the barley grain and malted barley [11].

Fat levels in barley malt rootlets have been reported in the range of 1.5–4.4% (Table 1). BR are lower in saturated fat than wheat flour [11]. Table 3 illustrates the fatty acid profile of the BR. Variations in the level of fatty acids may be attributed to differences in barley variety. Linoleic and linolenic acid are the dominant fatty acids present, followed by palmitic acid.

The ash content in BR ranges from 2.8–8.6% (Table 1). This is higher than the ash level present in the barley grain, malted barley, acrospire and husk [11,18]. The levels of micronutrients reported varied, which may be attributed to barley variety, however levels of calcium, potassium and phosphorus remained consistent as the highest micronutrients reported in BR [11,18,41]. Polyphenols and phytic acid have also been reported in barley rootlet composition (Table 1). Polyphenols are compounds which contain at least one phenol unit and originate as secondary metabolites of plants [52]. Phytic acid, also known as myo-inositol hexaphosphoric acid, is the principal storage form of phosphorus in plants [53]. It is widely accepted that both polyphenols and phytic acid have antioxidant properties in humans, however it is also known that they have antinutritional effects with respect to mineral bioavailability. Polyphenols and phytate bind to minerals, making them less bioavailable for absorption by humans and monogastric animals. However, in the case of BR, this may not pose a huge threat because polyphenol and phytic acid levels are relatively low (Table 1).

The quality of the BR can vary depending on the moisture content, storage, and processing of the rootlets. Moisture contents of rootlets have been reported in the range of 8.2–12.9%. The water contents of the BR are low post-kilning; however, barley rootlets are hygroscopic [9], meaning such moisture contents can fluctuate and are subject to change. In general, lower moisture contents lead to less microbial contamination but if uncontrolled storage conditions prevail, favouring water uptake, spoilage will occur. Production of mycotoxins in brewing

by-products has previously been highlighted as an area for concern. Cavaglieri et al. [54] found that *Fusarium verticilliodes* and *Aspergillus flavus* were the predominant microbes present in barley rootlets, with very little microbial diversity found. Although mycotoxin contamination has been found in all stages of the malting and brewing process, particular emphasis has been made on the level of mycotoxins present in barley rootlets [55]. Rootlets support the growth of Ochratoxin A, Aflatoxin B_1 [56] and Fumonisin B_1 [54] producing-fungi which are known to be harmful to human and animal health. In addition, rootlets have also been found to support the growth of deoxynivalenol and zearalenone producing-fungi [55]. Studies from Mastanjević et al. [57] and Krstanović et al. [58] also report significant levels of deoxynivalenol present in barley rootlets from malting. Ribeiro et al. [56], found changes in mycotoxin production with changing water activity, temperature, and storage time, indicating that such parameters need to be controlled to limit mycotoxin production on barley rootlets. Regular monitoring during storage may be necessary to consider rootlets as high-quality food ingredients. There is also potential for nitrosamines (carcinogenic compounds [59]) to accumulate in barley rootlets [9]. Under some conditions, such as interactions between the basic nitrogenous components of the rootlets and oxides of the nitrogen present in the kiln gases can occur, and result in the formation of nitrosamines can occur [9]. Although the introduction of low levels of sulphur dioxide at the beginning of kilning and indirect heating of kilns has significantly reduced the production of nitrosamines [60], it may still be a parameter to consider when assessing rootlet quality and suitability for their use in food.

Table 1. Chemical composition of malt barley rootlets.

Component	Hashitani Y. [41]	Hegazi et al. [44]	Salama et al. [18]	INRA-CIRAD-AFZ [50]	Aggelopoulos et al. [61]	Waters et al. [11]	Begea et al. [49]	Chiș et al. [62]
Protein	23.9	25.0	31.9	22.6	31.1	36.75	20.34	38.7
Fat	3.6	1.8	n.m.	1.8	4.4	1.7	1.9	2.1
Ash	3.4	8.0	8.7	5.9	6.8	2.8	3.78	8.4
Moisture (%)	10.2	8.5	12.6	10.2	12.9	n.m.	8.6	8.2
Carbohydrates	n.m.	n.m.	n.m.	n.m.	n.m.	60	n.m.	50.9
Total Fibre	n.d.	n.m.	n.m.	n.m.	n.m.	43.0	n.m.	n.m.
Crude fibre	20.5	9.7	10.7	13.9	n.m.	n.m.	n.m.	n.m.
Starch	n.m.	7.0	26.5	16.5	n.m.	2.6	n.m.	n.m.
Arabinoxylans (% T.F.)	n.m.	n.m.	n.m.	n.m.	n.m.	14.4	n.m.	n.m.
Polyphenols	n.m.	n.m.	0.35	n.m.	n.m.	0.0102	n.m.	n.m.
Phytic Acid	n.m.	n.m.	0.018	n.m.	n.m.	n.m.	n.m.	n.m.

All values are expressed in g per 100 g based on dry matter unless stated otherwise. % T.F.: percent of total fibre. n.m.: not measured.

Table 2. Essential and non-essential amino acids present in barley malt rootlets.

	Robbins et al. [17] (g/100g AA Rec)	Hegazi et al. [44] (mg/g N)	Salama et al. [18] (g/16g N)	Waters et al. [11] (g/100g Protein)
Essential Amino Acids				
Threonine	3.9	298	3.82	0.055
Methionine	2.0	101	n.m.	0.107
Tryptophan	n.m.	122	2.51	0.022
Phenylalanine	3.6	101	3.84	0.875
Isoleucine	3.9	n.m.	3.40	1.055
Leucine	5.8	n.m.	5.43	1.455
Lysine	5.5	244	5.29	n.m.
Histidine	2.2	260	6.16	7.589
Valine	5.5	268	6.09	1.334
Non-Essential Amino Acids				
Aspartic Acid	6.3	382	12.62	2.617
Glutamic Acid	13.1	596	11.32	3.025
Asparagine	n.m.	n.m.	n.m.	0.430
Serine	n.m.	306	3.9	0.882
Glutamine	n.m.	n.m.	n.m.	n.d.
Glycine	4.3	216	4.05	0.470
Arginine	5.2	493	4.78	1.117
Alanine	5.2	200	11.31	1.198
γ-Aminobutryic Acid	n.m.	n.m.	n.m.	7.302
Tyrosine	2.3	295	1.21	0.617
Proline	5.9	110	6.72	n.m.
Cystine	0.4	112	n.m.	n.m.

n.d.: not detected. n.m.: not measured. AA Rec: amino acid recovered.

Table 3. Fatty acid profile of barley rootlets.

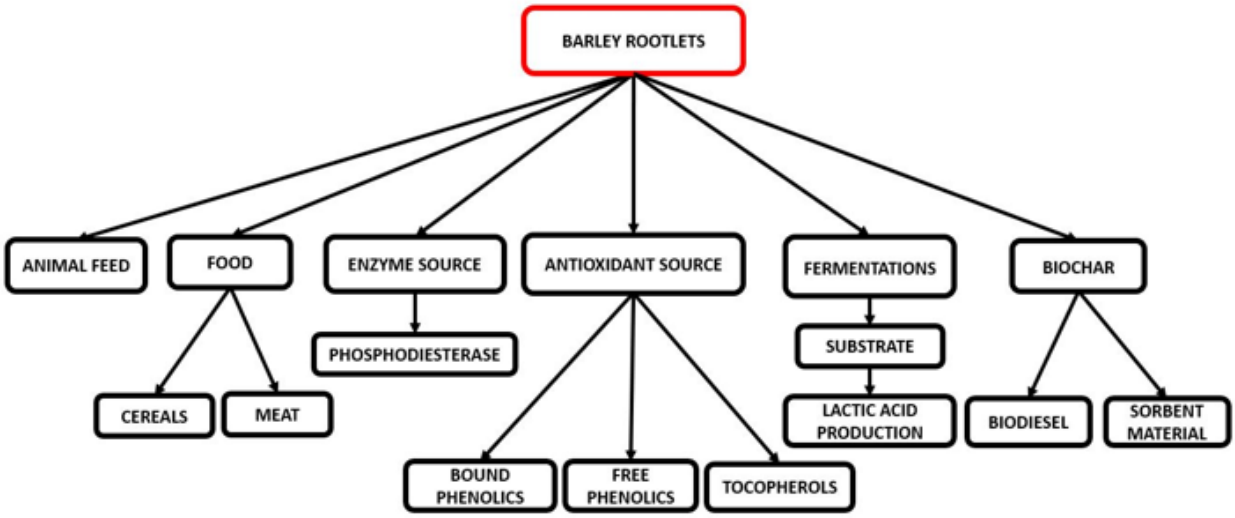
Components	Waters et al. [11]	Chiş et al. [62]
Fat %	1.7	1.9
Saturates	24.12	33.40
Monounsaturated fatty acids	8.39	14.15
Polyunsaturated fatty acids	69.47	70.20
Fatty Acids Present		
Caproic	0.02	n.m.
Caprylic	0.03	n.m.
Capric	0.15	0.31
Lauric	0.11	0.69
Myristic	0.65	n.m.
Pentadecanoic	0.30	0.42
Palmitic	14.81	30.50
Palmitoleic	0.26	0.26
Heptadecanoic	0.10	0.03
Stearic	1.40	1.45
Elaidic	0.09	0.09
Oleic	4.95	12.13
Cis-Vaccenic	1.15	n.m.
Linoleic	34.63	35.61
Linolenic	32.60	32.64
Arachidic	0.79	n.d.
Eicosenoic	0.79	n.m.
Eicosadienoic	0.26	n.m.
Heneicosanoic	0.06	n.m.
Arachidonic	n.d.	0.79
Behenic	1.12	n.m.
Docosenoic	0.16	0.38
Erucic	0.38	n.m.
Docosadienoic	0.12	n.m.
Tricosanoic	0.19	n.m.
Docosatetraenoic	0.61	n.m.
Lignoceric	0.82	n.m.
Docosapentaenoic DPA	0.09	n.m.
Docosahexaenoic DHA	1.16	1.16
Nervonic	0.70	n.m.
Obtusilic	n.m.	0.14
Vaccenic	n.m.	1.15

Based on % total fat. n.d.: not detected. n.m.: not measured.

2.6 Applications

Barley rootlets are an underused by-product. Their potential lies within their excellent composition and vast availability. Various patents (Table 4) are available which employ barley rootlets within their inventions. Studies from literature which use barley rootlets as raw materials are somewhat limited. They have primarily been used as animal feed, however; there has also been efforts to incorporate barley rootlets into food products, extract and utilise their enzymes and antioxidants, as substrates in fermentations (Figure 5), and also as a raw material in biochar production. Results from the studies are promising, which shines a positive light on the potential and diversity of barley rootlets for future applications.

Figure 5. Summary of applications of barley rootlets.



2.6.1 Animal feed

The primary application for barley rootlets is as animal feed because it is a valuable source of nutrients. Barley rootlets are incorporated as feed for both monogastric and ruminant animals and may be implemented as compound feeds or within feed mixtures. When compared to brewers' spent grain, another by-product of the brewing industry, rootlets have a higher rumen nitrogen degradability and lower nitrogen intestinal digestibility [63]. This implies that the rootlets have a lower proportion of rumen by-pass nitrogen (amount of

nitrogen which escapes degradation by ruminant microbes and available to be metabolised by the animal in the small intestine, more commonly referred to as “by-pass protein”). These parameters are important to consider when choosing feed for ruminant animals.

Alterations in the nutritional profile of animal food products depending on the feed the animals are consuming is known to occur, and barley rootlets can have a similar effect. Hashish and Abd El-Samee [64] found alterations in the fat profile in the yolk of eggs from laying hens with barley malt rootlets included in the hens diet. Incorporation of rootlets in the hen feed reduced the level of cholesterol and low-density lipoproteins in the egg yolk, with increasing levels of the rootlets in the diet having a greater impact in reducing these parameters. The enhanced fat profile of the egg yolk is associated with the favourable fat and fibre composition of the barley rootlets [64]. This effect may be true for other animal food products, however further investigations are required to confirm this.

Although barley rootlets are a valuable source of nutrition for animals, there is some caution advised with their usage in equine feeds because rootlets contain the protein hordenine [33,41]. Hordenine has been viewed in some countries as potentially an illegal drug in horse racing. The protein is classified as a naturally occurring prohibited substance (NOPS), and in some countries horses may be disqualified from racing if found in the urine [65]. Hordenine has been shown to induce a pharmacological effect in relation to respiratory function in horses [66]. It increases systolic and diastolic blood pressure and peripheral blood flow volume. Such effects are short lived and found after administration of high doses (2 mg/kg Body Weight) through IV injection [66]. Although there is no evidence for these effects from hordenine consumption in feed [66], barley rootlets and other feed materials containing hordenine should be avoided in equine diets.

2.6.2 Food Applications

Barley rootlets have a desirable nutritional profile, being high in protein and fibre. Such fractions are highly sought after for incorporation in the human diet, particularly fibre, due to the health benefits associated with it [67]. Utilisation of barley rootlets as a food ingredient

has been shown to enhance the nutritional profile of breads, described as flattened breads and pan breads [68]. Increased usage of barley rootlets in food applications may be a cost-effective way to improve the nutritional profile of these products.

Salama et al. [18], highlighted the technological properties of barley rootlets. To determine a suitable application for an ingredient, knowledge of the functional properties is needed. Barley rootlets had favourable outcomes in comparison to the acrospires, husks and malt sprouts (mixture of rootlets, husk and acrospires). The study revealed that rootlets had the highest water and oil absorption capacities, as well as the highest emulsification capacity [18]. The barley rootlets had the lowest foaming capacity, but the greatest foam stability in comparison to the husk, acrospire and malt sprouts [18].

Barley rootlets have been incorporated into bread, biscuit, and butcher sausage formulations. Waters et al. [11], examined the effect of substituting milled barley rootlets and fermented milled barley rootlets in wheat bread formulations at 5, 10, 15 and 20% addition. Barley rootlets were made into a flour using a mill feeder and a laboratory mill. The rootlets were fermented by *Lactiplantibacillus plantarum* FST 1.7 to prepare a rootlet sourdough. Waters et al. [11] postulated that the replacement of wheat flour with rootlets in bread formulations could improve the nutritional properties of the bread by enhancing the amino acid profile, increasing fibre levels, reducing saturated fat content, and lowering sodium intake coming from the flour. Increased addition of fermented and unfermented rootlets to bread formulations generally decreased the bread volume, increased hardness, and produced a darker-coloured bread. However, at 5% addition of the fermented rootlets, specific volume and hardness of the substituted breads were not statistically different from the wheat bakers flour control. Rootlet and unfermented rootlet breads with up to 5% inclusion were preferred by the sensory panel, however up to 10% was accepted. Inclusion of rootlets at 10% would likely enhance the overall protein and fibre contents in the bread. Increased intake of fibre improves human health, due to the health promoting benefits associated [67]. Many people around the world do not take in sufficient fibre in the diet and increasing fibre in a staple food product such as wheat bread may improve the overall fibre intake for a human. With regards

to effects on protein, there is potential for improvements in protein quality in wheat bread with rootlet substitution. Rootlet inclusion could create a better balance in the amino acid profile in wheat bread, as seen with the substitution of other plant based ingredients [43], however further analysis would be needed to confirm this. Inclusion of barley rootlets/fermented rootlets in bread appears to be an option up to 10% addition, and the use of fermented rootlets at a low level of inclusion could enhance bread characteristics [11]. Chiş et al. [62] studied the addition of barley rootlets up to 25% inclusion in biscuit formulations, with emphasis on the volatiles of the rootlets which may affect flavour perception. Increasing levels of barley rootlet addition caused a darker colour to occur as well as increasing intensities of odour, flavour, and taste. Panellists found an intense 'whiskey' or 'alcohol' note with barley rootlet addition, as well as citrus, pine, and mint notes [62]. The study outlined that inclusion of barley rootlets in biscuit formulations up to 15% was acceptable, because over this value an unpleasant aldehydic taste was perceived [62]. The results obtained from these studies were similar to those observed by Salama et al. [68] with the incorporation of rootlets into bread and biscuits. Salama et al. [68] reported enhancements to the nutritional profile of the bread at 5% addition of rootlets (approx. 1% increase in fibre and approx. 1% increase in protein contents). Additionally, barley rootlets were examined for their usage in sausage formulations as an extender/binder [68]. Successes were observed organoleptically with the inclusion of barley rootlets up to 10%. Incorporation of barley rootlets decreased cooking losses and the authors postulated a reduction in production costs with rootlet inclusion. In addition, the fibre content of the sausages was enhanced (1.18–3.25% Dry Basis (D.B)); however, there was a marked decrease in the relative protein content (65.9–61.7% D.B) and moisture levels (65.2–63.7% D.M) [68]. The reduction in protein content may be associated more to the decrease in the amount of meat used in the formulation, rather than the effects of the rootlet inclusion.

Overall, the incorporation of barley rootlets into food applications up to a certain level has had promising outcomes. Their inclusion in food may have a maximum point, because higher levels of inclusion resulted in various off-flavours in bread and biscuits [11,62]. Although

food studies are limited, the analysis available shows that barley rootlets have potential as a fibre fortifier and could possibly improve the protein profiles of foods and reduce production costs when used as extenders in sausage formulations, while also having potential as a fermented ingredient. This may encourage their usage in other food applications in the future. Additionally, the use of rootlets as a food ingredient may encourage industry evolution. Brewing and malting industries could shift focus to creating food ingredients from rootlets rather than producing them as an inherent by-product.

2.6.3 Enzyme Applications

Rootlets of pale malts are particularly rich in enzymes due to the reduced heat exposure during kilning [10]. Evidence from literature suggests that rootlets contain a variety of different enzymes, some of which include: invertase, superoxide dismutase, nucleases (RNase and DNase), 5′-phosphodiesterase, phosphotransferase and phosphomonoesterase [69–73]. 5′-phosphodiesterase (5′PDE) has been the predominant enzyme isolated and utilised in applications from barley rootlets. It has been found in appreciable amounts in the barley rootlets and in the malted barley grain [74,75]. The enzyme has been used commercially to hydrolyse RNA to make 5′-nucleotides. These 5′-nucleotides can be utilised as flavour enhancers, 5′GMP (5'Guanosine Monophosphate) and 5′IMP (5'Inosine Monophosphate) specifically, that have an umami-like taste, and are also used in the production of pharmaceuticals [76–78]. After the discovery of the flavour nucleotides (5′GMP and 5′IMP) and the synergistic flavour effects with monosodium-L-glutamate (MSG) [79], the flavour nucleotides have been produced as seasonings (mixed with MSG) for use in savoury foods such as soups and broths [80].

Processes for the extraction of 5′PDE from barley rootlets vary, with some patents (Table 4) also developed. The process generally requires a purification step, because various other undesired enzymes such as phosphatases, 5′-nucleotidase and nucleosidases may also be present in barley rootlets [74,81] that may produce unwanted products or inhibit 5′-nucleotide yield.

Hua and Huang [81] isolated 5′PDE from barley rootlets to form 5′-nucleotides using water extraction, gel filtration and freeze drying. Various parameters were found to affect the extraction rate of 5′PDE from barley rootlets. These included barley rootlet size, pH, temperature, volume of the extraction solvent, and extraction time. The optimum conditions for extraction of 5′PDE were pH 7, 20 °C and 7 h. The optimum solvent (water) and rootlet ratio for extraction was 16:1, along with a rootlet size larger than 120 mesh size. Hua and Huang [81] isolated two types of 5′PDE enzyme (termed 5′PDE (a) and 5′PDE (b)) of different optimum temperatures and pH (70 and 65 °C, 5 and 6, respectively). The study found that the 5′PDE enzymes showed excellent stability to heat (70 °C) over time (420 min). The purified enzymes contained fewer peaks vs the raw enzyme extract upon HPLC analysis. This indicates that the authors' purification step was successful in removing some of the undesired enzymes which could affect 5′nucleotide yield, however it was acknowledged that not all may have been removed [81]. Like Hua and Huang, Beluhan et al. [13] also used a purification step. The purification step involved thermal treatment and acetone precipitation, with the intention to reduce levels of phosphomonoesterase (PME). Beluhan et al. [13] found at least two 5′PDE isoenzymes which also differed in their optimum temperatures (55 and 70 °C).The variances in optimum temperature of the 5′PDE found between Hua and Huang [81] and Beluhan et al. [13] could potentially be linked to differences in extraction and purification methods, however this could also be linked with possible co-extraction of PME. Later studies by Beluhan et al. [82] reported an optimum temperature of 55 °C for PME and 70 °C for 5′PDE. Additionally, Beluhan et al. [82] found that a thermal treatment step could be a key factor in the purification of 5′PDE enzyme preparations; PME activity was significantly reduced after heat treatment. Beluhan et al. [13] highlighted the excellent storage stability of the 5′PDE in barley rootlets, with enzyme activity remaining almost constant for 90 days when stored at −18 °C. Hua and Huang, Beluhan et al. [82] and Beluhan et al. [13] were all in agreement with the excellent thermostability exhibited by 5′PDE preparations. Benaiges et al. [83,84] used a two-step purification process which included an acetone purification step and DEAE-Sepha-rose chromatography for the isolation of 5′PDE from barley rootlets. This

process was also successful in producing 5′-nucleotides. Laufer and Gutcho [74] found green malt rootlets, after oat rootlets, were the most effective in converting RNA to 5′-nucleotides in comparison to the rootlets, stems and kernels of other cereals and legumes tested (rye, oat, soy beans, mung beans, wheat, rice). Green malt rootlets are likely to perform better with regards to enzymatic activity rather than kilned rootlets, due to the reduced exposure to heat and risk of denatured enzymes. Further investigations were carried out into commercially available malt sprouts, which contain rootlets, to explore the 5′PDE activity in these. Laufer and Gutcho [74] found the addition of low levels of Zn^{2+} before a heat treatment of 72 °C for 5 min on washed malt sprouts was the best method for large scale production of 5′-nucleotides from RNA. Such observations are slightly conflicting with reports from Beluhan et al. [13,82], who noted an increase in 5′PDE activity with Mg^{2+} and slight to moderate inhibition with metal ions (Zn^{2+}). Laufer and Gutcho also showed that the heat applied to malt rootlets reduced the microbial load, which reduced the potential of microbial enzymes to participate in the RNA hydrolysis which may inhibit 5′-nucleotide production. A method to separate the flavour enhancing 5′nucleotides (5′GMP and 5′IMP) from the products of the RNA hydrolysis was also reported [74]. Sombutyanuchit et al. [85] used barley rootlets as a source of 5′PDE to produce 5′nucleotides from brewers' yeast with specific emphasis on the production of the flavour nucleotide, 5′GMP. The study concluded that significant levels of 5′GMP could be produced from a heat-treated extract (65 °C for 30 min or 70 °C for 7 min) containing 5′PDE sourced from barley rootlets and hydrolysed for 8–14 h. However, levels obtained for 5′GMP were 50% lower than commercial nucleotide extracts; the author related this more to the RNA source rather than a reduced activity of the enzyme [85]. Sombutyanuchit et al. [85] outlined the commercial nucleotide extracts were prepared using specially selected "high RNA" baker's yeast (*S. cerevisiae*) as the RNA source, which under standard autolysed procedure baker's yeast extracts have higher levels of guanine and 5′GMP. The author also highlighted that RNA levels are closely related to specific growth rate of the yeast, and brewers' yeast (*S. uvarum*) grows at a slower rate under low temperatures in lager production thus a lower level of RNA will be present [85]. Thus, the

reduction in RNA present during autolysis, which can often be linked with its source, may be a contributing factor to increasing or inhibiting the overall yield of 5′GMP.

Overall, barley rootlets prove to be a viable source of 5′PDE and can be used to produce 5′-nucleotides. This may be of interest for industry. The process requires purification steps and pre-treatments such as heat to maximise the 5′-nucleotide output and eliminate unwanted enzymes. Compared with other sources such as snake venom [86,87] and bovine intestine [88], rootlets could be more economical for use by industry, however further investigation into the cost effectiveness of using rootlets as an enzyme source for 5′PDE would need to be examined to confirm this.

2.6.4 Antioxidant source

Antioxidants are one of the main ingredients used to protect the quality of a food by preventing the oxidation of lipids which is deleterious for food quality [89]. They are also utilised in the cosmetic industry. In addition, antioxidants play an important role in the human diet and have a positive effect in controlling various diseases [90]. Antioxidants can be from natural or synthetic sources, with natural sources being more appealing to the consumer. Barley rootlets are potentially a plentiful source of natural antioxidants which may be utilised in food and cosmetics. Various levels of antioxidant compounds have been reported [11,14,18,91]. Variations in the levels reported may be linked to barley variety and malting practices. The term “antioxidant activity” and “antioxidant power” are used throughout this section. The terms appear to be interchangeable, however “antioxidant activity” is the more commonly used term in relation to the properties of an antioxidant when describing a compound’s capability to reduce or inhibit the process of oxidation [92].

Bonnely et al. [93] investigated three different extracts from barley rootlets which contained rootlet oil, free phenolic compounds and bound phenolic compounds (bound to lignin and arabinoxylans). The rootlet oil had a low level of tocopherols (α-tocopherol and γ-tocopherols) with little antioxidant activity. “Tocopherols” may also be referred to as vitamin E. Vitamin E has four tocopherol isomers existing in nature, namely α-tocopherol, β-

tocopherol, γ-tocopherol and δ-tocopherol [94].The bound phenolic extract (containing compounds such as trans-ferulic acid, cis-ferulic acid, cis-p-coumaric acid, trans-p-coumaric and hydroxycinnamic acid) had an appreciable antioxidant power but dry matter yield was low (2%). Bonnely et al. [93] deemed that these extracts were of little interest commercially due to the low quantity present versus the cost and complexity of the extraction. However, the extraction of free antioxidant compounds (containing 52% proteins, 33% sugars and 5.5% reducing compounds) from barley malt rootlets was promising, due to the antioxidant properties and yield associated with it. The free antioxidant extract was also found to have a synergistic effect with α-tocopherol in relation to antioxidant power. No loss in antioxidant activity was noted when the free antioxidant extract from barley rootlets was used to substitute part of an α-tocopherol mixture when compared with α-tocopherol alone [93]. Peyrat-Maillard et al. [95] produced two extracts, "a free rootlets extract" and "bound rootlets extract", and investigated the effect of vitamin E and vitamin C (also referred to as ascorbic acid) on antioxidant power. This was done to determine the antagonistic or synergistic effects of vitamin C and vitamin E on rootlet extract antioxidant power. The free rootlets extract was comprised of the free oxidoreduction agents, while the bound rootlets extract contained phenolic compounds which were previously attached to arabinoxylans and lignin. The study revealed the main phenolic compounds present were trans-p-coumaric acid and trans-ferulic acid, with a higher concentration of these found in the bound rootlets extract than the free rootlets extract. Like Bonnely et al. [93], Peyrat-Maillard et al. [95] found a positive synergistic effect with the malt rootlet extracts and α-tocopherol in relation to antioxidant power, however a negative effect was noted with regards to the malt rootlet extracts and ascorbic acid [95]. The authors outlined that ascorbic acid was a more efficient antioxidant than the rootlets extract, and the presence of the rootlet extracts, both bound and free, hindered the antioxidant power of vitamin C. The authors also highlighted that further investigation would be needed to explain this. The synergistic effect observed with the bound rootlets extracts and α-tocopherol was not linear, implying that higher doses did not further enhance the synergistic effects, but was linear with the free rootlets extract. The synergistic

effects noted was suggested to be due to two mechanisms: (1) the malt rootlets extracts preventing the oxidation of α-tocopherol; or (2) the malt extracts aiding in the regeneration of α-tocopherol in its radical form [95]. An optimised method for extraction of the antioxidant compounds in barley malt rootlets has also been investigated, using a statistical model known as response surface methodology (RSM) [96]. Three independent variables were found to affect antioxidant activity of barley rootlets, which included solvent composition (% v/w), temperature of extraction (°C) and extraction time (min). Meng et al. [96] found the extraction parameters for optimal total antioxidant activity were: 50% ethanol (v/w) solvent and an extraction temperature of 84 °C for 22 min. The predicted antioxidant activity value was 9.49 µmol TE, which was agreeable with the experimental value of 9.79 µmol TE [96]. Cheng et al. [97] determined the optimum conditions for the extraction of the alkali soluble components of barley malt roots (the term used in the study when referring to the malting by-product, likely implying rootlets) and determined its composition. The study concluded that 60 min, 40 °C, pH 9 and a solvent to raw material ratio of 25:1 were the optimal conditions necessary for the highest extraction yield of 29.2%. The composition of the extract was 33.72% protein, 0.1% polyphenols and 0.33% flavonoids [97]. Studies conducted on the barley rootlet proteome by Mahalingam [12], provide evidence for the presence of various antioxidant compounds in barley rootlets. Such compounds include ascorbic acid and glutathione. Analysis of the phenylpropanoid pathway enzymes during the study suggested that barley malt rootlets may also be a source of coumarin, cinnamaldehyde, sinapic acid and sinapyl alcohol [12]. The presence of such compounds present may be of interest in the future, and may potentially be used in food and pharmaceutical applications [98–100].

The effect of pre-treatments on the antioxidant compounds in barley rootlets has also been analysed. Pre-treatments of the barley rootlets, such as steaming, roasting, autoclaving, microwaving and enzyme treatment, has been investigated in relation to their effect on the antioxidant potential of the phenolic extracts from barley rootlets [14]. Budaraju et al. [14] investigated the effect of these pre-treatments on the free phenolic compound extracts and bound phenolic extracts. The use of pre-treatments generally enhanced the extraction yield

and antioxidant activity of the extracts, in comparison to the untreated samples [14]. Autoclaving had the greatest effect on total extraction yield and increasing total phenolic content of the extracts. The increase in total phenolic content was due to the enhancements observed in the free phenolic extract rather than the bound phenolic extract. Dudjak et al. [91] observed an increase in polyphenol content in barley roots upon treatment of the growing barley plant with cadmium in the growth medium. A 10.3% increase in polyphenol content was observed in the barley roots upon the addition of cadmium. However, such treatment had a greater impact on enhancing the polyphenol content in barley shoots (+16.7%) and barley leaf blades (+35.2%) than the barley roots. Treatment with cadmium could be applicable to barley rootlets for increased polyphenol content, however further investigation must be carried out to confirm this.

The studies outlined above indicate that barley rootlets are a potential source of natural antioxidants. They indicate more encouraging results for the potential of free phenolic extracts rather than the bound phenolic extracts. Thus, rootlets may be an abundant source of naturally occurring antioxidants which could be capitalised in the food and/or related industries and may contribute towards clean labelling of products.

2.6.5 Growth medium for fermentation

Barley rootlets can support the growth of micro-organisms, which makes it a potential substrate for microbial cultivation and fermentations. They have been employed as a substrate for lactic acid production as well as a growth and storage medium for lactic acid bacteria. Lactic acid bacteria have a prominent role in food and biotechnology industries as starter cultures for food production and as probiotic production. Lactic acid is a product of the lactic acid bacteria fermentation and is in the second tier of the 12 most promising value-added building blocks utilised in the production of numerous useful and specialty chemicals [101]. Incorporation of by-products streams as substrates in microbial fermentations to replace costly raw materials and reduce production costs has become increasingly desirable. Barley rootlets have a very low cost associated with them and are produced in high volumes

each year, which makes them an attractive substrate for utilisation in these applications. It is important to note, some studies throughout this section do not specify the grain source from which the rootlets originate. However, it is fair to assume the studies that do not specify the grain source could originate from barley and have been included in this review. Malt sprouts is the more common term used in this section because studies used such terminology.

Cejas et al. [102] investigated the use of barley malt sprouts and barley malt sprouts supplemented with 20% w/v fructo-oligosaccharides (MS FOS) as a substrate for the growth of two lactobacillus species, namely, *Lactobacillus salivarius* and *Lactiplantibacillus plantarum*. The results from the malt sprout media in relation to microbe lag times, change in pH and acidification rates were comparable to the MRS (DeMan, Rogosa and Sharpe) control. Twenty percent FOS addition enhanced the growth of *Lactobacillus salivarius* even more than the traditional MRS medium. The authors also found no loss in the culturability of the bacteria stored in malt sprout media after freeze drying and 60 days storage at 4 °C. This was attributed to the FOS present which likely had a protective role [102]. Laitila et al. [103] produced a malt sprout medium using a malt sprout extract instead of water in the preparation of the medium for growth of a *Lactiplantibacillus plantarum*. The malt sprout extract was a liquid extract which consisted of malt sprouts that had been soaked in water, autoclaved, centrifuged, and filtered. This extract was also supplemented with glucose and yeast extract. MRS agar was used as a control for the experiment. Results obtained indicated that the malt sprout extract medium supported the growth of the strain and could replace the MRS medium without affecting the cell count or the strains antimicrobial activity. The cost of the malt sprout extract medium was estimated at 20% of the cost for the MRS medium, which would considerably reduce production costs [103].

Radosavljević et al. [104] used malt rootlets as a carrier for the immobilisation of *Lacticaseibacillus rhamnosus* and found high cell viability during batch fermentations with the immobilised cells as well as a lactic acid yield of 93.3%. A brewery by-products mixture (brewers' spent grain and malt rootlets hydrolysate, brewers' yeast, soy lecithin) was utilised as the substrate for the fermentation. Continuing from this study, Radosavljević et al. [105]

determined the optimum levels of brewers' yeast and soy lecithin necessary for optimised lactic acid production with the brewers' spent grain and malt rootlets hydrolysate as the substrate. Both studies highlight the suitability of malt rootlets for utilisation in the growth of lactic acid bacteria and lactic acid production.

Investigation into barley rootlets as nitrogen source replacements in substrates for fermentation and bio stimulants has been conducted, with some patents available in this area (Table 4). Liu et al. [106] used malt sprouts as a nitrogen source during lactic acid fermentation and concluded that the malt sprouts could be used as an alternative nitrogen source at a concentration of 16 g/L in conjunction with corn steep liquor at a concentration of 12 g/L in the growth medium. Similar results were obtained by Hujanen and Linko [107], who also found that a barley malt sprout extract was capable of replacing most of the expensive nitrogen source for the fermentation without compromising on the level of lactic acid produced. Results from Göksungur and Güvenç [108] correlated with these findings; their study showed that malt sprouts were the most suitable alternative nitrogen sources after yeast extract.

Production of a bio-based concentrate from barley malt rootlets for utilisation as a stimulant in biotechnological processes for the vinegar industry at pilot scale has also been investigated [49]. A dark brown, viscous concentrate was produced, containing 51.3% dry matter (d.m.), 5.29% (d.m.) protein, 2.38% (d.m.) ash, 17.15% (d.m.) carbohydrates and 0.96% (d.m.) starch. The optimal conditions to produce this extract was 60 °C for 60 min using water as the extraction solvent, in a solvent to rootlet ratio of 8:1 [49]. The extract produced was intended to be used in the vinegar industry to increase the substrate concentration for the yeast fermentation that produces the ethanol. This also highlights another pathway for the potential of barley rootlets in the future.

Results from the studies employing barley rootlets as substrates in fermentations and lactic acid production are encouraging. The utilisation of a low-cost material in fermentations such as barley rootlets can reduce production costs [103], and from an economical point of view,

this may provoke further investigation of barley rootlets for use in this application and in others.

2.6.6 Biochar production

In more recent years, rootlets originating from the malting process have been used in biochar production. Biochar is produced by heating organic matter under oxygen limiting conditions and relatively low temperatures [109]. It may be utilised as an energy source, as an addition to soils for its fertiliser and carbon sequestrant properties, and as an absorbing agent in a range of applications [110]. Examination of rootlet biochar using microscopy techniques indicate that rootlets maintain their shape post biochar production and contain mineral deposits covering the external surface of the rootlets [111]. Various studies employ malt spent rootlet biochar in their investigations, however this review highlights the use of rootlet biochar as a possible sorbent material and as a catalyst in the biodiesel production process. Although the majority of the studies discussed in this section do not state the exact grain the malt rootlets come from post-malting (similar to Section 2.6.5), it is likely that some of the rootlets used for biochar production originated from the barley grain, hence their inclusion.

Rootlet biochar has been investigated as a potential sorbent for various types of water pollutants such as uranium, chlorine, chloroform, chromium, and methylene blue, with encouraging results observed [112–117]. Grilla et al. [111] used rootlet biochar as a platform to generate sulphate and hydroxyl radicals as well as an electron transfer mediator while exploring advanced oxidation processes to reduce the presence of trimethropin in water matrices. Rootlet biochar has also been used to activate sodium persulphate, which is needed in the oxidation and removal of sulfamethoxazole, an antibiotic microcontaminant which can be present in water supplies [118]. Manariotis et al. [119] and Valili et al. [120] found malt spent rootlet biochar had excellent sorption capacities for phenanthrene and mercury. Additionally, increases in the sorption capacity of rootlet biochar for phenanthrene and mercury was noted with varying pyrolysis temperatures [119,120]. Boutsika et al. [121], Anagnostopoulos et al. [122], and Boutsika et al. [123] also found promising results in

relation to the sorption capacity of mercury from aquatic solutions using malt spent rootlets biochar. These studies showed that a range of factors are involved in optimising sorption capacities of the biochar produced from rootlets. However, its use as a sorbent material in aquatic solutions or water treatment applications must also be monitored. Investigation into the toxicological effect of leachate from rootlet biochar by Tsouloufa et al. [124] revealed that washing of the biochar made from malt spent rootlets is a crucial step in the process to avoid any adverse effects.

The use of rootlet biochar as a catalyst in the transesterification reaction in biodiesel production has also been successful [125]. Ntaflou and Vakros [126] found that pre-treatments with NaOH of malt spent rootlet biochar enhanced transesterification activity of the biochar, showing activity similar to that of a homogenous catalyst, by increasing the basicity of the biochar. Similarly, Tsavatopoulou et al. [127] also had success in using rootlet biochar as the catalyst during transesterification, with the untreated biochar giving better conversion rates than H_2SO_4-treated biochar.

Malt rootlet biochar appears to have a promising future. The high sorption capacity of the biochar for pollutants highlights it as a potential option as a sorbent material, which may be useful in water treatment regimes. However, the potential leachate from the rootlet biochar in aquatic environments is something which must be monitored, and strict monitoring of this should be considered. Additionally, the use of rootlet biochar in biodiesel production as a heterogenous catalyst in the transesterification process may enhance the sustainability of the process. Heterogenous catalysts are viewed as more environmentally friendly catalysts because they can be easily separated and potentially reused in the process [128].

Table 4. Patents which utilise barley rootlets.

Barley Rootlet Patents			
Google Patent Number	**Title**	**Area of Usage**	**Summary**
US20070148317A1 [129]	Functional component-enriched barley malt rootlets and process for producing same	Food/cosmetic/medicinal ingredient	Process for the extraction of functional components from rootlets of barley which can be utilised as a raw material in food, cosmetic and medicinal formulations.
US9326542B2 [130]	Process for producing food and beverage products from malt sprouts	Food and beverage ingredient	Technology for utilising malt sprouts of a specific particle size as a raw material in food or beverages
US5034325A [131]	5'Phosphodiesterase enzyme preparation and method for its production	Enzyme preparation	An extraction method to obtain 5'phosphodiesterase from barley malt sprouts which is stable in storage
US3304238A [132]	Enzymatic material and method of preparing same	Enzyme preparation	Preparation of an aqueous enzyme medium from barley (and other grains) rootlets and stems capable of producing mainly 5'nucleotides
US3459637A [133]	Enzyme digestion of nucleic acids	Enzymatic production of 5'nucleotides	Method for enzymatically digesting RNA to primarily form 5'nucleotides using the aqueous extract of plant rootlets and stems (including barley)
US2925345A [134]	Preparation of an antioxidant from rootlets	Antioxidant extract	Method to limit auto-oxidation in a fatty material which involves the mixing of pulverised rootlets with the fatty material.
US2694011A [135]	Poultry and swine feeds containing rootlets of germinated barley	Animal feed	Utilisation of barley rootlets within animal feeds for poultry and swine.
US4613507A [136]	Malt-like flavour from cereal grain root cultures	Food and beverage flavour ingredient	Method of creating malt-like flavour ingredient from roots of grains (including barley) which can be used in food and beverage formulations
WO2019238928A1 [137]	Process for preparing a cereal-based beverage with malt and malt rootlets	Beverage ingredient	Utilisation of barley rootlets in wort to obtain a malt-based beverage
US20200178580A1 [138]	Malt sprouts extracts and their uses	Extract	Use of malt sprouts as raw materials in extract production for various uses
WO2018104531A1 [139]	Compositions and methods for stimulating plant growth	Extract	Incorporation of malt sprouts in extract preparation and use as a bio stimulant

2.7 Brewers spent grain and the use of fermentation technology as a processing technique

BSG is the most abundant by-product generated during the brewing process, accounting for approximately 85% of the by-products produced, and is recovered during the lautering step of brewing. It is comprised of protein (19-30%), fibre (cellulose (12-25%), hemicellulose (20-25%)), lignin (12-28%), minerals (2-5%) and low levels of fat (10%) [3]. Due to the attractive nutrient composition and the urgency to find more sustainable food resources to satisfy the ever-growing global population, BSG holds great prospects for incorporation into the human food chain. Fibre is of particular interest in BSG, with arabinoxylan being the fibre present in the most significant quantity in BSG. Arabinoxylans sourced from BSG have previously shown to positively impact the gut microbiome (*in vitro*) when extracted and solubilised [140], indicating BSG as a functional food ingredient could aid in maintaining a healthy microbiome. However, it is also important to note the basic composition of BSG can vary greatly and is dependent on a variety of factors including barley variety; malt type; grain cultivation; the brewing process employed; the point at which the BSG is retrieved; and even the area within the filter cake in which the BSG is sampled from [3,141–143].

To date, BSG has been implemented as a flour replacer in a variety of foods (primarily bakery products) including bread, biscuits, cookies, muffins, cakes, waffles, pancakes, tortillas, snacks doughnuts and brownies [144,145,154,146–153]. From a compositional perspective, the general consensus from most studies concluded inclusion of BSG over 10% inclusion enhanced fibre/protein contents and reduced starch values. However, from a techno-functional viewpoint, higher levels of BSG in these food products often resulted in product quality defects such as reduced volumes, harder textures, darker product colours and altered sensorial characteristics, ultimately reducing consumer acceptance [3]. In reference to breadmaking, increased inclusion of native BSG has resulted in higher water absorptions for flours; increased dough development times; increased crumb hardness; and reduced loaf volume [155]. Waters et al. [145] also concluded harder bread crumbs; increased chewiness; lower bread specific volumes; and increased staling kinetics with BSG inclusion when compared to wheat flour,

with higher levels of inclusion resulting in more significant effects. Thus, inclusion of BSG in a food matrix is challenging and additional processing aids are required to enhance the utilisation of BSG as a food ingredient.

In a review carried out by Lynch et al. [3], BSG was highlighted as a promising substrate for microbial fermentation, with carbohydrates and proteins at the forefront of its composition. LAB fermentation as a bioprocessing technique has sparked interest, in a bid to improve nutritional, techno-functional and sensory properties of food processing side-streams [156]. The technology has been applied across a wide variety of side streams/raw materials as an upcycling technique, some of which include wheat-milling side streams; maize-milling side streams; surplus bread; apple by-products; and legumes [156,157,166,167,158–165]. With regard to the exploitation of LAB fermentation in BSG, various studies have found successes with fermentation technology, improving the sensory experience, altering techno-functional characteristics of the applications, extending product shelf lives; enhancing product digestibility; and also, positively impacting the gut microbiome (*in vitro*). Waters et al. [145] found inclusion of a sourdough BSG up to 10% inclusion reduced crumb hardness and increased crumb springiness compared to native BSG and a wholemeal control. Similarly, a study conducted by Aprodu et al. [155] concluded inclusion of sourdough fermentation of BSG significantly improved bread loaf volume and decreased bread crumb hardness compared with unfermented BSG, even at up to 20% inclusion. From a sensory perspective, Ktenioudaki et al. [154] observed changes in the BSG bread aromas with inclusion of sourdough BSG, attributing this to the liberation of amino acids necessary for volatile compound formation. In addition, phytic acid was found to be reduced in the sourdough BSG breads and likely increasing the bioavailability of minerals [154]. Verni et al. [168] documented an enhanced antioxidant potential of BSG post fermentation and xylanase treatment, distinguished by higher radical scavenging activity; extended inhibition of linoleic acid oxidation; and enhanced defence against oxidative stress on human keratinocytes NCTC 2544. Moreover, the use of fermented BSG in pasta increased protein digestibility; reduced the glycaemic effect of the pasta (*in vitro*); and alleviated some of the sensory defects associated with BSG inclusion [169]. Additionally, a positive effect on the gut microbiome was noted *in vitro* with fermented BSG (fermented using *Weissella confusa* A16) incorporated into a wheat bread matrix, owing the positive influence as a result of the dextrans and oligosaccharides present in BSG after LAB fermentation [170].

Thus, BSG represents an attractive source of nutrition for the human food chain. However, inclusion in a food matrix poses challenges, negatively impacting on the techno-functional characteristics of the food product, especially at higher levels of inclusion. Consequently, a deterioration of food quality will result in reduced consumer acceptance. However, fermentation represents a promising valorisation tool for BSG rejuvenation which could improve food product quality and aid in consumer acceptance. Thus, the following chapters (3 & 4), explore the effects induced by two processed BSG ingredients, namely BSG and FBSG, on pasta and bread matrixes. The studies comprehensively examine the fundamental effects of BSG and FBSG inclusion on a variety of features of the cereal matrixes, which aids in fully understanding the effects of BSG inclusion on the technological aspects of these cereal matrixes and how LAB fermentation may alter the effects.

2.8 Conclusion

Increased focus has been placed on the recycling of food processing by-products, for applications in food and other industries, to enhance sustainability. BR and BSG are produced in large volumes each year as a by-product of the malting, brewing and distilling industry, but their primary use to date has been as animal feed. In reference to BR, evidence suggests a promising potential for BR to be used in food products and fermentations due to their nutritive value, but also as sources of enzymes, antioxidants and in biochar production. The use of BR as a nutrient-rich food ingredient may be of great interest in the future, with its high fibre content and interesting protein quality. Although studies are limited, successes have mainly been observed in their ability to enhance the nutritive value of cereal-based products, but increased attention and knowledge of their potential may provoke more investigations into their use in other food products. However, a key element which needs to be considered and addressed is the quality and safety of the BR, because evidence suggests that mycotoxins are prevalent. A system to regulate the quality of rootlets will be necessary to monitor this and ensure that a safe food ingredient is produced. A need for this has been stressed previously in relation to the consumption of rootlets and other brewing by-products in animals [11]. Thermal food processing and controlled storage conditions of BR may be an option to counteract this challenge. In regard BSG, a rather well explored area fundamentally, the inclusion of BSG in the human diet is of great interest due to the appealing nutritional composition. However, as conclusively highlighted, inclusion of BSG in food matrixes faces challenges with quality defects often observed particularly at higher levels of inclusion. Nevertheless, LAB fermentation technology has the potential to minimise quality defects and improve the sensory experience, techno-functional characteristics, and digestibility of BSG food products.

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Chapter 3

FUNDAMENTAL STUDY OF THE APPLICATION OF BREWERS SPENT GRAIN AND FERMENTED BREWERS SPENT GRAIN ON THE QUALITY OF PASTA

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3.1 Abstract

Upcycling and repurposing of side streams from food processing have become a necessity to merge our world into a more sustainable future. Brewers spent grain (BSG) is a highly abundant and nutrient rich by-product of the brewing industry. The aim of this study was to investigate the effect of fermentation on BSG (FBSG) while also examining the effects of including fibre rich BSG and FBSG ingredients on techno-functional and nutritional properties of semolina-based pasta. The gluten network formation, starch gelatinisation, texture, cooking loss, optimal cooking time, *in vitro* starch digestibility and ultrastructure of the pasta was investigated. BSG and FBSG inclusion weakened gluten network properties versus the semolina control but was more favourable than the wholemeal control. Addition of BSG and FBSG produced pasta with a greater nutritional profile, having a higher fibre content and lower predicted glycaemic index compared to semolina pasta. BSG and FBSG addition enhanced tensile strength and pasta firmness versus wholemeal pasta. An increased reduction in the predicted glycaemic index was noted with FBSG inclusion at the higher level of addition compared to BSG, suggesting fermentation of BSG may further enhance nutritional properties of the BSG ingredient.

3.2 Introduction

Brewers' Spent Grain (BSG) represents approximately 85% of the total by-products produced from brewing. Following beer production, on average, about 100 kg-130 kg of wet BSG (water content of approx. 80%) is generated from 100 kg of malt [10]. The increase in mass compared to malt is due to the high water content of BSG. BSG has attracted considerable attention due to the vast quantities of waste associated with it. The current primary use of BSG is animal feed; however, increased awareness of the nutritional profile of BSG has sparked investigation of its potential use as a food ingredient [141].

BSG is a lignocellulosic material rich in cellulose, hemicellulose, lignin, proteins, minerals and a low level of fat [3]. The composition of BSG can vary. Variations in BSG composition may be due to differences in barley grain type; malt type; grain cultivation; brewing process and equipment; the stage in brewing at which BSG is collected; and the location at which the BSG sample is taken from the filter cake as protein, fat and fibre contents are not evenly distributed [143]. In addition, some brewing processes may incorporate other cereal adjuncts within their process, and remnants of these adjuncts may also be present in BSG [141,142]. However, fibre and protein are the predominant fractions in BSG [3,171]. Protein constitutes approximately 19-30% of BSG, while fibre (cellulose, hemicellulose, lignin) represents 30-50% of the BSG composition [141]. The hemicellulose fraction of BSG is mainly comprised of arabinoxylans, which can be present at levels of up to 40%. The arabinoxylans consist of a xylose backbone with substituted arabinose residues and ferulic acid esterified to the arabinose residues [3].

Evidence exists to link fibre consumption with helping in controlling body weight, type-2-diabetes, and possibly lowering the risk of developing some cancers and coronary heart disease [67]. With fibre holding a large proportion of the composition of BSG, it is of interest to incorporate into the human diet. Previous attempts have been made to incorporate BSG into food products, such as snack foods, bread and pasta [145,149,152,172]. Improvements in nutritional profiles of foods have been noted, particularly in relation to the increase in fibre [172].

Fermentation of foods and ingredients has previously enhanced features such as sensory, shelf life, functionality and nutritional properties [11,173,174]. Successes have been found with BSG and brewers spent grain sourdough supplemented in wheat bread, with BSG fortified breads showing more favourable outcomes than the wholemeal control [145]. Fermentation

improved textural properties of the bread and proved acceptable by a sensory panel up to a 10% addition level [145].

A review carried out by Lynch et al., [3] highlights BSG as a suitable material for inclusion in cereal-based products while also being an attractive substrate for fermentation. The aim of this study was to determine the effects of fermentation on BSG at a molecular level and investigate the effects of the inclusion of BSG and fermented brewers spent grain (FBSG) ingredients in pasta formulations. Semolina and wholemeal flour were used as controls throughout the study. Analysis focussed on the effects of increasing fibre contents of pasta using BSG and FBSG ingredients; with ingredients added to pasta formulations according to Regulation (EC) No 1924/2006 [175], where fibre levels present in the final pasta product had 3 g/100g (Source of Fibre) and 6 g/100g (High in Fibre).

3.3 Materials and methods

3.3.1 Raw Materials

Semolina (East End Foods PLC, West Bromwich, UK) and stone grinded Wholemeal flour (WM) (Odlum Group, Dublin, Ireland) were used as control flours for this experiment. Salt (Glacia British Salt Limited, Cheshire, UK) and tap water were also incorporated into pasta recipes. Milled and spray-dried BSG and FBSG were produced and provided by Anheuser-Busch (Anheuser-Busch InBev, Leuven, Belgium). FBSG was produced according to patent number WO/2018/033521 [176].

3.3.2 Compositional Analysis of Raw Materials

Compositional analysis for semolina, WM, BSG and FBSG were performed by Concept life Sciences Ltd (Bar Hill, UK). Protein was determined using the Dumas principle (conversion factor= 6.25); moisture was evaluated using oven drying (105 ºC) for a minimum of 16 h; fat was determined using low resolution proton nuclear magnetic resonance; ash content was calculated by oxidation at 550 ºC to remove organic matter, leaving the mineral residue. Total carbohydrates were calculated by difference; sugars were determined on hot water extraction of the sample by ion chromatography with pulsed amperometric detection using a gold electrode and a calibration against an internal standard. Dietary fibre values for semolina and wholemeal flours were analysed in accordance with AOAC method 991.43. The dietary fibre values for BSG and FBSG were provided by the supplier, Anheuser Busch (Leuven, Belgium) and were determined according to AOAC method 2011.25**.** Digestible and resistant starch values of the ingredients were measured using the Megazyme kit K-RAPRS (Bray, Ireland). Total starch was calculated as the sum of digestible starch and resistant starch. Starch analysis was performed on cooked freeze-dried pasta and calculated based on moisture content of cooked vs freeze-dried pasta samples.

3.3.3 Alpha-amylase and Beta-amylase activity of fibre ingredients

The alpha-amylase activity of the ingredients was determined using the alpha-amylase assay kit (ceralapha method) supplied by Megazyme (Bray, Co. Wicklow, Ireland). Beta-amylase activity was determined using K-BETA3 assay kit also supplied by Megazyme.

3.3.4 Protein Profile Analysis

The protein profile of BSG and FBSG were analysed to investigate the effect of the fermentation process on proteins present in BSG. An Agilent Bioanalyzer 2100 Lab-on-a-Chip capillary electrophoresis system was used to analyse the protein profile and estimate molecular

weights of the samples. Samples were prepared according to Amagliani et al. [177], with slight modifications: ingredients were dispersed in 2% SDS, 2 M thiourea, and 6 M urea, to give a protein concentration of 2% w/v. Dispersions were shaken for 2 hours at room temperature and then centrifuged to remove insoluble material. Samples were analysed using an Agilent Protein 80 kit and Protein 230 kit according to the instructions within the ranges of 5–80 and 14–230 kDa, respectively. The protein 230 kit did not show any differences; hence data not shown. For stronger reducing conditions, Dithiothreitol (DTT) was included in the sample buffer according to kit instructions.

3.3.5 Addition levels of the fibre ingredients to pasta formulas

Inclusion of fibre was adjusted in accordance with "source of fibre" (SF) and "high in fibre" (HF) claims [175], referring to cooked pasta. The claim applies to the final food product; therefore, BSG and FBSG were adjusted with uptake of water by the pasta during cooking considered. Water uptake was calculated by determining the difference in moisture content between raw and cooked pasta formulations. Moisture was measured using Moisture Analyser LJ16 (Mettler Toledo, Ohio, US). Fibre ingredient additions (Table 5) were calculated based on the water taken up and adjusted to reach 3g/100g and 6g/100g claims.

Table 5. Pasta recipes expressed as percentage-based on flour, "Source of Fibre" (SF) and "High in Fibre" (HF) recipes shown. BSG represents Brewers Spent Grain and FBSG represents Fermented Brewers Spent Grain

	Control Semolina	**Control Wholemeal**	**BSG (SF)**	**FBSG (SF)**	**BSG (HF)**	**FBSG (HF)**
Flour	100.00	100.00	97.50	98.00	85.04	87.84
Ingredient	-	-	2.50	2.00	14.96	12.16
Salt	0.50	0.50	0.50	0.50	0.50	0.50
Water	30.00	36.50	30.00	30.00	36.52	36.52

3.3.6 Impact of fibre ingredients on gluten network

Analysis of gluten aggregation in the flours was investigated using GlutoPeak (Brabender GmbH and Co KG, Duisburg, Germany). 9 g of sample (based on 14% moisture) was added to deionised water (36 ºC) to a total volume of 18 g in the device sample cup. Flour blends endured a hand premixing step to ensure a homogenous blend was added to the deionised water. The sample slurries were subjected to high shear (2750 rpm: 36 ºC).

3.3.7 Effect of fibre ingredients on starch pasting properties

Pasting temperature, peak viscosity, final viscosity, and breakdown values were measured using a Rapid Visco Analyser (RVA Super 3, Newport Scientific, Warriewood, Australia). Three grams of the solid sample (based on 14% moisture) was added to deionised water to a total volume of 28 g. Flour blends were premixed before addition to water. The samples were mixed at a constant shear rate (160 rpm), and a temperature profile was applied as reported by Horstmann et al., [178].

3.3.8 Pasta Preparation

Recipes for pasta production are illustrated in Table 5. For each formulation, a total dough volume of 1 kg was prepared. Dry ingredients were premixed using a Kenwood chef mixer (Kenwood Ltd., New Hampshire, UK) with a K-beater for 2 mins. An adjusted volume of tap water (30 ºC) was added and mixed for 10 mins. For fibre enriched recipes, the amount of water added was adjusted by adding water at different levels to obtain an optimal crumbly dough consistency. The dough was transferred to a single screw extruder (PN 300 extruder, Haussler, Heiligkreuztal, Germany) equipped with a spaghetti die (internal diameter 2mm). Pasta samples of a length of 20 cm were produced. Fresh pasta was used in the analysis.

3.3.9 Pasta Characterisation

Analysis of each batch of fresh pasta was conducted on the same day of production.

3.3.9.1 Optimal Cooking Time

Optimal cooking time (OTC) is the time (mins) it takes for the core of the spaghetti strand to gelatinise fully. OTC is measured as the time it takes for the spaghetti core to become opaque when pressed between two glass slides and was determined according to AACC Approved Method 16–50 [179], as reported by Hager et al., [180]. This was performed before texture parameters of the pasta were analysed.

3.3.9.2 Cooking Loss

Cooking loss (CL) indicates the content of dry matter lost from the pasta during cooking, with a low cooking loss desired. This was determined using AACC Approved Method 16–50, as previously reported by Hager et al., [180].

3.3.9.3 Texture properties of cooked pasta

Firmness, tensile strength and stickiness were analysed on cooked pasta using a TA.XT*plus* texture analyser (Stable Micro Systems, Godalming, Surrey, UK) set with a 5 kg load cell.

The pasta firmness represents the resistance the pasta strand exhibits to a force and indicates the degree of the "al dente" mouthfeel. Firmness was determined according to the AACC spaghetti firmness method 66-52.01 and expressed as max cutting force (N). Firmness of the pasta was determined using the heavy-duty platform with a light knife blade and transparent Perspex plate (Stable Micro Systems, Godalming, Surrey, UK). Five spaghetti strands were aligned parallel on the centre of the texture analyser platform with a perspex blade attached. A trigger force of 0.05 N, test speed 0.17 mm/sec and a 4.5 mm distance were the testing parameters used. The test was repeated five times for each pasta batch produced.

Tensile strength reveals the elasticity of pasta strands and is defined as the resistance to uniaxial extension (expressed as maximum breaking strength). This was measured using the tension test A/SPR spaghetti/noodle tensile rig with a trigger force of 0.05 N, a test speed of 3 mm/sec and a 100 mm distance (Stable Micro Systems, Godalming, Surrey, UK). The analysis was performed on 10 strands of pasta strands (10 cm) per batch.

Pasta stickiness is an indication of the cooking quality of pasta, with excessive stickiness being undesired. It is defined as the max peak force (N) when the probe is retracted from the sample and was recorded using the pasta stickiness rig (HDP/PFS, Stable Micro Systems, Godalming, Surrey, UK). Five spaghetti strands were aligned in the centre of the raised platform of the texture analyser, under a rectangular aluminium probe, held by a plate with a rectangular opening. Test parameters included a trigger force of 0.2 N, test speed 0.5 mm/sec and distance of 25 mm. The analysis was repeated 10 times per batch produced.

3.3.9.4 Scanning Electron Microscopy (SEM)

Freeze-dried pasta was mounted on stubs (G 306; 10 mm x 10 mm Diameter; agar scientific, UK) and fixed using carbon tape (G3357N; Carbon Tabs 9 mm; agar scientific, UK). Mounted pasta samples were sputter coated with a gold-palladium alloy (ratio of 80/20), using a Polaron

E5150 sputter coating unit, and imaging was captured with a JEOL Scanning Electron Microscope (JSM-5510, Jeol Ltd., Tokyo, Japan). Settings were implemented as follows: 5 kV voltage, 20 mm working distance and a magnification factor of 1000.

3.3.10 *In vitro* starch digestibility as an indication of glycaemic index

In vitro starch digestibility determination is based on enzymatic degradation of digestible starch to reducing sugars over time.

An *in vitro* digestion assay for fibre enriched products was conducted as reported by Brennan & Tudorica [181]. Samples endured proteolytic treatment using a pepsin solution, followed by a 5 h incubation with pancreatic α-amylase solution within a dialysis tube. The amount of reducing sugars (maltose) released from the dialysis tubing system into the buffer was determined spectrophotometrically using 3,5-dinitrosalicylic acid (DNS) solution. Samples were taken every 30 min. 100 µl DNS was added to 100 µl of the sample taken, heated on a dry heating block at 100 °C for 15mins and diluted with 1 ml of distilled water. The absorbance at wavelength 546 nm was determined. All analysis was completed in duplicate. The reducing sugar release (RSR); the maltose diffusion in presence of the sample (DIFF sample); and the sugar diffusion index (SDI) were determined as reported by Brennan & Tudorica [181]. The predicted glycaemic index (pGI) was calculated using the following formula:

$$GI_{predicted} = 105.52 \times \frac{Fibre}{Carbohydrate} - 76.46 \times \frac{protein}{carbohydrate} + 1.23 \times RSRI_{at150\,min} + 69.41 \times SDI_{at\,270\,min} - 83.87 \quad (1)$$

3.3.11 Statistical Analysis

All experimental analysis was carried out in triplicate unless stated otherwise. One-way ANOVA test using a Tukey test ($p \leq 0.05$) was performed using Minitab version 19 (Minitab LLC., State College Pa.). Correlation analysis was carried out using Microsoft Excel.

3.4 Results & Discussion

3.4.1 Compositional Analysis of main ingredients used.

Results from compositional analysis of the ingredients used in the analysis are represented in Table 6.

Table 6. Compositional results of the flour ingredients incorporated in experimental analysis. "WM", "BSG" and "FBSG" denoting for wholemeal flour, brewers spent grain flour and fermented brewers spent grain flour, respectively.

Component	**Semolina**	**WM**	**BSG**	**FBSG**	**Literature values for BSG**
Protein	13.2	11.4	31.4	32.4	14.2 - 31.0
Moisture	11.7	12.0	4.7	5.0	n.m.
Fat	1.3	1.6	10.3	6.53	3.0 - 13.0
Ash	1.0	1.3	3.7	3.7	1.2 - 4.6
Total carbohydrate by difference	72.8	73.7	49.9	52.37	n.m.
Of which dietary fibre	5.0	7.1	42.6	49.4	Total Fibre 48.22
Of which sugars	1.4	1.2	0.2	2.9	n.m.
Beta-amylase (cu/g)	49.30 ± 0.09^{a}	35.38 ± 0.35^{b}	3.36 ± 0.01^{c}	3.73 ± 0.20^{c}	n.m.
Alpha-amylase (cu/g)	0.18 ± 0.01^{b}	0.12 ± 0.02^{c}	0.12 ± 0.00^{c}	0.24 ± 0.00^{a}	n.m.
Starch Analysis					
Total Starch	62.88 ± 0.37^{a}	55.55 ± 2.65^{b}	2.31 ± 0.05^{c}	3.75 ± 0.06^{c}	1 - 12
Digestible Starch	56.77 ± 0.40^{a}	48.32 ± 3.02^{b}	1.34 ± 0.04^{c}	2.47 ± 0.02^{c}	n.m.
Resistant Starch	6.11 ± 0.01^{b}	7.22 ± 0.37^{a}	0.97 ± 0.01^{c}	1.27 ± 0.04^{c}	n.m.

Values expressed in g/100g. N.m.= not measured. Literature values sourced from Lynch et al., [3] and Waters et al., [145].

3.4.1.1 Protein content

Protein content for semolina was 13.2%. Semolina (made from durum wheat) is the preferred raw material for pasta making, and protein levels measured for semolina in this study were similar to previous findings [182,183]. WM flour had a lower protein content (11.4%), which could be attributed to differences in wheat variety or cultivar [184,185]. BSG contained 31.4% protein. BSG is naturally high in protein, and levels of protein measured in this study were similar to protein concentrations previously reported (Table 6). The protein content of FBSG (32.4%) was slightly higher than the protein level measured in BSG and could be linked with the combined effect of batch variations of BSG and potential differences in the point at which the BSG sample was collected from the filter cake, as protein contents can vary within the filter cake [143]. However, the difference in protein concentration was minimal and was comparable with the level of protein expected (Table 6).

3.4.1.2 Protein profile

Figure 6 illustrates the results of the protein profile analysis of BSG and FBSG. The main proteins found in BSG are hordeins [186]. The hordeins may be separated into subunits, A hordeins <20kDa, B hordeins 35-50kDa, C hordeins 55-80kDa and D hordeins 96kDa based on previous publications [186–188]. Differences were observed in the size of the proteins present in BSG versus FBSG; indicating the fermentation process influenced the protein profile. FSBG contained a greater amount of low molecular weight proteins (particularly in the range of ~ 15 – 46 kDA) than BSG, likely due to proteolysis during the fermentation process. DTT addition induces stronger reducing conditions to ensure breakup of inter/intra disulphide bonds in proteins. DTT addition for BSG and FBSG resulted in higher amounts of low molecular weight protein versus without DTT addition, indicating BSG and FBSG proteins consist of smaller subunits. The enhanced luminous intensity in the lower region of FBSG with DTT addition indicates a greater number of smaller molecular weight proteins were present after fermentation and may be influencing outcomes in gluten network formation discussed in later sections (section 3.4.2).

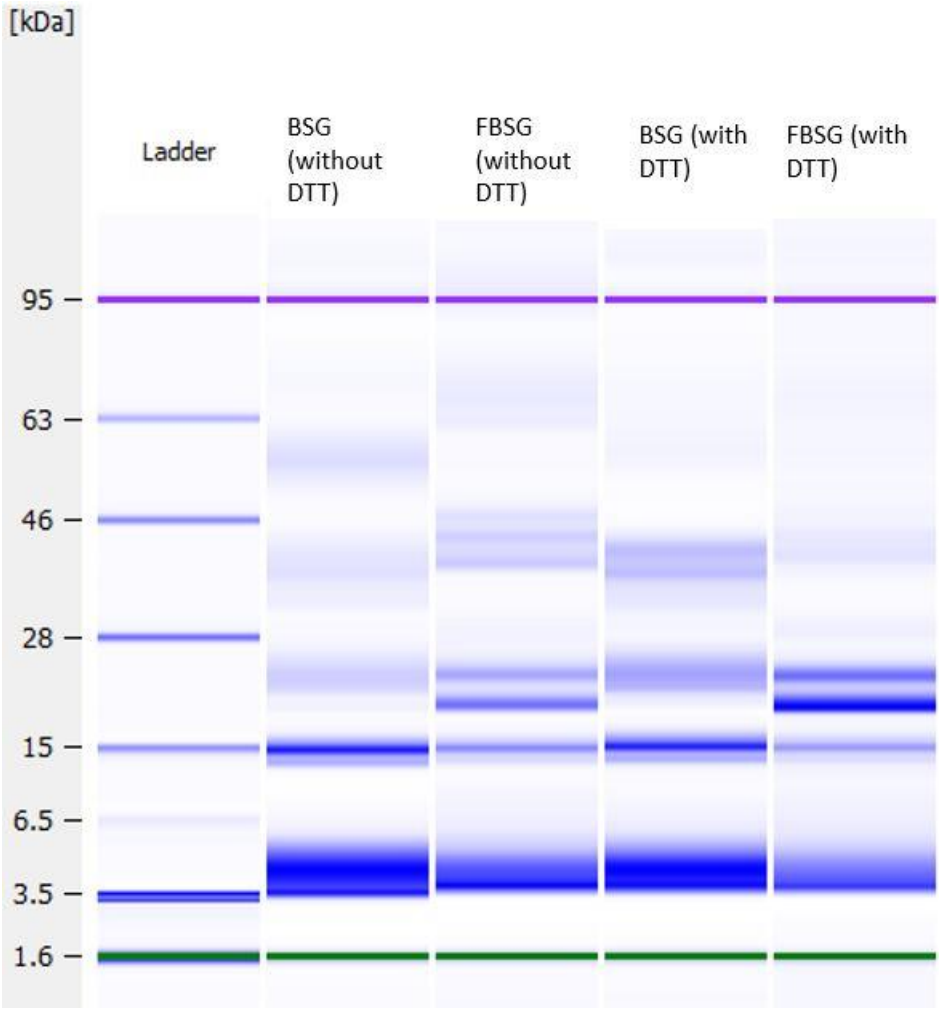

Figure 6. Protein profiles for brewers spent grain (BSG) and fermented brewers spent grain (FBSG) with and without DTT, in the range of 5-80kDa.

3.4.1.3 Minerals

Ash content for WM flour and semolina were 1.3% and 1%, respectively. Higher levels of minerals were present in BSG (3.7%) and FBSG (3.7%). BSG and FBSG are comprised of the outer layers of the barley grain (pericarp, seed coat and husk material), where minerals are concentrated in grains, hence the high levels present [5]. Levels of minerals present in BSG were in line with literature values for BSG (Table 6). Fermentation of BSG had no effect on mineral contents. However, some of the minerals in FBSG may be more bioavailable post fermentation [189]. Lactic acid bacteria produce lactic acid during fermentation, which creates

an acidic environment and enhances phytase activity. This contributes to the reduction of phytates, making more minerals available for absorption [189–191].

3.4.1.4 Fat

Semolina had the lowest fat content (1.3%) followed by WM flour (1.6%). Fat contents of BSG and FBSG were 10.3% and 6.53% respectively. Lipid content for BSG and FBSG were within range of previously reported values for BSG (Table 6). The variances in fat content observed between BSG and FBSG could be due to batch-to-batch variations in the brewing process and potential differences in BSG sample collection from the lauter tun. Lipid contents can be inhomogeneous within the filter cake in brewing and may account for the differences in fat observed [143].

3.4.1.5 Carbohydrates

3.4.1.5.1 Sugars

Sugar levels reported for semolina and WM were 1.4% and 1.2%, respectively. Sugar levels in BSG (0.2%) were low. Sugars are lost to the wort during the mashing process in brewing [192]; hence the very low levels found**.** An increased level of sugars was reported for FBSG (2.9%) in comparison to BSG (0.2%), which may be linked to the combined hydrolysis and fermentation process employed for FBSG production. Fibres and starch are degraded during this process, which liberates small chain polysaccharides and monosaccharides [193,194].

3.4.1.5.2 Dietary Fibre

Semolina contained 5% dietary fibre, while WM flour had a dietary fibre value of 7.1%. WM flour contains higher levels of dietary fibre than semolina due to the increased prevalence of the bran and germ layers in the flour. Dietary fibre levels in BSG (42.6%) and FBSG (49.4%) were significantly higher than the control flours. BSG is naturally high in fibre, namely insoluble fibre [145], with arabinoxylans being the predominant fibre present [3,195]. A higher dietary fibre content was observed in FBSG (49.4%) than BSG (42.6%). The differences observed in dietary fibre content may be attributed to batch variation of BSG or potential differences in BSG sample collection [143]. However, the combination of microbial enzymes and the mixture of enzymes added to FBSG may solubilise some dietary fibre in FBSG vs BSG, particularly in relation to the arabinoxylans [196].

3.4.1.5.3 Starch Analysis

Total starch levels are reported for semolina and WM in Table 6. Starch levels in BSG (2.31 $\pm$ 0.05%) and FBSG (3.75 $\pm$ 0.06%) were much lower. BSG consists of the outer layers of the

barley grain which include minimal levels of starch [3]. Starch is lost to the wort during mashing; therefore, a low level of starch was expected. Starch values recorded were in line with previous findings for BSG in Table 6 [3]. A high proportion of the starch present in BSG and FBSG was resistant starch. The term resistant starch refers to the starch, which is not broken down in the small intestine but rather slowly fermented in the large intestine. In BSG, 41.9% of the total starch was resistant starch, while in FBSG, 33.9% of the total starch was resistant starch. Variances in starch levels observed in the BSG ingredients could be linked with diversities found in BSG composition due to sample collection [143] as well as batch variations of BSG.

3.4.1.6 Alpha and Beta amylase results

Alpha and beta-amylase activities for semolina and WM are outlined on Table 6. The slightly higher amylase activity in semolina vs WM flour could be linked with some sprouting occurring which tends to increase amylase activity [197]. Minor differences in beta amylase activity indicated the fermentation did not have a major impact on residual beta-amylase activity. FBSG contained almost double the amount of alpha-amylase (0.24 $\pm$ 0.00 CU/g) than BSG (0.12 $\pm$ 0.00 CU/g). This is likely due to the addition of alpha-amylases during the fermentation process and the potential production of amylases from lactic acid bacteria during fermentation [198].

3.4.2 Impact of fibre ingredients on gluten network development

The incorporation of fibre-rich ingredients affected the torque maximum (TM) and the peak maximum time (PMT) of the gluten network development in semolina-based pasta (Figure 7).

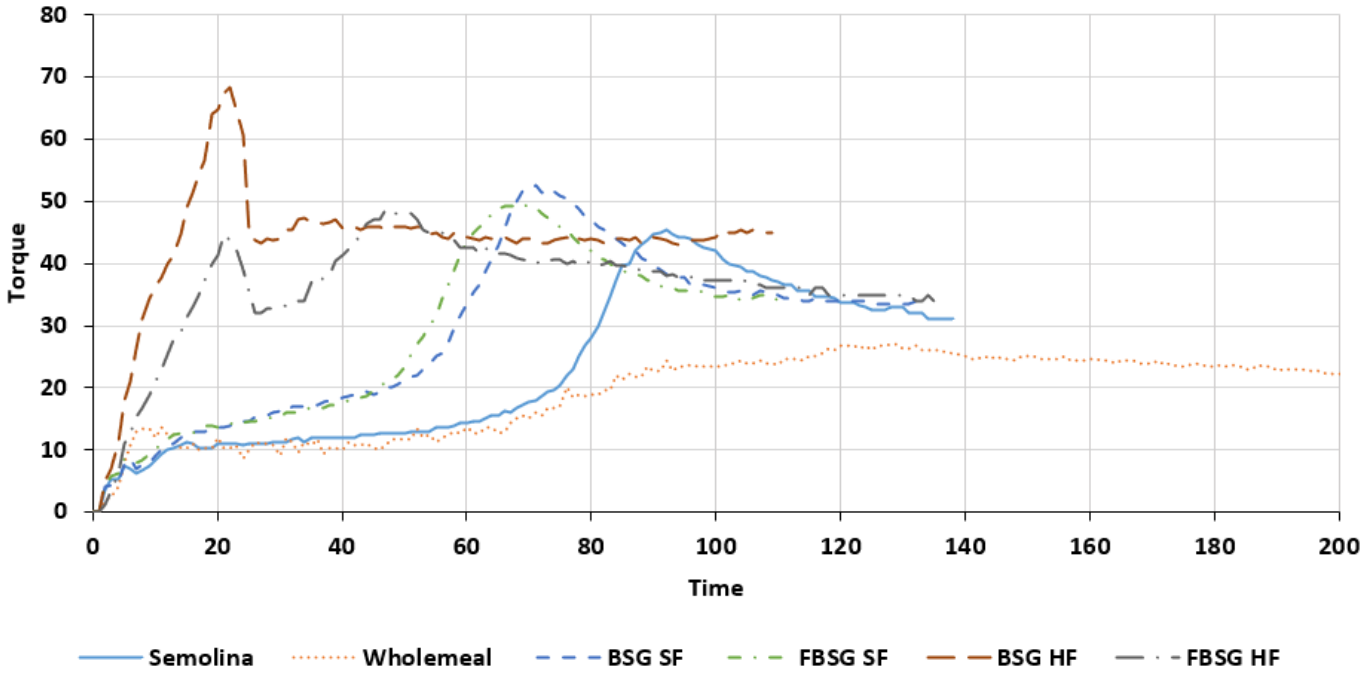

Figure 7. Graphical representation of GlutoPeak results from controls and flour mixtures with brewers spent grain (BSG) and fermented brewers spent grain (FBSG) at source of fibre (SF) and high in fibre (HF) addition levels.

Comparing the controls with each other, semolina showed a continuous increase in torque reaching a TM at 45 BU after 91.3 $\pm$ 0.6 sec, while WM resulted in a slower increase in average torque with a TM at 27.7 $\pm$ 1.2 BU after 126 $\pm$ 7.5 sec. The significantly weaker gluten network occurred due to the presence of coarse bran particles which interfered with the gluten network development [199–201].

The replacement of semolina with BSG and FBSG to achieve SF claim led to a significantly faster and stronger gluten network development than the semolina control, with FBSG causing the fastest development (65.3 $\pm$ 6.1 sec). Furthermore, an increase in TM was observed in samples including BSG (52 $\pm$ 1 BU) and FBSG (52.3 $\pm$ 0.6 BU). The ingredients BSG and FBSG contain a significant amount of proteins (Table 6), which amplified the development of a protein network, mainly by their charged amino acids [145], resulting in a stronger network. Moreover, BSG and FBSG are rich in minerals (Table 6). Minerals induce a charge screening

effect and the exposure of apolar protein residues, which causes stronger hydrophobic interaction in the protein and leads to increased aggregation [202].

The higher addition level of fibre ingredients resulted in curves which were not comparable with any of the control flours. The inclusion of BSG at HF level showed a pronounced peak after 21 sec, followed by a steady torque at 45 BU. The ratio of glutenin's to gliadins is known to be a factor in determining the strength of gluten network [203]. Semolina flour from durum wheat contains a higher proportion of gliadins, which results in a slightly weaker gluten network [182,204]. Melnyk et al. [205] reported an increase in gluten strength with increasing levels of glutenin inclusion. The inclusion of BSG at the higher level of addition is likely to be shifting the balance of glutenin and gliadins present, enhancing the glutenin proportion and causing an increase in torque.

The replacement of semolina by FBSG resulted in two peaks at the HF addition level. An initial torque of 43 BU was reached after around 21 seconds, followed by a TM of 50.3 $\pm$ 0.6 BU after 45 $\pm$ 3 seconds. The presence of two peaks indicates additional protein aggregation, other than gluten, which occurred at a different time. FBSG includes 32.40% of proteins (Table 6), which underwent modification during the fermentation process, including proteolysis (Fig 6) and changes in tertiary structure due to the drop in pH post lactic acid production. Gluten aggregation is hindered in acidic conditions, and alterations in charges facilitates the formation of new secondary bonds [206]. In addition, these modified protein/peptides may show differences in solubility compared to gluten, which also affects the protein aggregation [40] and contributes to the formation of two peaks during the measurement.

3.4.3 Starch pasting properties

Utilisation of fibre rich ingredients BSG and FBSG in semolina-based pasta formulations influenced starch pasting properties (Table 7).

Table 7. Rapid Visco Analyser, GlutoPeak and pasta characterisation results for "source of fibre" (SF) and "high in fibre" (HF) recipes. BSG and FBSG represent brewers spent grain and fermented brewers spent grain, respectively. WM indicates wholemeal control. Values are given as the average + standard deviation. No significant difference occurred between values in the same row which share the same letter ($p < 0.05$).

	Semolina	**WM**	**BSG SF**	**FBSG SF**	**BSG HF**	**FBSG HF**
Rapid Visco Analyser						
Peak visc. (cP)	789 ± 33.6^{a}	599 ± 33.3^{c}	685 ± 12.1^{b}	701 ± 7.0^{b}	431 ± 14.2^{d}	322 ± 25.4^{e}
Breakdown (cP)	101.0 ± 7.6^{b}	101.0 ± 2.0^{b}	91.6 ± 4.2^{b}	134.0 ± 6.6^{a}	41.7 ± 3.5^{c}	104.3 ± 5.9^{b}
Final visc. (cP)	1527 ± 66.5^{a}	$1317 \pm 28.0^{b,c}$	1403 ± 31.0^{b}	1253 ± 18.6^{c}	967 ± 20.1^{d}	540 ± 25.9^{e}
Paste Temp (°C)	$62.1 \pm 5.8^{a,b}$	73.7 ± 6.8^{a}	$60.7 \pm 8.9^{a,b}$	$64.2 \pm 0.5^{a,b}$	$61.8 \pm 5.5^{a,b}$	50.7 ± 0.4^{b}
GlutoPeak						
Peak Max Time (sec)	91.3 ± 0.6^{b}	126.0 ± 7.5^{a}	72.0 ± 2.0^{c}	65.3 ± 6.1^{c}	21.6 ± 0.6^{d}	45.0 ± 3.0^{e}
Torque Maximum (BEM)	45.0 ± 0.0^{c}	27.7 ± 1.2^{d}	52.0 ± 1.0^{b}	52.3 ± 0.6^{b}	67.3 ± 3.1^{a}	50.3 ± 0.6^{b}
Pasta Characterisation						
Total Average Fibre in Cooked pasta (%)	3.08	4.25	3.68	3.51	6.44	6.12
Optimal Cook time (mins)	5.5 ± 0^{e}	4.0 ± 0^{f}	6.0 ± 0^{d}	6.5 ± 0^{c}	7.0 ± 0^{b}	7.0 ± 0^{a}
Cook Loss (%)	5.44 ± 0.82^{a}	5.20 ± 0.96^{a}	4.95 ± 0.45^{a}	5.14 ± 0.17^{a}	4.88 ± 0.39^{a}	5.44 ± 0.68^{a}
Firmness after cooking (N)	2.17 ± 0.37^{bc}	1.47 ± 0.25^{d}	2.27 ± 0.40^{abc}	2.54 ± 0.38^{ab}	2.62 ± 0.65^{a}	1.85 ± 0.16^{cd}
Tensile Strength (N)	0.29 ± 0.03^{a}	-	0.27 ± 0.03^{a}	0.24 ± 0.04^{b}	0.16 ± 0.04^{c}	0.15 ± 0.03^{c}
Stickiness (N)	4.79 ± 0.40^{a}	5.23 ± 0.71^{a}	4.26 ± 0.99^{a}	5.04 ± 0.73^{a}	3.46 ± 0.59^{b}	4.64 ± 0.78^{a}
Predicted Glycaemic Index	55.09 ± 1.41^{a}	38.99 ± 5.30^{bc}	46.86 ± 3.86^{ab}	50.50 ± 2.44^{ab}	27.42 ± 0.73^{cd}	18.57 ± 1.52^{d}
Resistant Starch (DWB g/100)	1.00 ± 0.00^{b}	0.99 ± 0.04^{b}	1.20 ± 0.02^{a}	1.04 ± 0.04^{b}	0.80 ± 0.04^{c}	1.02 ± 0.03^{b}
Digestible starch (DWB g/100)	69.47 ± 0.65^{a}	61.01 ± 0.06^{b}	68.07 ± 3.2^{b}	68.47 ± 0.26^{a}	59.22 ± 1.61^{b}	61.32 ± 0.73^{b}
Total Starch (DWB g/100)	70.47 ± 0.66^{a}	61.99 ± 0.02^{b}	69.27 ± 3.2^{a}	69.50 ± 0.21^{a}	60.02 ± 1.64^{b}	62.34 ± 0.77^{b}

DWB represents Dry weight basis. (–) denotes "not measurable". No significant difference in values was found between values in the same row which share the same letter ($p<0.05$).

As a general trend, a reduction in peak and final viscosities was noted upon inclusion of the fibre ingredients. This is consistent with previous findings [207,208]. Peak viscosity values represent the level of water taken up by starch granules in the presence of heat and shearing. Semolina exhibited the highest peak viscosity (789 ± 33.6 cP). WM had a significantly lower peak viscosity (599 ± 33.3 cP) than semolina due to the increased prevalence of bran particles in WM, which have a higher water-binding capacity and compete with starch for hydration [209,210]**.** The addition of BSG and FBSG significantly decreased the peak viscosity. The higher the fibre addition level the lower the peak viscosity (Table 7). Semolina is replaced by low starch, high fibre BSG and FBSG ingredients; therefore the amount of starch present to absorb water and contribute to viscosity is lower in these formulations [208,211]. The peak viscosity for FBSG HF (322 ± 25.4 cP) was significantly lower than BSG HF peak viscosities, putatively due to the increased level of alpha-amylase activity in the FBSG ingredient (Table 6), which hydrolyses the starch polysaccharides and causes a further reduction to viscosity [212].

Similar trends were observed for the final viscosity, which represents the level of starch retrogradation and paste formation. Final viscosity tends to increase with increasing levels of starch [213]. The higher the inclusion level of BSG or FBSG the lower the amount of retrogradation (BSG (SF: 1403 ± 31.0 cP; HF: 967 ± 20.1 cP) and FBSG (SF 1253 ± 18.6 cP; HF: 540 ± 25.9 cP)). Again, semolina was replaced by low starch, high fibre ingredients which dilutes the starch available to retrograde during analysis. Collar et al., [208] suggested the increased fibre concentration negatively influences the intermolecular association which occurs in the starch network upon cooling via physical disruption; interference in secondary forces; and sterical hindrance. The higher level of amylase activity in the FBSG ingredient is likely to be influencing the lower final viscosities in FBSG formulations compared to BSG. Alpha-amylases have an anti-retrogradation effect and delay the rate of starch retrogradation [214,215]. However, the exact mechanism of how this effect occurs is somewhat unclear [216].

Breakdown values indicate the extent of amylose leaching from starch granules during heating and shearing. The breakdown values for semolina (101 ± 7.6 cP), WM (101 ± 2 cP), BSG SF (91.6 ± 4.2 cP) were comparable with no significant differences observed. At the HF addition level for BSG, a significantly lower breakdown value was recorded (41.7 ± 3.5 cP) due to the greater reduction in starch present; therefore a lower level of amylose leaching occurred [208]. Interestingly, the FBSG ingredient showed a different trend to the BSG ingredient at both inclusion levels. Both breakdown values for FBSG SF (134 ± 6.6 cP) and FBSG HF (104.3 ±

5.9 cP) were significantly higher in comparison to BSG. The increased amylase activity and the resulting starch hydrolysis products produced in the fermented formulations is likely to be a contributing factor to the greater breakdown values observed during heating and shearing.

Starch paste temperatures occur at the onset of the sharp increase in suspension viscosity upon heating. Increases have been noted in paste temperatures with fibre inclusion and were attributed to the restrictive nature of fibre inclusion on swelling and amylose leaching [208]. However, in this study, paste temperatures for all formulations were aligned with the semolina paste temperature (62.1 °C). This indicates the addition of fibre ingredients BSG and FBSG did not have a major effect on starch pasting temperatures at either addition level.

3.4.4 Effect of fibre ingredient addition on pasta structure

Analysis of pasta ultrastructure was performed on cooked pasta, which are represented in Figure 8.

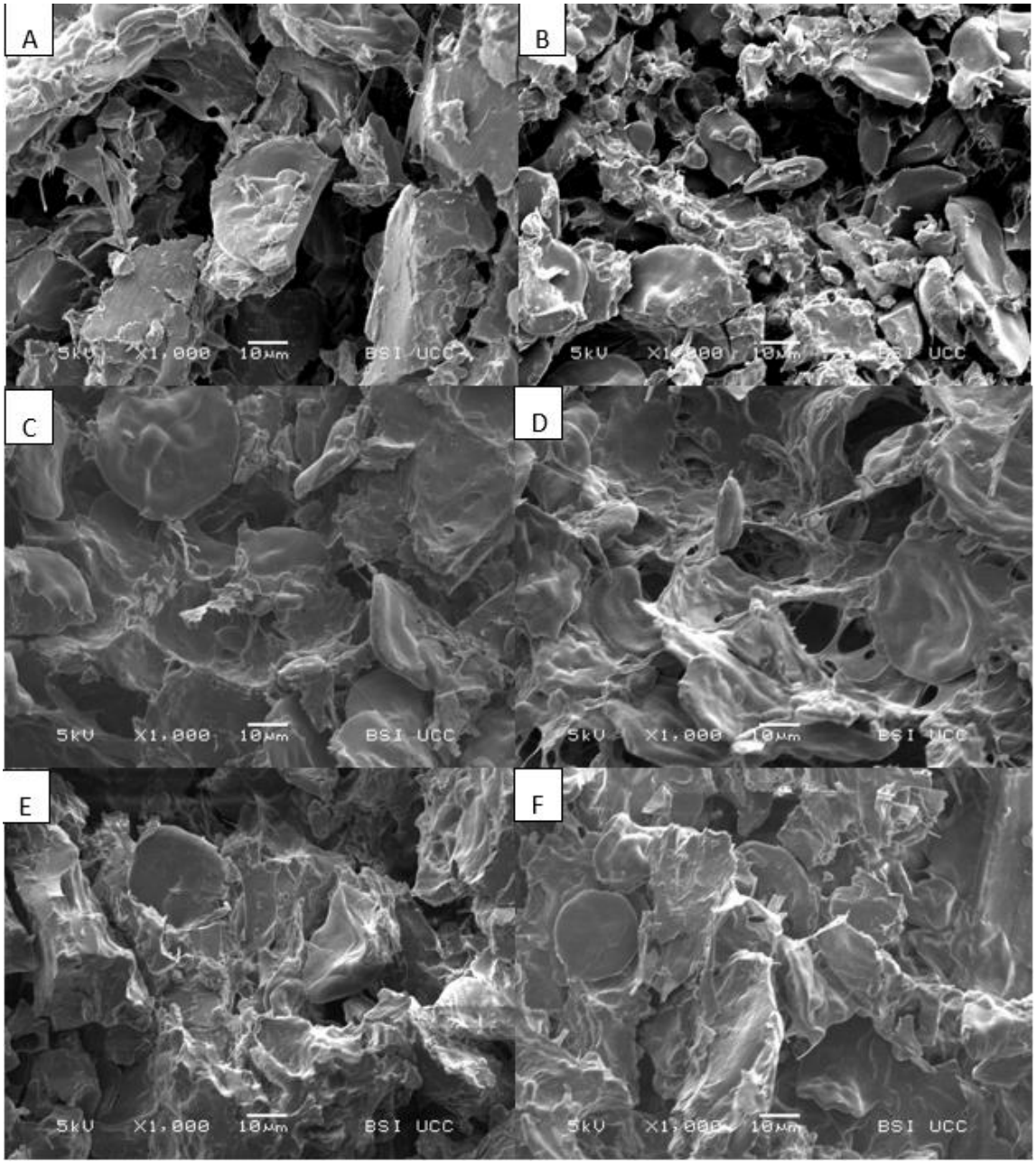

Figure 8. Ultrastructure of cooked pasta samples. Image A-F represents semolina (A), wholemeal (B), brewers spent grain "source of fibre" (C), fermented brewers spent grain "source of fibre" (D), brewers spent grain "high in fibre" (E) and fermented brewers spent grain "high in fibre" (F) pasta formulations, respectively.

Semolina pasta (Fig 8.A) contains gelatinised starch granules along with a well-integrated protein matrix. This is consistent with previous reports [217,218]. WM pasta (Fig 8.B) has exposed starch granules and lacks the prevalence of string-like gluten structures, which is also reflected in the weak gluten network highlighted during GlutoPeak analysis. The introduction of bran and germ particles from WM flour caused a disruption to gluten network formation, thus effecting it's continuity [199,219].

BSG SF (Fig 8.C) and FBSG SF (Fig 8.D) pasta showed the gluten string-like structures similar to those in semolina pasta. This also coincides with the GlutoPeak analysis in these pasta recipes. However, a different trend was observed in relation to the starch granules. The starch granules appear to have a layer surrounding them, creating a gel-like structure. This gel like layer is amplified in micrographs for BSG HF (Fig 8.E) and FBSG HF (Fig 8.F) due to the increased addition level. BSG contains arabinoxylans [3], which have the unique capability to crosslink and form a gel-like structure when sufficient concentration are present [48,220]. The gel-like layer/aggregates observed in BSG and FBSG ultrastructure could be due to interactions between arabinoxylan chains. BSG HF and FBSG HF also lack the distinct gluten structures putatively due to the presence of the arabinoxylans, which negatively affect gluten formation through a physical effect (increasing viscosity and depleting protein interactions) and a chemical mediated effect (interactions between ferulic acids) [201]. The similarity in SEM micrographs for BSG and FBSG indicates fermentation of BSG did not have a major impact on pasta ultrastructure.

3.4.5 Impact of fibre ingredients on pasta properties

The effect of fibre fortification on semolina pasta using BSG and FBSG was investigated by evaluating pasta characteristics, such as tensile strength, firmness, stickiness, optimal cooking time and cooking loss (Table 7).

3.4.5.1 Tensile strength

Tensile strength for semolina pasta was 0.29 $\pm$ 0.03 N, which was comparable to previous reports [217,221]. WM pasta tensile strength was immeasurable. The WM pasta strands broke whilst attempting to conduct the measurement, highlighting the weak structure of the pasta. This was due to the physical disruption of the large bran and germ particles within the gluten network, which had a negative effect on the continuity of the gluten network and is reflected in SEM images.

The tensile strength of BSG SF pasta (0.27 $\pm$ 0.03 N) was not significantly different to the semolina control. A reduction in tensile strength was observed for FBSG SF (0.24 $\pm$ 0.04 N), BSG HF (0.16 $\pm$ 0.04 N) and FBSG HF (0.15 $\pm$ 0.03 N). Tudorică et al. [217] and Brennan et al. [222] also found a reduction in tensile strength with addition of inulin and guar gum. The lower tensile strengths in these formulations are potentially due to the presence of the arabinoxylans in the BSG and FBSG ingredients. Arabinoxylans hinder gluten properties negatively, making it less extensible [201], hence the negative effects observed in the elasticity of the pasta. The relatively comparable tensile strength recorded for BSG and FBSG pasta indicates fermentation did not influence the elastic properties of the pasta.

3.4.5.2 Firmness

Torque values from GlutoPeak analysis correlated positively with firmness values for the final pasta ($r=0.871$, $p\leq0.03$), suggesting gluten network strength influenced the firmness of the final pasta (Table 7).

Semolina pasta had a firmness after cooking value of 2.17 $\pm$ 0.37 N, while WM pasta had a significantly lower firmness value (1.47 $\pm$ 0.25 N). This aligns with previous reports [219,223]. The lower firmness value is likely to be linked with the weaker gluten network formed (GlutoPeak), which allows for a more open, porous structure (SEM) and contributes to the reduction in pasta firmness.

The firmness after cooking of BSG SF (2.27 $\pm$ 0.40 N) and FBSG SF (2.54 $\pm$ 0.38 N) pasta were marginally higher than the semolina control. This is likely due to the stronger gluten network formed (GlutoPeak) and the increase in protein content with inclusion of high protein

ingredients BSG and FBSG. Enhanced protein contents have previously been linked with increased pasta firmness [219,224]. BSG HF pasta had a significantly higher firmness value (2.62 $\pm$ 0.65 N) than the semolina control. Again, the stronger gluten network (GlutoPeak) is likely to be influencing this, as well as the further increase in protein concentration with higher levels of BSG. The incorporation of FBSG at the high fibre level decreased the pasta firmness (1.85 $\pm$ 0.16 N) compared to BSG, indicating the fermentation of BSG reduced pasta firmness. This could be due to the variations observed in gluten aggregation properties (GlutoPeak) which negatively impacted the firmness of the pasta.

3.4.5.3 Stickiness

Pasta stickiness has been associated with starch pasting properties [225]. Furthermore, a strong positive correlation was found with breakdown values from RVA analysis and pasta stickiness ($r=0.9$, $p \leq 0.02$), indicating the level of amylose leaching during cooking influences the stickiness of the final pasta. Additionally, GlutoPeak torque values and stickiness in pasta correlated positively ($r=0.825$, $p \leq 0.05$), suggesting gluten network strength also affects the stickiness of the final pasta (Table 7).

Stickiness of semolina pasta (4.79 $\pm$ 0.4 N) and WM pasta (5.23 $\pm$ 0.7 N) were not significantly different. Similar stickiness values were obtained for BSG SF (4.26 $\pm$ 0.99 N) and FBSG SF (5.04 $\pm$ 0.73 N) pasta. An increase in BSG addition showed a significantly reduced pasta stickiness (3.46 $\pm$ 0.59 N) putatively due to the lower amount of starch available to gelatinise, the stronger gluten network and the lower level of amylose leaching. The stickiness value for FBSG HF pasta (4.64 $\pm$ 0.78 N) was significantly higher than the stickiness of BSG HF pasta. This result coincides with the enhanced breakdown values observed in RVA trials. Chamberlain et al. [226], found an increase in crumb stickiness in bread with increased alpha-amylase activity and production of high molecular weight dextrins. The higher amylase activity in FBSG may enhance the production of starch degradation products such as dextrins which may increase the stickiness of the surface of the pasta. Additionally, the alterations in protein network formation (GlutoPeak) with FBSG inclusion could negatively influence pasta structure and allow for a greater amount of amylose to leach onto the pasta surface compared to BSG HF pasta. However, the stickiness of the FBSG HF pasta was not significantly different to the semolina control.

3.4.5.4 Optimal Cooking Time

Changes were observed in optimal cooking time (OCT) with the inclusion of fibre ingredients. A strong positive correlation was noted in OCT and torque values ($r=0.9$, $p \leq 0.02$), as well as OCT and PMT values ($r=0.96$, $p \leq 0.03$) from GlutoPeak analysis. This indicates the strength and speed of gluten formation influences the optimal cooking time of the pasta.

Semolina pasta had an OCT of 5.5 $\pm$ 0 mins. A shorter OCT was noted in WM pasta (4 $\pm$ 0 mins). These results are in agreement with previous findings [219,223,227]. The reduction in OCT may be attributed to the disruptive nature of the bran and germ particles in WM flour to the protein network. This provides a clear pathway for water to enter the spaghetti core gelatinise the starch and reduce OCT [219].

Inclusion of BSG and FBSG ingredients increased OCT in comparison to the semolina control. BSG SF and FBSG SF had an OCT of 6 $\pm$ 0mins and 6.5 $\pm$ 0 mins, respectively. A further increase in OCT was noted with inclusion of higher levels of BSG and FBSG ingredients, with both BSG HF and FBSG HF pasta having an OCT of 7 $\pm$ 0 mins. This most likely occurred due to the stronger gluten networks formed in these pasta formulations. Conflicting results have been noted in literature with both increases and decreases in OCT found with fibre addition. Variations in OCT have been attributed to pasta structure and gluten network formation [228–231]. The inconsistent results suggest OCT may be reliant on fibre type and gluten network formation. The similarity in OCT for BSG and FBSG pasta formulations indicate fermentation of BSG did not influence the cooking quality of the pasta.

3.4.5.5 Cooking Loss

Cooking loss has been linked with pasta structure and the ability of the protein network to retain amylose [219,229].

No significant differences were observed in cooking loss between semolina (5.44 $\pm$ 0.82%) and WM (5.20 $\pm$ 0.96%) pasta. This is in agreement with Manthey & Schorno [219] and Vignola et al. [227]. The addition of BSG or FBSG did not significantly influence the cooking loss, regardless of the inclusion level (BSG SF (4.95 $\pm$ 0.45%), FBSG SF (5.14 $\pm$ 0.17%), BSG HF (4.88 $\pm$ 0.39%), FBSG HF (5.44 $\pm$ 0.68%)). Aravind et al. [228], also found similar cooking losses with semolina pasta and semolina pasta substituted with pollard (up to 30 %). Pasta with FBSG showed a marginally higher cooking loss than BSG pasta which may be associated with the slightly higher level of amylose leaching in FBSG formulations, reflected in the higher breakdown values.

3.4.6 Impact of fibre ingredient on Glycaemic Index

Inclusion of different fibre ingredients, both soluble and insoluble, have previously shown to be capable of reducing the predicted GI of pasta products, with increasing levels of fibre added having a greater effect [181,222]. Values for predicted GI analysis are illustrated in Table 7.

Semolina pasta had a predicted GI value of 55.09 ± 1.41, which is slightly higher than previous reports [181] but was still within range of the expected GI for pasta [232]. WM pasta had a significantly lower predicted GI value (38.99 ± 5.30) than semolina, putatively due to the lower level of digestible carbohydrates and higher fibre concentration in the pasta (Table 7), which reduces GI values [181].

Predicted GI values for BSG SF (46.86 ± 3.86) and FBSG SF (50.50 ± 2.44) did not differ significantly from the semolina control, most likely due to similar level of available carbohydrates in these pasta formulations (Table 7). BSG HF (27.42 ± 0.73) and FBSG HF (18.57 ± 1.52) pasta had significantly lower predicted GI values than the semolina control, which may be due to the dilution effect of digestible starch with increasing levels of fibre added (Table 7). The differences observed in BSG HF and FBSG HF predicted GI values indicate fermentation of BSG had a greater effect in reducing the predicted GI. Further reductions in starch hydrolysis with inclusion of fermented ingredients versus an unfermented ingredient has previously been noted [164,166]. This may be due to the combined effect of the slightly higher level of resistant starch (1.3% in BSG HF vs 1.6% in FBSG HF pasta) as well as the presence of lactic acid in the fermented ingredient. Östman et al. [233], investigated the possible mechanisms responsible for the lower availability of starch for amylolysis in bread and concluded the presence of lactic acid during heat treatment promotes interactions between starch and gluten and reduces the bioavailability of starch.

3.5 Conclusion

The incorporation of spray-dried BSG and FBSG ingredients improved the nutritional properties of semolina pasta in several aspects. In comparison to the semolina control, the addition of BSG and FBSG created a pasta with an improved nutritional profile by achieving a high fibre claim; and further reducing the predicted glycaemic index of the pasta produced. Furthermore, the addition of BSG and FBSG showed a stronger gluten network formation compared to the wholemeal control, resulting in pasta with improved techno-functional properties such as a stronger tensile strength and firmness. Additionally, fermentation of BSG further improved the predicted glycaemic index of HF pasta. This study highlights the excellent potential of upcycling BSG, the main brewing by-product, to produce highly nutritious pasta and potentially further improve pasta nutritional quality using fermented BSG.

3.6 Acknowledgements

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Chapter 4

FERMENTATION AS A TOOL TO REVITALISE BREWERS SPENT GRAIN AND ELEVATE TECHNO-FUNCTIONAL PROPERTIES AND NUTRITIONAL VALUE IN HIGH FIBRE BREAD

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4.1 Abstract

Recycling of by-products from the food industry has become a central part of research to help create a more sustainable future. Brewers' spent grain is one of the main side-streams of the brewing industry, rich in protein and fibre. Its inclusion in bread, however, has been challenging and requires additional processing. Fermentation represents a promising tool to elevate ingredient functionality and improve bread quality. Wheat bread was fortified with spray-dried brewers' spent grain (BSG) and fermented brewers' spent grain (FBSG) at two addition levels to achieve "source of fibre" and "high in fibre" claims according to EU regulations. The impact of BSG and FBSG on bread dough, final bread quality and nutritional value was investigated and compared to baker's flour (BF) and wholemeal flour (WMF) breads. The inclusion of BSG and FBSG resulted in a stronger and faster gluten development; reduced starch pasting capacity; and increased dough resistance/stiffness. However, fermentation improved bread characteristics resulting in increased specific volume, reduced crumb hardness and restricted microbial growth rate over time. Additionally, the inclusion of FBSG slowed the release in reducing sugars over time during in vitro starch digestion. Thus, fermentation of BSG can ameliorate bread techno-functional properties and improve nutritional quality of breads.

4.2 Introduction

Brewers spent grain (BSG) has been labelled as the most abundant side-stream generated by the brewing industry and accounts for approximately 85% of the total by-products produced [171]. BSG is a lignocellulosic material comprised of the outer layers of the barley grain, namely the husk-pericarp-seed coat [171]. It is rich in dietary fibre (30-50%), mainly arabinoxylan, and protein (19-30%), contains low levels of fat and starch, as well as vitamins and minerals [3]. However, variations in the composition of BSG are common [3,141], which can be associated with numerous factors such as differences in barley variety, harvesting conditions, malt type, the adapted brewing process and the addition of adjuncts during brewing, the point at which BSG is retrieved from the brewing process and also where in the filter cake the BSG sample is retrieved [141–143,171]. The primary outputs for BSG are in animal nutrition and landfill. However, numerous alternative uses for BSG are emerging [3,141,171,194,234]. As BSG is considered a highly nutritious raw material, increased attention has been given to the use of BSG as an ingredient in different food products to elevate their nutritional value and pursue the goal of a more sustainable future.

The demand for high fibre foods has increased due to consumers' awareness of the health benefits associated with the intake of dietary fibre, such as reducing the risk of cardiovascular disease [67], lowering cholesterol [235] and preventing the development of colorectal cancer [236]. Even though the demand for foods rich in dietary fibre has increased, most people following a Western diet fail to meet the recommended daily intake of 25 g fibre [237]. With fibre holding a significant proportion of BSG composition, its use as a fibre fortifier in food in the future is of high interest. Previous studies implementing BSG into food such as bread [144,145,154,155,238], pasta [172,239], breadsticks [153], crisp-slices [147], cookies [240], extruded snacks [149,152] and meat [148], have shown promising outcomes with increasing fibre contents of foods. Although BSG elevates the nutritional value of the food products, it affects the techno-functional characteristics of the systems, particularly in bread, leading to a poor quality bread regarding the specific volume and crumb texture [145,155]. However, great successes have been observed in relation to nutritional and techno-functional properties of both bread and pasta with the inclusion of two refined BSG ingredients, EverVita Fibra and EverVita Pro, which highlights the potential of BSG as a food ingredient after additional processing [241,242].

Fermentation with lactic acid bacteria (LAB) has proven to be a valuable tool in compensating for quality loss of bread systems in various studies, leading to positively enhancing some technological aspects, such as extending microbial shelf life [174], improving dough quality [243], reducing staling [145,244] and increasing specific volume [244]. In addition to the technological benefits, improved flavour profiles [11], reduced glycaemic responses [159], and enhancements in antioxidant activity [168] have also been observed. Previous studies incorporating BSG in bread formulations in the form of sourdough fermentation reported a positive impact on bread crumb hardness [145,155], an increase in bread specific volume [155] and also altering the sensory profile of the breads [145,154]. Hence, fermentation technology represents a promising approach to overcome quality losses in cereal-based products fortified with BSG. In a previous study, the incorporation of fermented BSG in pasta showed superior product quality regarding technological and nutritional characteristics compared to wholemeal pasta [245].

The current study reveals the effect of partial replacement of wheat flour with spray-dried BSG (BSG) and spray-dried fermented BSG (FBSG) on dough rheology and techno-functional, structural, and nutritional characteristics of bread. BSG and FBSG were included in bread formulations in two different concentrations to achieve a ‘source of fibre’ and a ‘high in fibre’ health claims according to EU Regulation (EC) No 1924/2006 [175]. Baker’s wheat flour (BF) and wholemeal flour (WMF) were used as controls throughout the study.

4.3 Materials and methods

4.3.1 Raw Materials

Flour ingredients incorporated in bread recipes include: baker's flour (BF) supplied by Odlums Group, Dublin, Ireland; stone ground wholemeal flour (WMF) from Odlums Group, Dublin, Ireland; milled and spray-dried brewers spent grain (BSG); and milled and spray-dried fermented brewers spent grain (FBSG). BSG and FBSG ingredients were provided by Anheuser-Busch InBev SA/NV (Leuven, Belgium). FBSG was produced using a patented process (patent number: WO 2018/033521 A1) [176] using a combined saccharification and fermentation process using lactic acid bacteria *Lactiplantibacillus plantarum* f10 and/or *Lacticaseibacillus rhamnosus* GG (LGG®). Compositional analysis of ingredients used in the study are provided on Table 8. WMF, BSG and FBSG composition on Table 8 have been reported in a complimentary study (chapter 3) [245]. BF compositional analysis was completed by an accredited laboratory (Concept Life Sciences Ltd, Bar hill,UK). Alpha-amylase activity was determined in duplicate using the alpha-amylase assay kit (ceralpha method) supplied by Megazyme (Bray, Co. Wicklow, Ireland). Other ingredients used in bread recipes include instant active dried baker's yeast *Saccharomyces cerevisiae* (Puratos, Groot-Bijgaarden, Belgium), sugar (Siúcra, Dublin, Ireland), salt (Glacia British Salt Limited, Cheshire, UK), sunflower oil (Musgraves, cork, Ireland) and tap water. All chemicals used in experiments were purchased from Sigma Aldrich (St. Louis, Missouri, USA). The addition of BSG and FBSG was adjusted in accordance with "source of fibre" (SF) and "high in fibre" (HF) claims [175]. This claim applies to the final food product, implying the final food product contains 3g/100g fibre (SF) and 6g/100g fibre (HF).

Table 8. Compositional analysis of baker's flour (BF), wholemeal flour (WMF), brewers spent grain (BSG) and fermented brewers spent grain (FBSG) flour ingredients in g/100g.

	BF	**WMF**	**BSG**	**FBSG**
Protein	12.9	11.4	31.4	32.4
Moisture	12.6	12.0	4.7	5.0
Fat	0.86	1.6	10.3	6.5
Ash	0.70	1.3	3.7	3.7
Total Carbohydrate	72.9	73.7	49.9	52.37
Of which dietary fibre	3.1	7.1	42.6	49.4
α-amylase (cu/g)*	0.12 $\pm$ 0.01	0.12 $\pm$ 0.02	0.12 $\pm$ 0.00	0.24 $\pm$ 0.00

* Cu/g = ceralpha units / g flour. One unit represents the amount of enzyme needed to release 1 µmol of *p*-nitrophenol per min at 40 °C (in the presence of excess α-glucosidase) [246].

4.3.2 Dough Analysis

4.3.2.1 Water content adjustment

Farinograph-TS® (Brabender GmbH and Co KG, Duisburg, Germany), equipped with an automatic water dosing system (Aqua inject), was used to determine the water addition level of the different formulations. The target consistency was set to 500 $\pm$ 20 FU and the temperature of the kneading chamber was 30 °C. The formulations included the controls BF and WMF, as well as the blends of BF and BSG/FBSG in the proportions demonstrated in Table 9.

Table 9. Bread recipes expressed as % based on flour + fibre ingredient (=100%). BF and WMF represents Baker's flour and Wholemeal Flour, respectively. SF and HF represent "source of fibre addition level" and "high in fibre addition level", respectively. BSG and FBSG denotes "brewers spent grain" and "fermented brewers spent grain", respectively.

Ingredient	BF	WMF	BSG (SF)	FBSG (SF)	BSG (HF)	FBSG (HF)
Baker's flour	100	-	95.0	96.0	82.0	85.0
Wholemeal	-	100	-	-	-	-
Fibre ingredient	-	-	5.0	4.0	18.0	15.0
Salt	1.2	1.2	1.2	1.2	1.2	1.2
Sugar	2.0	2.0	2.0	2.0	2.0	2.0
Sunflower oil	3.2	3.2	3.2	3.2	3.2	3.2
Dry Yeast	2.0	2.0	2.0	2.0	2.0	2.0
Water	57.3	59.3	61.6	60.1	68.6	66.9

- represents "not applicable"

4.3.2.2 Gluten Aggregation analysis

GlutoPeak (Brabender GmbH and Co KG, Duisburg, Germany) was used to determine the quality of the gluten network. Before analysis, flour blends according to the flour/ingredient proportions given in Table 9 were prepared. Flour blends were premixed to ensure homogeneity before analysis. 9 g of sample (based on 14% moisture) was added to deionised water (36 °C) to a total volume of 18 g and the test was started using a shear speed of 2750 rpm. The chamber temperature was set to 36 °C. Torque was monitored over time (s). The

torque maximum (TM) in Brabender Units (BU), and the peak maximum time (PMT) in seconds (s) were evaluated.

4.3.2.3 Starch pasting properties

Starch pasting behaviour was analysed using a Rapid Visco Analyser (RVA) (RVA Super 3, Newport Scientific, Warriewood, Australia). Therefore, blends of BF and BSG/FBSG were prepared according to the inclusion level illustrated in Table 9. Three grams of sample (based on 14% moisture) was added to 25 g of deionised water in an aluminium cup. Before the test, the sample was dispersed in the water using the RVA-paddle. A temperature profile was applied under constant shear of 160 rpm, starting at 50 ºC for 60 s, followed by heating to 95 ºC at a heating rate of 0.2 ºC/s, holding the temperature for 162 s, cooling to 50 ºC at a cooling rate of 0.2 ºC/s and holding the final temperature for 120 s. Parameters analysed include peak viscosity, breakdown viscosity and trough viscosity and final viscosity in centipoise (cP).

4.3.2.4 Bread dough preparation

Bread doughs were produced by mixing the dry ingredients first, followed by the addition of yeast solution and sunflower oil. Yeast solution was prepared by adding instant active dried yeast in water (25 ºC) and allowing to activate for 10 min. After the addition of the liquids, the ingredients were mixed (MACPAN MX 10 spiral mixer, MACPAN SNC, Thiene, Italy) at speed 1 for 6.5 min, followed by a second stage of mixing at speed 2 for 5 min.

4.3.2.5 Evaluation of bread fermentation quality

The fermentation quality of each bread dough was analysed using a Rheofermentometer (Chopin, Villeneuve-la-Garenne Cedex, France). Three hundred grams of bread dough (prepared according to section 4.3.2.4) was placed into the fermentation chamber and a 1500 g cylindrical weight was placed on top of the dough. The chamber was closed, and the dough was left to ferment for 3 h at 30 °C. The maximum dough height (Hm) in mm, volume of CO_2 produced during fermentation in ml and CO_2 retention coefficient in % was evaluated.

4.3.2.6 Dough rheology

Viscoelastic properties of doughs (prepared according to section 4.2.2.4) were analysed using a Rheometer Physica MCR 301 (Anton Paar GmBH, Ostfildern, Germany). Yeast was omitted. Serrated plates were placed in parallel geometry. The lower plate was held at 35 °C throughout the analysis, accompanied by an upper plate of 50 mm in diameter. Dough samples were loaded onto plates and the linear viscoelastic region was determined using an amplitude sweep as described by Hager et al. [247]. Frequency sweeps were performed using a constant strain of

0.01% and a frequency range from 100 – 0.1 Hz (data obtained at angular frequency 2.58 Hz). Prior to analysis, dough samples were left to rest for 5 mins to allow for equilibration. The damping factor ($\tan\delta\ \frac{G''}{G'}$) was evaluated to investigate the extent of changes in viscoelastic properties of dough samples with fibre ingredient addition.

4.3.3 Bread Production

Bread dough was produced following section 4.3.2.4. A total dough volume of 2500 g was divided into five 450 $\pm$ 1 g pieces, moulded, transferred to greased tins, and proofed in a proofing chamber (KOMA SunRiser, Roermond, The Netherlands) for 90 min at 35 °C and 75 % humidity. After proofing, the bread loaves were baked in a deck oven (MIWE Condo, Arnstein, Germany) for 35 min at 220/230 °C top/bottom temperature. Before loading, 400 ml of steam was injected into the oven, leaving the draft open throughout baking. Following baking, bread loaves were left to stand for 1 h to cool before analysis. Each recipe outlined in Table 9 was baked and analysed in triplicate. Values reported represent the mean of three independent baking trials.

4.3.4 Bread Analysis

4.3.4.1 Bake loss

Bake loss was determined to investigate the amount of water lost due to baking. The bake loss of five bread loaves per batch was measured. This was calculated in percentage according to the following formulas:

$$\text{Weight of dough before baking (g)} - \text{Weight of bread after baking (g)} = \text{Moisture lost from bake (g)} \quad (2)$$

$$\frac{\text{Moisture lost from bake (g)}}{\text{Weight of dough before baking (g)}} \times 100 = \text{Bake loss \%} \quad (3)$$

4.3.4.2 Specific Volume

Specific volume was calculated using a Volscan Profiler (Stable Micro Systems, Surrey, UK), measured in ml/g. Two loaves per batch were analysed.

4.3.4.3 Crumb Structure

Analysis of bread crumb structure was completed using a C-Cell Imaging System (Calibre Control International Ltd, Warrington, UK). Two loaves per batch were sliced into 25 mm thick slices. The crust slices were omitted from the analysis and only centre slices (five per loaf) were considered. The C-Cell Imaging System was used to provide numerical data on slice area, number of cells and cell diameter.

4.3.4.4 Texture

Crumb texture was determined using a TA-XT2i Texture Analyser (Stable Micro Systems, Surrey, UK) equipped with a 25 kg load cell. A two-compression test with a strain of 40%, test speed of 5 mm/s, a trigger force of 0.05 N and a waiting time of 5 s between the two compressions was chosen. A 35 mm cylindrical probe was used in the analysis. Bread slices with a thickness of 25 mm were measured and the crumb hardness and the crumb resilience was analysed on the day of baking. Crumb hardness is calculated as the maximum force of the 1st compression and crumb resilience was calculated by dividing the upstroke energy of the first compression by the down stroke energy of the first compression. Bread staling was determined by measuring the crumb hardness over five days. The staling rate was determined as reported by Sahin et al. [248].

4.3.4.5 Colour

Crumb and crust colour were determined using a hand-held colorimeter (Minolta CR-331, Konica Minolta Holdings Inc., Osaka, Japan). The CIE L*a*b* colour system was used for colour evaluation. The differential colour index (ΔE) was determined according to the below equation to evaluate the changes in the colour of the crust and crumb with BSG and FBSG inclusion.

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (4)$$

Where $\Delta L^* = L^*_{control} - L^*_{sample}$, $\Delta a^* = a^*_{control} - a^*_{sample}$ and $\Delta b^* = b^*_{control} - b^*_{sample}$

4.3.4.6 Water activity and microbial shelf life

Water activity was determined using the water activity meter AquaLab series 3 (Decagon Devices Inc., Pullman, Washington, USA). The influence of fibre ingredients on microbial shelf life was analysed using the mould environmental challenge method indicated by Dal Bello et al. [244] and Sahin et al. [249] with slight modifications. Briefly, ten centre slices of 25 mm thickness (two bread loaves) per batch were placed on a sterile metal rack. The bread crumb of

both sides of the bread was exposed to the environment for 5 min. Bread slices were packed singly in sterile bags and heat-sealed. A filter pipette was placed in each bag to allow for consistent aerobic conditions to prevail. Bread samples were stored at 20 $\pm$ 1 °C and 50% relative humidity in a sterilised and temperature-controlled chamber (KOMA SunRiser, Roermond, The Netherlands) for 14 days. Mould growth of each bread slice was visually analysed daily, and mould growth was rated as "mould free", "mould growth < 10%", "10-24% mould growth", "25-49% mould growth" and "mould growth > 50%".

4.3.4.7 *In vitro* starch digestibility

An *in vitro* digestion assay based on enzymatic degradation of starch to reducing sugars over time designed for fibre enriched products was conducted as reported by Brennan and Tudorica [181]. Briefly, 4 g of crushed bread samples were exposed to a 30 min proteolytic treatment using pepsin solution. After this, samples were placed in 1-inch width dialysis tubing, suspended in sodium potassium phosphate buffer (pH 6.9) and incubated for 5 h with a pancreatic α-amylase solution. Samples were taken every 30 min and dialysis tubing were inverted every 15 min. To determine the amount of reducing sugars (maltose) released over time spectrophotometrically (wavelength 546 nm), 100 µl of the samples taken were diluted with 100 µl of 3,5-dinitrosalicyclic acid, heated to 100 °C for 15 min and then diluted with 1 ml of deionised water. Analysis was completed in duplicate. Reducing Sugar Release % (RSR%) overtime was calculated according to Brennan and Tudorica [181] using the following formula:

$$\mathrm{RSR\%} = \frac{\mathrm{A_{sample}} \times 500 \times 0.95}{\mathrm{A_{maltose}} \times \text{available carbohydrate}} \times 100 \quad (5)$$

Where: A_{sample} represents the active sample absorbance at 546 nm; 500 (ml) displays the solution total volume; 0.95 is the maltose to starch conversion factor, $A_{maltose}$ indicates the absorbance of 1 mg of pure maltose/ ml buffer; and available carbohydrate (in mg) represents sugar and digestible starch present in 4 g of sample. Available carbohydrate values were determined using the digestible carbohydrate values measured using the Megazyme kit K-RAPRS (Bray, Ireland). The starch digestibility is presented as the release of reducing sugars

over time in the form of a plot. The slope of the curves was calculated using Microsoft Excel after ensuring a linearity ($r^2 > 0.99$).

4.3.4.8 Bread Microstructure

Bread samples were freeze-dried, mounted on stubs (G 306; 10 mm x 10 mm Diameter; Agar scientific, Stansted, UK) and fixed using carbon tape (G3357N; Carbon Tabs 9 mm; Agar scientific, Stansted, UK). Mounted bread samples were sputter-coated with a gold-palladium alloy (ratio of 80: 20), using a Polaron E5150 sputter coating unit, and imaging was captured with a JEOL Scanning Electron Microscope (JSM-5510, Jeol Ltd., Tokyo, Japan). Settings for analysis were as follows: 5 kV 185 voltage, 20 mm working distance and a magnification factor of 1000.

4.3.5 Statistical Analysis

Analysis was conducted in triplicate unless stated otherwise. A one-way ANOVA with post hoc pairwise Tukey test (p value ≤ 0.05) was performed using statistical software SPSS to determine significant differences between groups. When equal variances were not assumed, a correction using welch test and Games-Howell post hoc test ($p \leq 0.05$) was applied. A two-way ANOVA was conducted to evaluate the effect of the type of ingredient and addition level on parameters using Statistical software Minitab version 19 (Minitab Inc., State College Pa.). Correlation analysis was carried out using Microsoft Excel.

4.4 Results

4.4.1 Dough Analysis

4.4.1.1 Water absorption

Determining the optimal water addition and dough consistency of the dough is necessary to ensure optimal conditions for dough hydration and gluten network formation. Results from farinograph water absorption (FWA) analysis are displayed in Table 10.

Significant differences in FWA capacities occurred between the controls BF (57.30 $\pm$ 0.2%) and WMF (59.33 $\pm$ 0.15%). Furthermore, the replacement of BF by BSG and FBSG caused an increase in water absorption, particularly in high in fibre (HF) formulations (BSG HF (68.60 $\pm$ 0.35%); FBSG HF (66.93 $\pm$ 0.31%)). BSG HF showed the highest water absorption among all samples.

Table 10. Results from the effect of Brewers spent grain (BSG) and fermented brewers spent grain (FBSG) addition at source of fibre (SF) and high in fibre (HF) inclusion levels on farinograph water absorption capacities, gluten aggregation properties, starch pasting behaviour, dough rheology properties, and fermentation capacity. BF and WMF represent results obtained for Baker's flour and Wholemeal Flour controls, respectively. The values provided represent the mean + the standard deviation. Values that share the same letter in the same row do not differ significantly.

	BF	**WMF**	**BSG (SF)**	**FBSG (SF)**	**BSG (HF)**	**FBSG (HF)**
Farinograph						
Water Absorption %	57.30 ± 0.2^{f}	59.33 ± 0.15^{e}	61.60 ± 0.1^{c}	60.10 ± 0.1^{d}	68.60 ± 0.35^{a}	66.93 ± 0.31^{b}
GlutoPeak						
Peak Max Time (Sec)	48.67 ± 1.53^{b}	141.33 ± 15.18^{a}	47.67 ± 1.15^{b}	40.33 ± 0.58^{c}	15.67 ± 2.08^{d}	14.67 ± 0.58^{d}
Torque Max (BU)	71.67 ± 0.58^{c}	29.0 ± 1.0^{e}	57.33 ± 0.58^{d}	60.67 ± 0.58^{d}	100.0 ± 2.0^{a}	84.0 ± 2.65^{b}
Rapid Visco Analyser						
Peak Viscosity (cP)	1007.67 ± 14.57^{a}	591.33 ± 30.85^{e}	911.0 ± 15.13^{b}	900.66 ± 13.05^{b}	701.33 ± 5.13^{d}	760.67 ± 10.69^{c}
Final Viscosity (cP)	1327.33 ± 26.58^{a}	1371.67 ± 27.61^{a}	1229.69 ± 23.69^{b}	1038.0 ± 11.79^{c}	992.67 ± 8.33^{c}	643.0 ± 6.24^{d}
Trough (cP)	607.0 ± 15.52^{a}	486.0 ± 25.71^{c}	543.67 ± 11.59^{b}	486.67 ± 13.58^{c}	438.67 ± 3.79^{d}	321.33 ± 10.26^{e}
Breakdown (cP)	400.67 ± 1.15^{b}	105.33 ± 10.96^{e}	367.33 ± 4.04^{c}	$414.0 \pm 3.61^{a,b}$	262.67 ± 1.53^{d}	439.33 ± 7.23^{a}
Rheology						
Damping factor	0.368 ± 0.015^{a}	0.341 ± 0.007^{b}	0.331 ± 0.004^{b}	0.330 ± 0.004^{b}	0.264 ± 0.005^{d}	0.280 ± 0.003^{c}
Rheofermentometer						
Height max (mm)	53.33 ± 1.7^{a}	20.3 ± 0.44^{c}	35.03 ± 0.6^{b}	38.4 ± 3.48^{b}	0 ± 0^{d}	0 ± 0^{d}
Total Vol CO_2 (ml)	2159.3 ± 132.03^{a}	2237.7 ± 71.93^{a}	2139.6 ± 118.5^{a}	2124.0 ± 62.81^{a}	2114.6 ± 76.8^{a}	2047.6 ± 89.51^{a}
CO_2 retention coefficient (%)	$98.73 \pm 0.74^{a,c}$	$99.60 \pm 0.10^{a,b}$	$99.36 \pm 0.15^{a,c}$	99.20 ± 0.10^{c}	99.73 ± 0.06^{a}	99.70 ± 0.10^{a}

4.4.1.2 Gluten network formation

Ingredients rich in dietary fibre influence the gluten network formation. The gluten network development time (PMT) and the torque maximum (TM) of the different formulations are illustrated in Table 10. In addition, Figure 9 graphically displays the effect of BSG and FBSG inclusion on the network development.

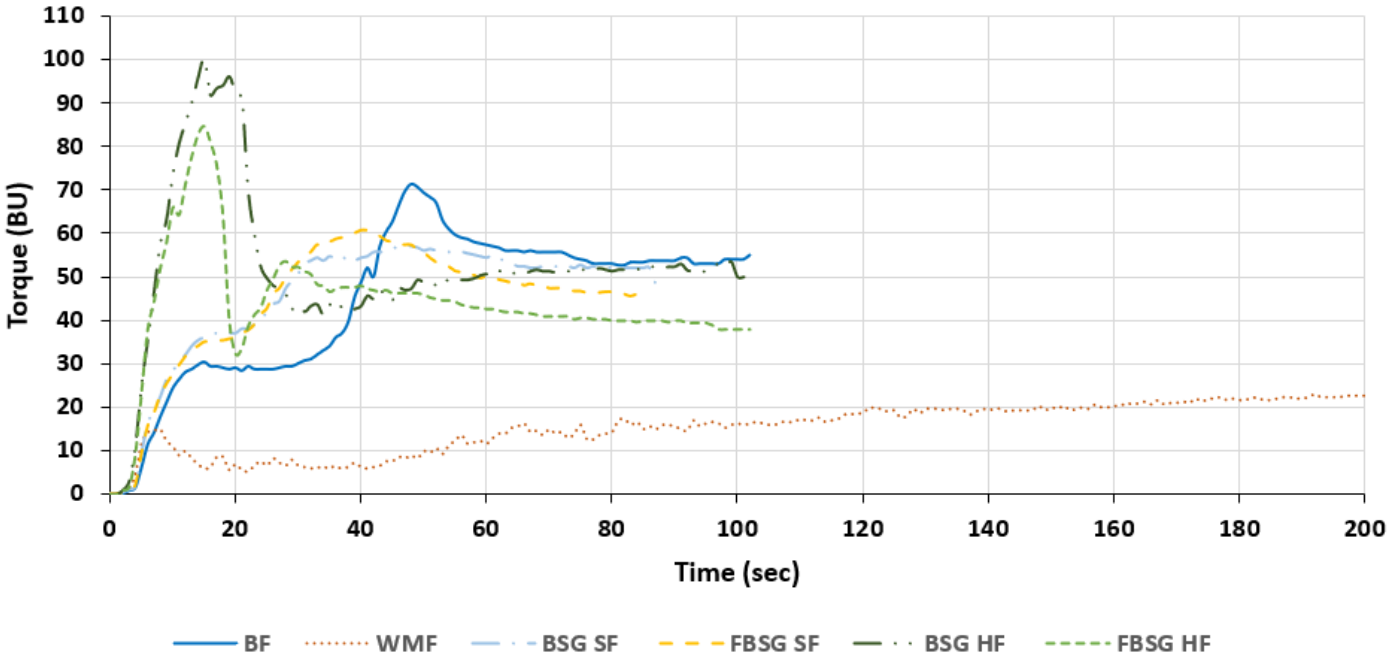

Figure 9. Graphical representation of results from GlutoPeak analysis using baker's flour (BF), wholemeal flour (WMF), brewers spent grain (BSG) and fermented brewers spent grain (FBSG). SF and HF denotes "source of fibre addition level" and "high in fibre addition level", respectively.

BF showed a typical wheat flour gluten aggregation curve with a PMT of 48.67 ± 1.53 s and a TM of 71.67 ± 0.58 BU. In comparison, WMF displayed a prolonged increase in torque with a PMT of 141.33 ± 15.18 s and a weak gluten network (TM = 29.0 ± 1.0 BU).

The incorporation of BSG and FBSG in source of fibre levels weakened the gluten network significantly, resulting in TM of 57.33 ± 0.58 BU and 60.67 ± 0.58 BU in BSG SF and FBSG SF formulations, respectively. However, SF formulations showed stronger gluten aggregation than WMF. A faster gluten network formation occurred in BSG SF (47.67 ± 1.15 s) and FBSG SF (40.33 ± 0.58 s) recipes compared to BF.

Inclusion of BSG and FBSG at the HF level resulted in gluten aggregation curves that were not aligned with BF or WMF (Figure 9). Such formulations caused a rapid protein network formation followed by a fast breakdown. The replacement of BF by BSG in high in fibre level showed the strongest gluten network (TM = 100.0 ± 2.0 BU), followed by FBSG HF (84.0 ± 2.65 BU). In addition, the high fibre formulations showed two peaks, indicating the aggregation of two protein groups. Furthermore, the network formation occurred at an earlier time point compared to BF (BSG HF = 15.67 ± 2.08 s; FBSG HF = 14.67 ± 0.58 s).

4.4.1.3 Effect on Starch behaviour with fibre ingredient addition

The impact of BSG and FBSG in two inclusion levels on starch pasting properties are displayed in Table 10. The results show the peak viscosity (PV), final viscosity (FV), trough and breakdown viscosity (BV) of the different formulations.

The PV indicates the increase in viscosity during heating. The addition of fibre caused a decrease in PV. The highest PV occurred in BF (1007 ± 14.57 cP), while WMF caused the lowest PV (591 ± 30.85 cP). The incorporation of BSG and FBSG weakened the pasting behaviour of the system, resulting in a decrease in PV with the increase of addition level. Comparing BSG and FBSG with each other, FBSG showed a slightly lower peak viscosity at source of fibre levels (BSG SF (911.0 ± 15.13 cP), FBSG SF (900.66 ± 13.05 cP)), whereas BSG caused a lower PV at high in fibre levels ((BSG HF (701.33 ± 5.13 cP) and FBSG HF (760.67 ± 10.69 cP)).

The FV indicated the degree of retrogradation of the system after gelatinisation. BF and WMF showed the highest final viscosity with 1327.33 ± 26.58 cP and 1371.67 ± 27.61 cP, respectively. The addition of BSG or FBSG decreased the degree of retrogradation with increasing addition level. Comparing those two fibre ingredients with each other, FBSG caused a lower FV than BSG, particularly at high in fibre addition level (BSG HF (1038.0 ± 11.79 cP); FBSG HF (643.0 ± 6.24 cP)).

Trough results reflect the viscosity of the suspension after the rupturing of the starch granules and indicates the system's holding strength before the retrogradation process begins. The highest trough was noted in BF (607.0 ± 15.52 cP). Compared to BF, a reduction in trough viscosity was noted in WMF (486.0 ± 25.71 cP), BSG SF (543.67 ± 11.59) and FBSG SF (486.67 ± 13.58). The reduction in trough values was amplified at the HF addition level (BSG HF: 438.67 ± 3.79 cP, FBSG HF: 321.33 ± 10.26). Comparing values obtained for BSG and FBSG a greater reduction in trough viscosity was noted in FBSG formulations.

The breakdown viscosity represents the decrease in viscosity caused by the disruption of the gelatinised starch granules due to heat and shear after the peak viscosity has been reached. The highest BV was noted in FBSG HF (439.33 ± 7.23 cP), followed by FBSG SF (414.0 ± 3.61 cP) and BF (400.67 ± 1.15 cP). A reduction in BV was noted in WMF (105.33 ± 10.96 cP) and BSG formulations (BSG SF: 367.33 ± 4.04 cP, BSG HF: 262.67 ± 1.53). Comparing BSG and FBSG formulations, a higher BV was observed for FBSG formulations.

4.4.1.3 Dough Rheology

The oscillatory damping factor (DF) indicates changes in the viscous and elastic proportion of the bread dough system. A system is defined as an ideal elastic, if the DF is 0, meaning no viscous parts are present. Hence, the higher the DF the more viscous behaviour the system has. The DF of the different formulations are illustrated in Table 10.

BF dough showed the highest DF (0.368 ± 0.015), indicating the highest viscous behaviour among all formulations, followed by WMF (0.341 ± 0.007). A significant reduction in DF values occurred with inclusion of BSG (SF: 0.331 ± 0.004; HF: 0.264 ± 0.005) or FBSG (SF:

0.330 ± 0.004; HF: 0.280 ± 0.003). Comparing both fibre ingredients with each other, the addition of BSG caused a greater shift towards elastic dough behaviour.

4.4.1.4 Fermentation capacity of doughs

The fermentation capacity of the bread dough was determined using a Rheofermentometer and the results are demonstrated in Table 10.

Hm represents the maximum dough height achieved during dough fermentation. BF bread dough reached the highest dough height (53.33 ± 1.7 mm), whereas WMF bread dough resulted in a significantly lower Hm (20.3 ± 0.44 mm). The substitution of BF by BSG or FBSG resulted in a significant decrease in Hm with BSG SF and FBSG SF resulting in 35.03 ± 0.6 mm and 38.4 ± 3.48 mm, respectively. Comparing BSG and FBSG with each other, FBSG showed a slightly higher Hm at source of fibre addition level. No dough rise occurred in high in fibre BSG/FBSG formulations.

The volume of CO_2 produced for BF dough was 2159.3 ± 132.03 ml. No significant differences in the volume of CO_2 produced during fermentation were noted in WMF and BSG/FBSG formulations at either addition level compared to the BF control. The volume of CO_2 produced during fermentation for these formulations was in the range of 2047.6 – 2228.3 ml (Table 10).

The CO_2 retention coefficient represents the percentage of CO_2 retained in the bread dough. BF dough had a CO_2 retention coefficient of 98.73 ± 0.74%, while WMF dough had a slightly higher CO_2 retention (99.60 ± 0.10%). No significant differences were noted CO_2 retention coefficients in comparison to BF for BSG and FSBG ingredients and were in the range of 99.20% – 99.73%.

4.4.2 Bread Analysis

4.4.2.1 Bake loss

Bake loss (BL) results are reported on Table 11. The highest bake loss was observed in BF (15.04 ± 0.53 %), followed by FBSG (13.00 ± 0.69 %) and BSG SF (12.89 ± 0.40 %). An increased addition level of BSG and FBSG resulted in the lowest bake loss with 10.23 ± 0.26 % and 10.47 ± 0.40 %, respectively. BSG and FBSG affected the baking loss of the breads to the same extent.

Table 11. Results from analysis of the techno-functional properties of bread with inclusion of brewers spent grain (BSG) and fermented brewers spent grain (FBSG) at source of fibre (SF) and high in fibre (HF) addition levels. BF and WMF represents results obtained from Baker's flour and Wholemeal Flour breads, respectively. The values shown represent the mean + the standard deviation. Values which have the same letter in the same row do not differ significantly.

	BF	**WMF**	**BSG (SF)**	**FBSG (SF)**	**BSG (HF)**	**FBSG (HF)**
Fibre Content (g/100)	2.10	4.76	3.32	3.27	6.41	6.37
Digestible Starch content of breads (g/100)	38.74 ± 0.55^{a}	33.81 ± 0.21^{c}	37.03 ± 0.97^{a}	35.70 ± 0.97^{b}	29.38 ± 0.74^{d}	31.37 ± 0.43^{c}
Bake loss (%)	15.04 ± 0.53^{a}	12.03 ± 0.51^{c}	12.89 ± 0.40^{b}	13.00 ± 0.69^{b}	10.23 ± 0.26^{d}	10.47 ± 0.40^{d}
Specific Volume (ml/g)	5.49 ± 0.11^{a}	2.07 ± 0.11^{d}	3.49 ± 0.13^{c}	3.86 ± 0.21^{b}	1.45 ± 0.05^{f}	1.69 ± 0.03^{e}
Slice Area (mm^2)	11654 ± 361^{a}	5127 ± 361^{d}	8060 ± 313^{c}	8788 ± 471^{b}	4776 ± 278^{e}	5214 ± 234^{d}
Number of cells	6472 ± 282^{a}	3250 ± 168^{c}	5556 ± 225^{b}	5593 ± 246^{b}	5483 ± 426^{b}	5441 ± 387^{b}
Cell diameter (mm)	2.28 ± 0.07^{a}	2.18 ± 0.14^{b}	1.72 ± 0.08^{d}	1.86 ± 0.10^{c}	1.09 ± 0.06^{f}	1.15 ± 0.07^{e}
Bread Texture						
Hardness T2 (N)	2.99 ± 0.36^{f}	30.13 ± 6.15^{c}	10.91 ± 1.32^{d}	7.91 ± 1.31^{e}	79.22 ± 5.88^{a}	47.24 ± 3.97^{b}
Resilience (T2)	0.49 ± 0.02^{a}	0.41 ± 0.02^{c}	0.46 ± 0.02^{b}	0.47 ± 0.02^{b}	0.34 ± 0.02^{d}	0.34 ± 0.02^{d}
Stale rate	$2.20 + 0.48^{a}$	$0.95 \pm 0.46^{c,d}$	$1.22 \pm 0.36^{b,c}$	1.59 ± 0.34^{b}	0.72 ± 0.24^{d}	$1.04 \pm 0.21^{c,d}$
Colour						
ΔE Crust (Bakers Flour)	-	-	8.90 ± 1.03^{b}	8.39 ± 1.30^{b}	9.26 ± 1.25^{b}	11.99 ± 1.25^{a}
ΔE Crust (Wholemeal flour)	-	-	20.91 ± 1.11^{a}	20.59 ± 1.07^{a}	12.85 ± 0.96^{b}	10.52 ± 0.71^{c}
ΔE Crumb (Bakers Flour)	-	-	14.64 ± 1.16^{c}	15.24 ± 0.95^{c}	29.18 ± 0.55^{a}	28.31 ± 0.86^{b}
ΔE Crumb (Wholemeal flour)	-	-	8.54 ± 0.66^{d}	9.17 ± 0.75^{c}	11.61 ± 0.77^{a}	10.50 ± 0.75^{b}
Water Activity	0.95 ± 0.01^{b}	$0.96 \pm 0.01^{a,b}$	0.97 ± 0.01^{a}	0.97 ± 0.01^{a}	0.97 ± 0.01^{a}	0.97 ± 0.01^{a}

"-" Represents not applicable

4.4.2.2 Specific Volume

Superior bread quality is often characterised by a bread with a high specific volume (SV). The results of the SV of the different bread formulations are illustrated in Table 11.

The SV for BF (5.49 $\pm$ 0.11 g/ml) was significantly higher than the SV recorded for the WMF (2.07 $\pm$ 0.11 g/ml). The inclusion of BSG and FBSG ingredients caused a decrease in SV of breads, with higher inclusion levels having more significant effects when compared to the BF control. However, in comparison to WMF, the SV of BSG SF (3.49 $\pm$ 0.13 g/ml) and FBSG SF (3.86 $\pm$ 0.21 g/ml) breads were significantly higher. The incorporation of BSG and FBSG in high in fibre concentrations resulted in the lowest SV with 1.45 $\pm$ 0.05 g/ml and 1.69 $\pm$ 0.03 g/ml recorded for BSG HF and FBSG HF, respectively. Comparing BSG and FBSG with each other, FBSG resulted in a higher SV.

4.4.2.3 Crumb Structure

The crumb structure of the different bread formulations was investigated by the determination of the slice area, the number of cells, and the cell diameter. The results are depicted on Table 11.

The biggest slice area occurred in BF bread (11654 ± 361 mm^2), followed by source of fibre breads including FBSG (8788 ± 471 mm^2) and BSG (8060 ± 313 mm^2). The smallest slice area was detected in breads including BSG and FBSG in high in fibre addition levels with 4776 ± 278 mm^2 and 5214 ± 234 mm^2 reported, respectively.

Cells are created within the dough due to the production of CO_2 during proofing. BF bread had the highest number of cells (6472 $\pm$ 282), while WMF bread showed the lowest number of cells (3250 $\pm$ 168). A significant decrease in the number of cells occurred in bread fortified with BSG or FBSG at both addition levels, which were, however, significantly higher than the WMF control.

BF had the largest cell diameter (2.28 $\pm$ 0.07 mm), followed by WMF (2.18 $\pm$ 0.14 mm). However, the result of WMF bread cannot be taken into account due to the imaging system potentially recognising larger bran particles as cells due to the dark colour. The WMF bread

crumb image (Figure 10. D) shows a dense crumb with limited gas cells embedded in the bread matrix versus BF. The inclusion of BSG and FBSG at the SF addition level reduced the cell diameter, resulting in 1.72 $\pm$ 0.08 mm and 1.86 $\pm$ 0.10 mm, respectively. The increase in addition level to HF amplified the reduction in cell diameter, leading to 1.09 $\pm$ 0.06 mm and 1.15 $\pm$ 0.07 mm in BSG HF and FBSG HF breads, respectively.

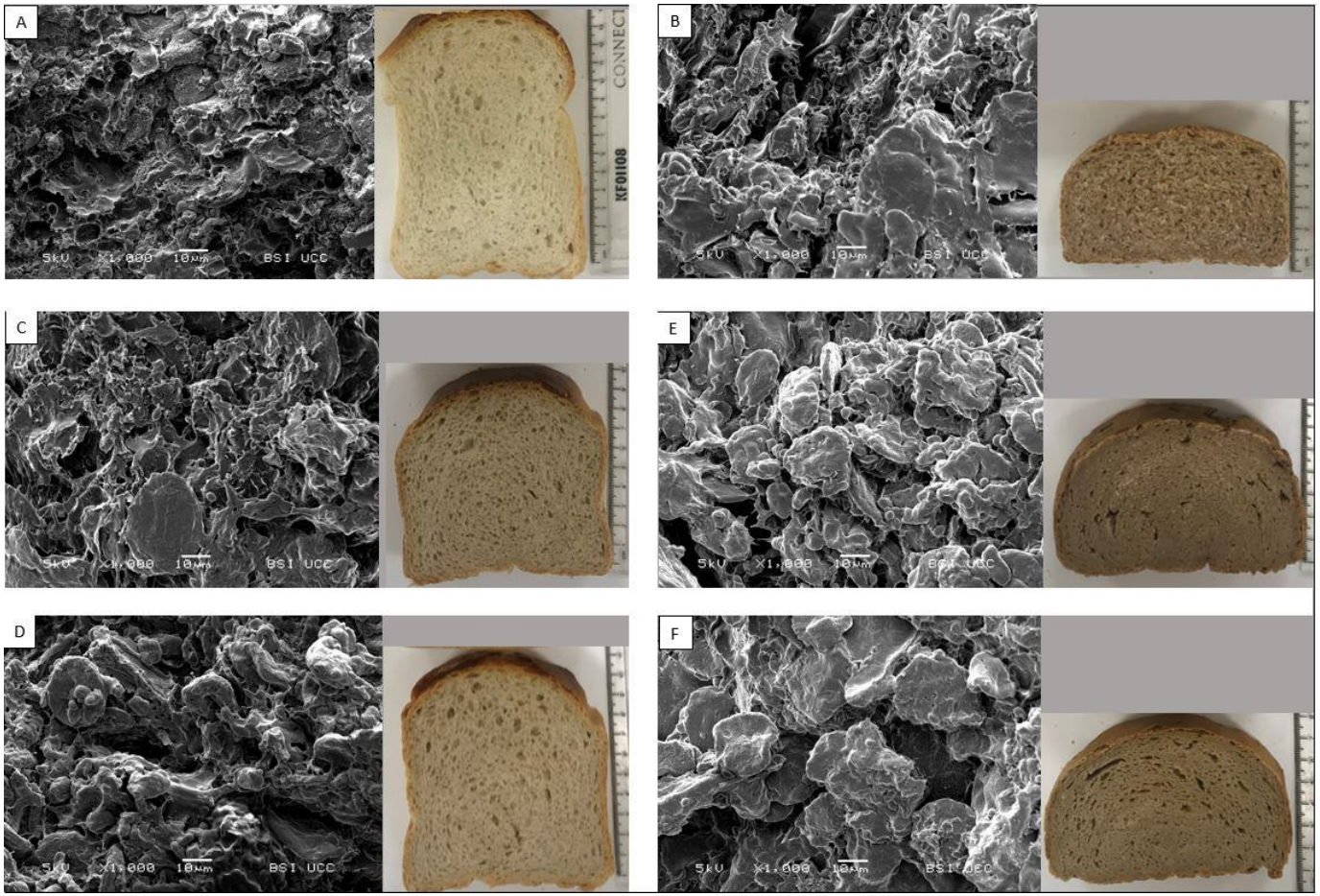

Figure 10. SEM micrographs of freeze-dried breads and images of their respective bread crumbs on day of baking. Pictures (A-F) illustrate baker's flour (A), wholemeal flour (B), brewers spent grain "source of fibre" (C), fermented brewers spent grain "source of fibre" (D), brewers spent grain "high in fibre" (E) and fermented brewers spent grain "high in fibre" (F) breads.

4.4.2.4 Bread Texture and staling

Crumb texture is considered an important parameter to analyse to ensure optimal bread quality. Values for crumb hardness, crumb resilience and the bread staling rate are presented in Table 11.

The softest crumb was determined in BF bread (2.99 ± 0.36 N), while WMF bread showed a significantly harder crumb (30.13 ± 6.15 N). The replacement of BF by BSG and FBSG at a source of fibre level increased crumb hardness to 10.91 ± 1.32 N and 7.91 ± 1.31 N, respectively. The increase in inclusion level of BSG and FBSG amplified the elevation in crumb hardness, resulting in the highest values (BSG HF: 79.22 ± 5.88 N; FBSG HF: 47.24 ± 3.97 N). Comparing BSG and FBSG with each other, FBSG caused a softer crumb.

The bread crumb with the highest resilience was determined in BF bread (0.49 ± 0.02 N), while a reduction in bread crumb resilience was observed in WMF bread (0.41 ± 0.02 N). The inclusion of BSG and FBSG at the SF addition level further reduced the resilience of the crumb (0.46 ± 0.02 N and 0.47 ± 0.02 N, respectively). Increased levels of BSG and FBSG inclusion resulted in a greater decrease in crumb resilience (BSG HF: 0.34 ± 0.02 N, FBSG HF: 0.34 ± 0.02 N). No difference was observed between BSG and FBSG concerning the resilience of the bread crumb.

The staling of bread is the changes in crumb hardness over time due to retrogradation and moisture migration. BF bread had the fastest staling rate (2.10 ± 0.49) while a reduction in the rate of staling occurred in WMF bread (1.0 ± 0.12). The replacement of BF with BSG and FBSG decreased the rate of staling to 1.34 ± 0.35 and 1.73 ± 0.13, respectively. Increased addition of BSG and FBSG resulted in an even lower staling rate (BSG HF: 0.70 ± 0.14, FBSG HF: 1.08 ± 0.06). When comparing staling results from BSG and FBSG formulations, a slightly lower staling rate was observed in BSG formulations; however, this was not statistically significant.

4.4.2.5 Crust and crumb colour

Differences in the crust and crumb colour of breads were evaluated using ΔE values compared to BF and WMF bread, considering the differences in colour values L*, a* and b* compared to the controls.

Compared to BF, FBSG HF (11.99 ± 1.25) showed the greatest difference in crust colour, while BSG HF (9.26 ± 1.25), BSG SF (8.90 ± 1.03) and FBSG SF (8.39 ± 1.30) resulted in a more similar crust colour. Compared to WMF bread, a significant difference in crust colour was observed in BSG SF (20.91 ± 1.11) and FBSG SF (20.59 ± 1.07). The addition of higher amounts of BSG or FBSG caused a lower ΔE-value, however (BSG HF: 12.85 ± 0.96, FBSG HF: 10.52 ± 0.71).

The greatest difference in ΔE values for crumb colour compared to BF was observed in BSG HF (29.18 ± 0.55), followed by FBSG HF (28.31 ± 0.86). A reduction in ΔE crumb values occurred at the SF addition level (BSG SF:14.64 ± 1.16, FBSG SF 15.24 ± 0.95). In comparison to WMF crumb colour, the BSG HF had the highest ΔE (11.61 ± 0.77), followed by FBSG HF (10.50 ± 0.75), FBSG SF (9.17 ± 0.75) and BSG SF (8.54 ± 0.66).

4.4.2.6 Water Activity and Microbial Shelf life

The water activity (A_w) of the bread crumb of the different formulations is illustrated in Table 11. BF (0.95 ± 0.01) and WMF (0.96 ± 0.007) bread crumbs exhibited a lower Aw than BSG and FBSG breads. The incorporation of BSG and FBSG led to an increase in Aw-value to 0.97 regardless the addition level or type of fibre ingredient.

The microbial shelf life of the breads over time is demonstrated in Figure 11. The first mould growth on BF bread occurred on day 4, while the shelf life of WMF bread was 5 days. The inclusion of BSG SF did not affect the microbial shelf life, whereas FBSG SF resulted in a prolonged shelf life by one day. Additionally, the inclusion of HF levels of both, BSG or FBSG, resulted in breads with a shelf life of 5 days. Even though the day of the first mould growth was very similar, the kinetics of mould growth were different, particularly when FBSG was used as a fibre ingredient. Figure 11 shows slower microbial growth in breads containing FBSG compared to BF bread, WMF bread or bread containing BSG.

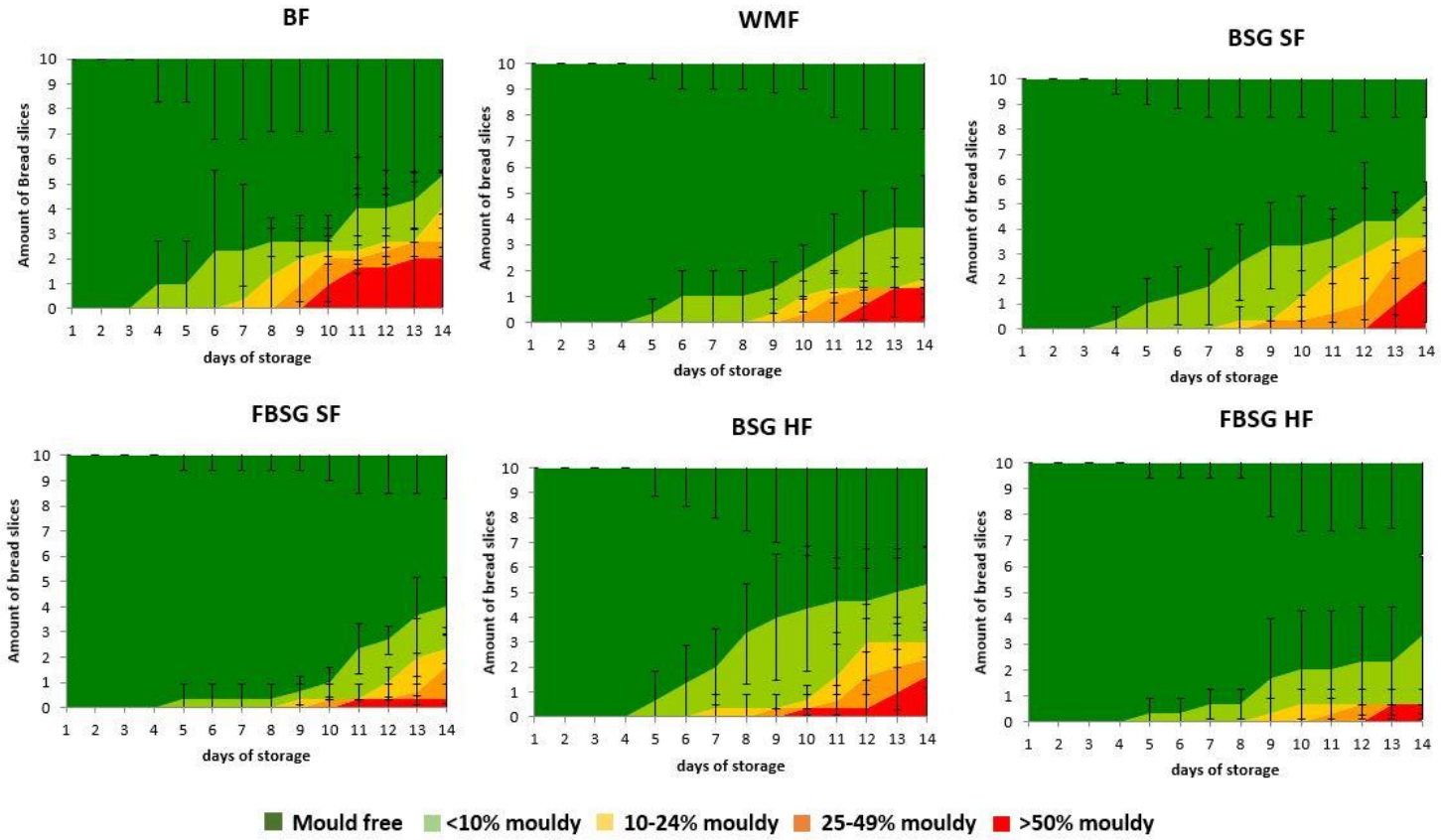

Figure 11. Shelf-life plots from 14-day analysis of breads. The amount of bread slices which contained each mould group ("mould free, <10% mouldy, 10-24% mouldy, 25-49% mouldy and >50% mouldy) was counted over a period of 14 days. BF and WMF represent bakers flour wheat control and wholemeal bread, respectively. BSG and FBSG denote brewers spent grain and fermented brewers spent grain breads, respectively. SF and HF stand for "source of fibre" and "high in fibre" addition levels, respectively. The graph represents mean values obtained across three independent batches with standard deviations included as error bars.

4.4.2.7 In vitro starch hydrolysis

Starch digestibility was determined using an *in vitro* model system, and the release of reducing sugar (RSR) during digestion was investigated (Figure 12).

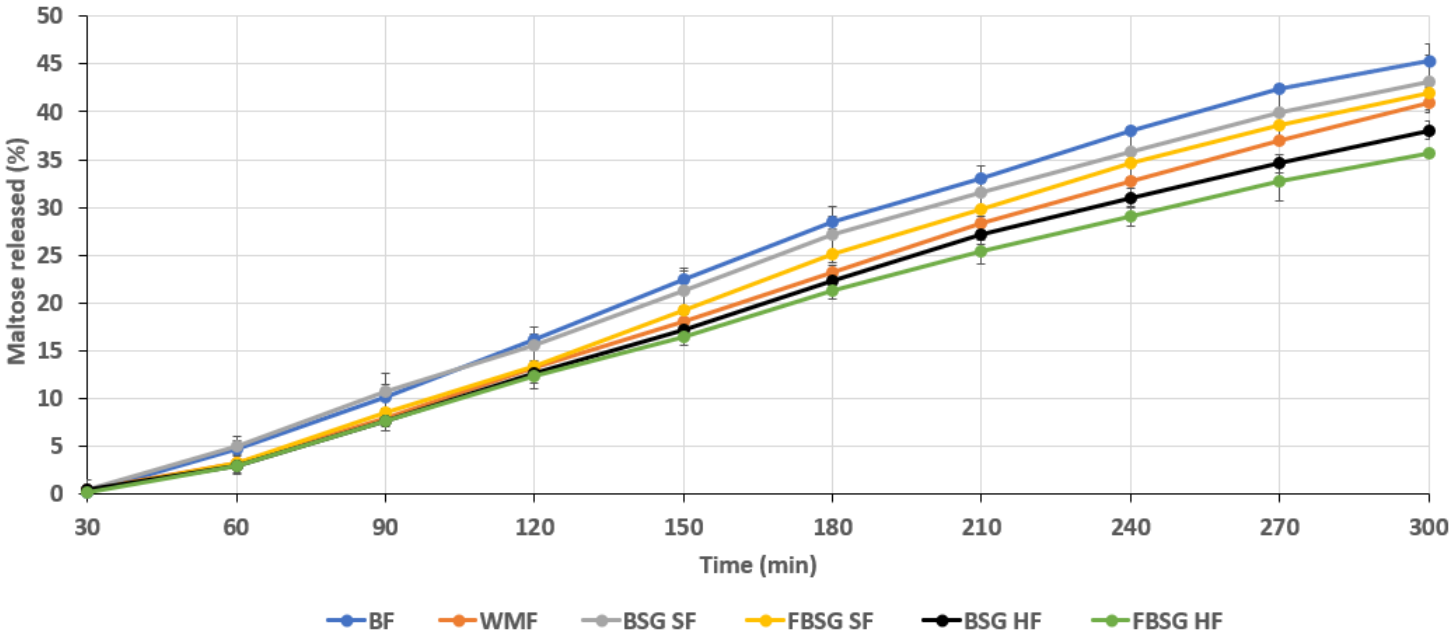

Figure 12. Comparison of the release of maltose over time from baked bread samples. BF and WMF denote "baker's flour" and "Wholemeal flour" breads, respectively. BSG and FBSG indicate "brewers spent grain" and "fermented brewers spent grain breads", respectively. SF denotes "source of fibre addition level" and HF represents "high in fibre addition level". Graphs show mean values of duplicate samples with standard deviations represented as error bars.

The highest release of reducing sugars over time was observed in BF bread, indicated by the highest slope (0.175 maltose released (%) / min). WMF bread showed a lower release of reducing sugars over time (slope: 0.157 maltose released (%) / min). The inclusion of BSG and FBSG decreased the starch digestibility of the breads, resulting in a lower release of sugars, particularly at high addition levels. FBSG HF breads showed the lowest degree of starch digestibility, leading to a slow sugar release with a slope of 0.137 maltose released (%) / min.

4.4.2.8 Bread Ultrastructure

Scanning electron microscopy (SEM) was used to analyse changes in crumb structure with BSG and FBSG inclusion. Images of freeze-dried bread crumbs are illustrated in Figure 10.

The BF crumb (Figure 10. A) displays partially gelatinised, porous starch granules embedded in a protein matrix. In contrast, the WMF bread crumb (Figure 10. B) demonstrates a higher level of intact and more defined starch granules, indicating a lower level of starch gelatinisation.

BSG SF (Figure 10. C) and FBSG SF (Figure 10. D) exhibit similar findings to those observed in WMF crumb structure. A higher level of intact starch granules is evident. BSG HF (Figure 10. E) and FBSG HF (Figure 10. F) amplify the trends observed in SF formulations. In addition, a film associated with the starch granules occurred. Negligible differences were apparent in crumb structure in SEM images of BSG and FBSG formulations; however, FBSG HF (Figure 10. F) images showed a smoother crumb matrix than BSG HF.

4.5 Discussion

The inclusion of BSG in bread is challenging, with higher inclusion levels leading to bread with significant inferior quality than standard wheat bread. This study shows that processing of BSG using fermentation technology is a promising approach to aid in maintaining dough and bread quality. BSG and FBSG were included in bread formulations at two addition levels, “source of fibre” and “high in fibre”, with both the type of fibre ingredient and ingredient addition level significantly impacting dough quality and bread characteristics.

The gluten network strength and the gluten network development time are significant parameters in the breadmaking process to ensure a desirable dough and bread quality. The inclusion of BSG and FBSG, particularly at higher inclusion levels, resulted in a stronger network that developed faster than in the BF control. The inclusion level, as well as the type of ingredient, significantly impacted gluten network strength ($p < 0.001$) and time to develop ($p < 0.001$). Fermentation alleviated the impact of BSG-derived ingredients on gluten network compared to unfermented BSG. Flours displaying a rapid gluten network aggregation and fast breakdown are regarded as poorer flours with weakened technical capacity [250–252]. BSG and FBSG ingredients contain a high amount of minerals (3.7%), proteins (31.4% and 32.4%, respectively) and fibres (42.6% and 49.4%, respectively), all of which can influence the strength and development time of gluten. Minerals can facilitate a charge screening effect, exposing apolar protein side-chains, causing greater hydrophobic interaction [202,253] and hence could result in a stronger network displayed by the higher torque. Furthermore, incorporating higher levels of protein could shift the balance of glutenin and gliadin present, leaning more towards a higher level of glutenin and increasing gluten strength [205]. Additionally, the inclusion of fibres has previously shown to enhance the kinetics of the gluten network [250], interact with the secondary structure of gluten proteins (primarily glutenin) and restrict hydration of the gluten network [254]. Arabinoxylans, the main fibre in BSG [3], have also been reported to be of particular hindrance to gluten formation [145,146,155,199,201]. The two peaks noted in Figure 9 at HF addition levels highlights potential secondary networks forming with the inclusion of BSG and FBSG at higher levels of addition. Results from chapter 3 [245] revealed the presence of low molecular weight peptides present in BSG and FBSG, and with the introduction of charged amino acids from BSG [145], these conditions may facilitate the formation of secondary networks at a different time point to gluten formation [40]. The weaker gluten network and the more pronounced second peak in FBSG HF highlight a further

modification to proteins post-fermentation. Proteins in FBSG undergo modifications during the fermentation process due to proteolysis and changes in pH with lactic acid production [206,255]. The weaker gluten network and more pronounced second peak observed could be linked with the acidic environment created with the introduction of the fermented ingredient. This could increase the positive charges present, which initially favours gluten network formation through the unfolding of the gluten proteins and enhancing hydrophobic interactions; however, the strong intermolecular forces cause a rapid breakdown of gluten and inhibits the formation of further bonds necessary for strong gluten formation [206,255,256]. The modified proteins/peptides in FBSG may also differ in charge and structure post-fermentation; which may have induced further unfolding of proteins, exposing more hydrophobic regions and facilitating co-networking with gluten proteins via hydrophobic interactions to a greater extent [162].

As well as changes in gluten network development, differences in the viscoelastic behaviour of doughs were noted with BSG and FBSG inclusion, resulting in an increase in elastic parts in the dough. The damping factor was influenced by both the type of fibre ingredient ($p < 0.001$) and ingredient addition level ($p < 0.001$). Replacement of BF with BSG and FBSG ingredients reduced the amount of gluten and starch available within the dough matrix, resulting in a stiffer dough with greater resistance to deformation. However, replacement of BF with FBSG, predominantly at HF addition levels, led to a dough with more viscous parts than unfermented BSG, which emphasises the great potential of fermentation as a tool to functionalise BSG. As mentioned previously, the acids present in the fermented ingredient putatively induces an environment lower in pH, causing a weaker gluten network and reduces dough firmness [256] compared to unfermented BSG. In addition to this, the acidic environment can also enhance the proteolytic activity within the dough system, further reducing the elasticity and stiffness of the dough [255,256]. The damping factor also correlated positively with Hm ($r= 0.9169$, $p < 0.01$), highlighting that the more elastic properties of the doughs restricted their ability to rise and expand during proofing. This could be the reason for BSG HF and FBSG HF showing no dough rise (Hm=0). Neither addition level ($p < 0.345$) nor type of fibre ($p < 0.446$) affected the volume of CO_2 produced during proofing and did not differ significantly from BF. Hence, the yeast fermentation was not affected by the inclusion of BSG and FBSG, and the adverse effects observed during the dough's rise are related to the dough rheology/structure imposed by the ingredients.

Dough rheology parameters such as Hm and the damping factor also significantly impacted bread quality characteristics. Both Hm and the damping factor correlated positively with specific volume ($r= 0.96$, $p <0.002$, $r= 0.82$, $p <0.04$, respectively). This highlights the significant effect of dough rheology on final bread volume. Specific volume was influenced by both ingredient addition level ($p < 0.001$) and the type of ingredient ($p < 0.001$). Fermentation of BSG led to an increase in specific volume, putatively due to the reductions observed in dough elasticity, which facilitated the dough's expansion and rise. The reductions in specific volume with BSG and FBSG inclusion caused changes in crumb texture. Correlations between specific volume and crumb hardness ($r= -0.85$, $p \leq 0.03$) as well as crumb resilience ($r=0.92$, $p \leq 0.01$) occurred, highlighting bread texture is dependent on the extent of the dough rise. Crumb hardness was influence by both ingredient type ($p < 0.001$) and level of addition ($p < 0.001$). Fermentation reduced crumb hardness, which was likely the result of the greater dough rise achieved during proofing ($r = -0.92$, $p \leq 0.01$) combined with the more viscous nature of the dough with FBSG inclusion ($r=-0.90$, $p \leq 0.01$) [256,257]. The impact of dough characteristics on crumb structure is highlighted by the positive correlation between cell diameter and Hm ($r= 0.83$, $p \leq 0.001$) as well as the damping factor ($r = 0.97$, $p \leq 0.001$). Bread crumb resilience is also an important bread quality parameter and was affected mainly by ingredient addition level ($p < 0.001$) rather than the type of fibre ingredient. Both BSG-derived ingredients weakened crumb resilience, putatively due to the changes observed in the gluten network formation, which impacts gluten quality and the adverse effects noted in dough rheological properties.

Apart from the gluten network and the viscoelastic properties of the dough, changes in the viscosity of the formulations during heating also influenced bread quality. As a general trend, replacement of BF with more fibrous ingredients resulted in a reduction in peak viscosities, likely due to the reduction in the overall starch content, as well as the increase in competition for hydration by the fibre and protein fraction of BSG and FBSG [146,208,258]. Both ingredient type ($p < 0.006$) and addition level ($p < 0.001$) influenced the evaluated pasting parameters. Peak viscosity represents the highest viscosity reached during heating and correlated positively with specific volume ($r=0.88$, $p \leq 0.02$). Higher degrees of starch swelling facilitate a greater expansion in starch granules, which aids in achieving a higher specific volume [259]. The incorporation of FBSG resulted in a higher peak viscosity than BSG, putatively due to the slightly lower amount of BF replacement, resulting in a higher total amount of starch susceptible for pasting. Furthermore, as mentioned before, the inclusion of BSG-derived ingredients leads to an increase in competition for water with starch, resulting in

a lower degree of starch gelatinisation. This can be observed in the micrographs, showing higher amounts of intact starch granules in the crumb of high in fibre BSG/FBSG breads.

After the peak, a breakdown of viscosity occurs due to starch leaching, resulting in the trough viscosity before cooling. FBSG showed a higher breakdown than BSG, most likely due to the higher amylase activity in the FBSG ingredient, resulting in reduced starch granule rigidity and enhancing sensitivity to deformation [260]. The final viscosity indicates the degree of retrogradation of the system during cooling. BSG and FBSG caused a lower final viscosity, most likely due to the higher amounts of fibre present, which interrupts the realignment of the macromolecular matrix during the cooling process through physical disruption, obstruction of secondary forces and sterical hindrance [208]. Furthermore, the significantly lower final viscosities observed in formulations including FBSG may be linked with the higher amylase activity of the FBSG ingredient, decreasing the degree of retrogradation [214,261,262]. Amylases partially degrade amylopectin and amylose, negatively influencing their rearrangement during retrogradation [263]. Moreover, the inclusion of FBSG introduces lactic acid to the system, which has previously shown to restrict starch retrogradation [264] and increase solubility of amylopectin, which may further inhibit the realignment process [265]. However, higher bread staling rates occurred in breads including FBSG compared to BSG, which is putatively due to the lower replacement level of BF by FBSG. This led to a higher total starch level in formulations containing FBSG compared to BSG. Furthermore, it needs to be mentioned that the dense crumb structure caused high crumb hardness already after baking. Hence, the increase in hardness over time was less pronounced.

The denser crumbs might also be the reason for the extended microbial shelf life observed in high fibre breads, with a denser crumb potentially restricting the aeration needed for microbial growth [266]. Furthermore, the inclusion of FBSG appears to exhibit some anti-microbial properties and slows the kinetics of microbial growth overtime compared to BSG. Sourdough technology using a variety of different lactic acid bacteria has previously shown to induce an anti-microbial effect, which has been attributed to the combined acidification and the synergistic effect of the various anti-microbial metabolites produced during lactic acid bacteria fermentation [267,268].

Besides extending microbial shelf life, bread fortified with BSG-derived ingredients resulted in a lower sugar release during starch digestion. This likely occurred mainly due to the reduction of available carbohydrates caused by wheat flour replacement. Furthermore, the

incorporation of fibre and protein is known to restrict the extent of enzyme hydrolysis [269,270]. More densely-packed food structures can also be limiting factors to enzyme activity [271,272], and the dense crumb structure of BSG and FBSG may further inhibit enzyme-substrate affinity. The micrographs of high fibre breads including BSG or FBSG also revealed a film in association with the starch granules, which could be the product of potential protein-starch-fibre interactions [199,217,269,271]. This film could act as a further barrier for enzyme hydrolysis leading to a lower sugar release [269,271,273,274]. Fermentation of BSG resulted in a lower release of sugars during *in vitro* starch digestions. This may be attributed to the lactic acid produced during fermentation which creates a more acidic environment and hinders starch hydrolysis [166,275–277]. Studies from Östman et al., [233] proposed a potential mechanism for this, indicating lactic acid present during heat treatment induces interactions between starch and gluten and limits the bioavailability of starch for enzyme hydrolysis.

4.6 Conclusion

Rejuvenating BSG for bakery application, particularly breadmaking, is challenging because of its high impact on dough rheology and bread quality characteristics. Hence, new approaches which involve further processing are needed. The results from this study highlight the great potential of fermentation as a tool to functionalise BSG and turn it into a food ingredient, which elevates the nutritional value of bread by increasing protein and fibre content and simultaneously ensuring higher bread quality. The inclusion of FBSG reduced dough stiffness and affected the gluten network formation to a lesser extend compared to unfermented BSG. These changes in the dough system positively enhanced bread techno-functional properties, resulting in an increase in bread specific volume and reduction in crumb hardness. In addition to the improved bread quality, fermentation of BSG resulted in an ingredient that prolonged microbial shelf life and reduced the staling of bread. Furthermore, the fermentation of BSG can positively enhance the nutritional value of the ingredient by decreasing the release of sugar during digestion. Further investigations related to the optimisation of the baking process by adjusting the mixing process, for example, would be of great importance. Moreover, additional baking aids, such as dough improvers, might ameliorate the dough rheology and result in higher bread quality. This work epitomises the excellent potential of fermentation technology as a processing aid that could further valorise BSG as a food ingredient in the future.

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Chapter 5

FOOD INGREDIENTS FOR THE FUTURE: IN-DEPTH ANALYSIS OF THE EFFECTS OF LACTIC ACID BACTERIA FERMENTATION ON SPENT BARLEY ROOTLETS

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5.1 Abstract

By-product repurposing to alternative applications has become a vital part of food research. Barley rootlets (BR) are a side-stream of malting and brewing industries. This study focuses on BR processing into food ingredients, using fermentation with five lactic acid bacteria (LAB) as a valorisation technique. The strains used were *Lactiplantibacillus plantarum* FST 1.7, *Lactobacillus amylovorus* FST2.11, *Weissella cibaria* MG1, *Leuconostoc citreum* TR116 and *Limosilactobacillus reuteri* R29. The influence of fermentation on sugar/FODMAP/acid compositions and microbial metabolites in BR was analysed. A variety of techno-functional properties were also evaluated. Results showed BR were a suitable substrate for LAB, particularly for *Lactiplantibacillus plantarum* FST 1.7 and *Lactobacillus amylovorus* FST2.11. Sugar, acid and FODMAP composition of the fermented BR demonstrated various traits imparted by LABs, including high mannitol production from *Leuconostoc citreum* TR116 and *Limosilactobacillus reuteri* R29. *Limosilactobacillus reuteri* R29 also produced fructans using BR as a substrate. Techno-functional analysis of BR showed a significant reduction in α-amylase activity post sterilisation and fermentation. Fermentation reduced water binding capacity and significantly increased oil binding capacity. The LAB used displayed great potential in improving the functionality of BR as a food ingredient while also showcasing LAB fermentation as a viable processing aid for BR valorisation.

5.2 Introduction

By-product valorisation has become an important part of food research throughout the last number of years. Rootlets are the main by-product of the malting industry and can be generated in quantities of 3-5% of the weight of the malt produced [9,10]. As the malting industry is intrinsically associated with the brewing industry, rootlets are also considered a by-product of the brewing industry [6]. Rootlets, often referred to as malt coombes/culms/sprouts/germs, are produced during the germination stage of malting and are collected in the deculming process, which follows malt kilning [278]. Depending on the type of malt produced, rootlets may be sourced from a variety of grains [279]. However, barley (*hordeum vulgare*) is the most common grain used for brewing purposes [6], therefore the vast majority of rootlets originate from barley.

BR are an excellent source of nutrients, containing high levels of fibre (9.7 - 43%), protein (20.34-38.7%), minerals (2.8-8.7%) and minor amounts of fat (1.7-4.4%) [278]. In addition to this, BR are a natural source of a diverse set of enzymes and antioxidants while also serving as a suitable substrate for lactic acid bacteria (LAB) fermentations and biochar production [278]. However, the main use of BR to date has been confined to animal feed [280], with limited studies available investigating their use as a food ingredient [11,18,62,68]. Hence, further processing and exploration of BR is required to make them a viable food ingredient.

Heat treatment is widely used as a processing aid in the food sector, with a variety of thermal treatments used depending on the end goal requirements. Much like other brewing by-products, high microbial loads and high enzymatic activity is expected in BR [281]. Thus, an initial thermal treatment is required to aid in the development of BR as a food ingredient. In recent years, LAB fermentation has been explored as a processing aid for the valorisation of a variety of side-stream products, including brewers spent grain [145,154,155,168,169,245,282], bran and germ from wheat and maize milling side streams [156,157,159,163], surplus bread [158] and apple by-products [166]. These studies showed that fermentation of the by-products improved ingredient properties as well as the techno-functional, sensory and nutritional aspects of bread and pasta products formulated with the fermented ingredients. In relation to BR specifically, a previous study by Waters et al. [11] included fermented BR sourdough in a bread system, which reported improvements in bread texture and flavour perception with inclusion levels of 5-10% when compared to a wholemeal control, further showcasing the potential of fermentation as a processing aid.

This study investigates the use of batch LAB fermentation as a processing aid for the rejuvenation of BR using five different LAB strains. This study differs from previous literature as it provides a fundamental study on the effects of processing (sterilisation and fermentation) on BR and showcases the potential of the developed BR ingredients as ingredients for food applications, particularly in the cereal and bakery industry.

5.3 Materials and methods

5.3.1 Raw materials

BR were provided by Anheuser-Busch InBev (Leuven, Belgium). All chemicals used in the experimental analysis were purchased from Sigma-Aldrich (Missouri, USA) unless stated otherwise. BR were sieved, milled and fermented with *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) FST 1.7 (BR-FST1.7), *Lactobacillus amylovorus* FST2.11 (BR-FST2.11), *Weissella cibaria* MG1 (BR-MG1), *Leuconostoc citreum* TR116 (BR-TR116) and *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) R29 (BR-R29). Unfermented (BR-UnF) and heat-treated BR (BR-Ster) ingredients were used as controls. *L. plantarum* FST 1.7, *L. amylovorus* FST2.11, *W. cibaria* MG1 and *L. reuteri* R29 belong to the culture collection stock of the Cereal and Beverages Research Group in of the School of Food and Nutritional Sciences, University College Cork, Ireland (Table 12). *L. citreum* TR116 belongs to the culture collection of the Department of Biological Sciences, Munster Technology University, Ireland. LAB isolates were maintained as frozen stocks in 40% glycerol at -80 °C. The strains were routinely subcultured on deMan Rogosa Sharpe (MRS) agar plates supplemented with 0.05 g/L bromocresol green under anaerobic conditions using AnaeroGen gas packs (Thermo Fisher Scientific, Waltham, MA, USA) at 30 °C for 48 h.

Table 12. Attributes of the lactic acid bacteria used for experimental analysis

Species	***Leuconostoc citreum***	***Lactobacillus amylovorus***	***Weissella cibaria***	***Limosilactobacillus reuteri***	***Lactiplantibacillus plantarum***
Strain	TR116	FST 2.11	MG1	R29	FST 1.7
Metabolism	Heterofermentative	Homofermentative	Heterofermentative	Heterofermentative	Heterofermentative
Fermentation substrate	Fructose	Sucrose	Sucrose	Sucrose	Sucrose
Source	Yellow pea sourdough	Brewing environment	Sourdough	Human intestine	Malted barley
Special traits	Mannitol producer, antifungal producer	Antimicrobial producer, high acid producer	Dextran exopolysaccharide producer	Mannitol producer, Antifungal producer	Antifungal producer, high acid producer
Reference	[160,162,174,283,284]	[285–287]	[277,288–293]	[294–296]	[244,277,287,296,297]

5.3.2 Rootlet preparation

Approximately 5 Kg of the BR mixture was sieved using an Endecotts Titan 450 sieve shaker (Endecotts, London, UK) at power level 5 for 30 min to remove husks, barley dust, broken barley kernels, acrospires and debris within the BR mixture. Sieves of size 1 mm, 500 µm, 250 µm, 500 µm and 150 µm were used to fractionate the BR mixture. The BR with a size between 499 µm and 250 µm were milled using a laboratory scale disk mill (Buhler group, Uzwil, Switzerland) with the distance between disks set to 3 mm. Sieved and milled rootlets (BR-UnF) were frozen until usage.

5.3.3 Rootlet fermentation

Prior to fermentation, cell suspensions of the single LAB strains were prepared by inoculating one single colony into MRS broth, incubated at 30 °C for 24 h, and subculturing (1%) into fresh MRS broth at 30 °C for 16 h. Cells were harvested by centrifugation (5000 rpm, 10 min, 4 °C), the supernatant was discarded, and the cell pellet was resuspended in sterile Ringers' solution. Cells were washed twice by repeating the centrifugation and resuspension step. Rootlet fermentations were performed in 1 litre batch bioreactor vessels on the DASGIP Bioblock combined with the DASGIP TC4SC4 and DASGIP PH8 modules (Eppendorf, Stevenage, UK) for temperature and agitation control. BR-UnF were defrosted and mixed with water at 12.5% (w/v) addition level. A total volume of 800 mL of substrate was placed in the bioreactor, to which 40 g (5% w/v) of the appropriate sugar (Table 12) was added. Sucrose was added as a sugar source for *L. plantarum* FST 1.7, *L. amylovorus* FST2.11, *W. cibaria* MG1 and *L. reuteri*, while fructose was used in the case of *L. citreum* TR116 to trigger mannitol production [162,174,283,284,298]. The mixture was sterilised by heating at 90 °C for 30 min, after which it was cooled to 30 °C. LAB strains were inoculated into the rootlet mixture to give a cell concentration of 10^7 CFU/mL. Fermentations were performed at 30 °C with constant mixing (400 rpm) for 96 h. Aliquots were taken at time point 0 and at 24 h intervals and pH, titratable acidity (TA) and viable cell count was determined. After fermentation, the mixture was pasteurised at 72 °C for 15 min. Untreated rootlets (BR-UnF) and rootlets which just underwent sterilisation at 90 °C for 30 min (without the addition of sugar) were used as controls. Fermented rootlets and sterilised control rootlets were frozen and freeze-dried to produce dried ingredients.

5.3.3.1 Acidification of rootlets and microbial growth

A 10 g sample of the fermentate was mixed with 95 mL of distilled water and 5 mL of acetone using a magnetic stirrer (Stuart, Thermo Fisher Scientific, Waltham, MA, USA). The pH of the solution was measured using a digital pH meter (Mettler Toledo, Columbus, Ohio, USA) [11]. TA was determined by titration of the mixture with 0.1 M NaOH until a pH of 8.5 was reached [11]. Microbial growth was determined by serial dilution of 10 g of fermentate in 90 mL of sterile Ringers' solution and plating of the dilutions on MRS agar supplemented with 0.05 g/L bromocresol green. Plates were incubated anaerobically using AnaeroGen gas packs (Thermo Fisher Scientific, Waltham, MA, USA) at 30 °C for 48 h and colony forming units were determined by counting [11].

5.3.4 Compositional Analysis of ingredients

5.3.4.1 Basic composition of unfermented barley rootlets

Compositional analysis of the BR-UnF was performed by an accredited lab (Chelab S.r.l, Merieux NutriSciences Corporation, Resana TV, Italy). Protein was measured by Dumas method using a modified version of AOAC 992.23 1992. Fat was determined using the Soxhlet method according to the ISTISAN report [299]. Ash in samples was calculated using AOAC 923.03. Moisture was analysed using ISO 712:2009. Total carbohydrates for rootlets measured by difference based on AOAC 986.25. Fibre was determined using AOAC 2017.2016.

5.3.4.2 Quantification of sugars, organic acids and FODMAPs

All ingredients were lysed using QIAGEN Tissue Lyser II (QIAGEN, Hilden, Germany) for 30 s at a frequency of 30 Hz prior to extraction. All extractions were performed in triplicate unless stated otherwise.

5.3.4.2.1 Extraction and detection of mono-, di-, tri-saccharides and organic acids

Mono-, di-, and tri-saccharides present in BR ingredients were extracted using a protocol outlined previously [162], with the exception that 2 g of sample was exposed to the extraction method instead of 1 g. Glucose, fructose, sucrose, maltose, and maltotriose were quantified via high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) on a Dionex ICS-5000^{+} system (Thermo Fisher Scientific, Sunnyvale, CA) and external standard calibrations between 0.05 – 1 mg/L and 1 – 20 mg/L using the conditions described by Ispiryan et al. [300]. Two different columns were used for the chromatographic separation of the different saccharides, the Dionex CarboPac PA200 (3

mm x 250 mm) and the Dionex CarboPac PA1 columns (2 mm x 250 mm) with their corresponding guards, respectively. The same extracts were used to analyse the organic acids (lactic acid, acetic acid) present in the BR ingredients. The organic acids were quantified on a Dionex Ultimate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA), with ultraviolet light/ diode array detection (UV/DAD; Thermo Fisher Scientific, Waltham, MA, USA) at 210 nm using an external calibration between 0.03 – 6 g/L. Separation of the analytes was achieved using a Hi-Plex H column (8µm, 7.7 mm x 300 mm; Agilent Technologies, Santa Clara CA, USA) with isocratic elution with 5 mM sulfuric acid and a flow rate of 0.5 mL/min at 60 °C.

5.3.4.2.2 Extraction and detection of fermentable oligo-, di- and monosaccharides and polyols (FODMAPs)

FODMAP (Fermentable oligosaccharides, disaccharides, monosaccharides and polyols) levels in BR ingredients were determined according to Ispiryan et al. [300] via HPAEC-PAD using the same analytical set-up as described above for the analysis of the mono-,di- and tri-saccharides. Sample preparation and analysis of the ingredients were performed according to the method described by Ispiryan et al. [300] without any modifications, except for the fructan analysis, which required an additional assay. First according to the method described by Ispiryan et al. [300] two 500 µL aliquots of the diluted sample extracts were hydrolysed with 150 µL of two enzyme mixtures, respectively (A, containing 1:1:1 mixture of amyloglucosidase, α-galactosidase, 0.1 M sodium acetate buffer and B, containing 1:1:1 mixture of amyloglucosidase, α-galactosidase, inulinase). The fructan contents of BR-UnF and BR-Ster were calculated based on the glucose and fructose released from the fructans, as described by Ispiryan et al. [300]. The fructan contents of the fermented BR ingredients were calculated based on only fructose released from fructans (unknown interference impairing accurate determination of glucose released from fructans). Therefore, a third and a fourth 500 µL aliquot of the same diluted extract were incubated with 150 µL sucrase (30 U/mL in 0.1 M sodium maleate buffer) and 150 µL of 50 mg/L sodium azide solution, respectively, for 30 min at 30 °C, providing information on free eluting and matrix associated sucrose in the samples (matrix associated sucrose is not eluting as free sucrose but contributing to an increase in glucose and fructose upon sucrase treatment). The fructan content was then calculated according to equations 6-10. The concentration of fructose released from fructans was calculated according to equation 7, where $F_{A/B}$ are fructose concentrations (µmol/ L) from hydrolysates A and B, and F_S is the fructose released from free and matrix associated sucrose,

calculated according to eq 10; F_{Suc} is the fructose concentration after sucrase treatment and F_{free} represents the fructose in the unhydrolysed sample; 180.16 is the molecular weight (g/mol) of fructose, DF the dilution factor, V_E the extract volume (100 mL) and M_S the sample mass (400 mg). The total fructan content was calculated according to equation 10, with a glucose correction factor (G_f) and water correction factor (k), calculated based on an average degree of polymerisation (DP_{av}) of 4.5 determined from the unfermented and sterilised rootlets (eq 8 and 9).

$$F_S\ (\mu mol/L) = F_{Suc} - F_{free} \quad (6)$$

$$F_f(\%) = \frac{(F_B - F_A - F_S) \times 180.16 \times DF \times V_E}{10\,000 \times M_s} \quad (7)$$

$$k = \frac{180 + 162 \times (DP_{av} - 1)}{180 \times DP_{av}} = 0.92 \text{ (for } DP_{av} = 4.5) \quad (8)$$

$$G_f = \frac{F_f}{DP_{av} - 1} \quad (9)$$

$$\text{Fructan } (\%) = 0.92 \times (G_f + F_f) \quad (10)$$

5.3.4.3 Metabolomic analysis

BR ingredients were lysed with a QIAGEN Tissue Lyser II (Hilden, Germany) for 30 s using a frequency of 30 Hz to optimise extraction. Extractions were performed in duplicate. For extraction, 200 mg of each ingredient was suspended in 1200 µL of autoclaved ultrapure water and vortexed thoroughly. Samples were centrifuged at 16,000 x *g* for 30 min and the supernatant was retained. Three centrifugation cycles were performed, and the pooled supernatants were transferred to micro-centrifuge filters (Merck Ultrafree-CL GV 0.22 µm) for a final centrifugation step (4800 x g, 20 min, 20 °C). The sample supernatants were retained and analysed by MS-Omics, Denmark. Briefly, samples were derivatized with methyl chloroformate using a slightly modified version of the protocol described by Smart et al. [301]. The analysis was conducted using gas chromatography (7890B, Agilent, California, USA) accompanied with a quadrupole mass spectrometry detector (5977B, Agilent, California, USA). Several metabolites typical of the tricarboxylic acid cycle were analysed (pyruvic acid,

succinic acid, fumaric acid, malic acid, α-ketoglutaric acid, cis-aconitic acid, citric acid, isocitric acid and lactic acid). Lactic acid values were also determined in this analysis and included in the metabolomics analysis for completeness. Pyruvic acid values are given as normalised peak areas to an internal standard of deuterium labelled alanine. Data was evaluated using Chemstation (Agilent, California, USA) and Matlab R2018b (Mathworks Inc., Natick, MA, USA).

5.3.4.4 Alpha and beta amylase activity

Alpha-amylase activity of all ingredients was measured in duplicate using the ceralpha method assay kit supplied by Megazyme (Bray, Ireland) with minor modifications. Sample quantity used in the analysis was 1.5 g to accommodate high buffer absorption and to allow for sufficient sample mixing. An additional filtering step was also employed using a HPLC grade polyamide filter (0.2 µm, Thermo Fisher scientific, Waltham, MA, USA) after enzyme extraction to improve clarity of extracts before photometric analysis. The beta amylase activity of all ingredients was measured in duplicate using K-BETA3 assay kit supplied by Megazyme (Bray, Ireland) following assay kit instructions.

5.3.5 Scanning Electron Microscopy

Ingredients were mounted on stubs (G 306; 10 mm x 10 mm Diameter; Agar Scientific, Stansted, UK) and fixed with carbon tape (G3357N; Carbon Tabs 9 mm; Agar Scientific, Stansted, UK). The mounted ingredients were then sputter coated (Polaron E5150 sputter coating unit) using a gold-palladium alloy (ratio of 80/20) and images were captured using a JEOL scanning electron microscope (JSM-5510, Jeol Ltd., Tokyo, Japan). The following settings were used for the analysis: 5 kV 185 voltage, 20 mm working distance and a magnification factor of 1500.

5.3.6 Functional properties of rootlet ingredients

5.3.6.1 Water and oil binding capacity

Water and oil binding capacity measurements were determined using modified methods based on Salama et al [18] and Traynham et al [302] and was expressed as the amount of distilled water/sunflower oil retained per 100 g of sample. Briefly, 0.125 g $\pm$ 0.005g of the BR ingredients were suspended in 6 g of distilled water/sunflower oil and contents of the tubes were vortexed for 3 min. The tubes were allowed to stand for 1 h to allow sufficient uptake of distilled water/sunflower oil and then centrifuged at 4000 x *g* for 30 min at 20 °C. Following centrifugation, the supernatant was carefully removed from the tubes and the tubes

were inverted for 30 min to allow for sufficient drainage. The weight of the tubes content was determined, and water/oil binding capacity was calculated according to the following formula (Equation 11):

$$\frac{(Weight\ of\ tube\ and\ pellet - weight\ of\ tube - sample\ weight)}{sample\ weight} \times 100 \quad (11)$$

5.3.6.2 pH and titratable acidity

The pH and titratable acidity (TA) of each ingredient was carried out using the method outlined by Waters et al. [11], in line with the methods previously described for pH and TA in section 5.3.3.1.

5.3.6.3 Colour

Ingredient colour was measured using a hand-held colorimeter (Minolta CR-331, Komica Minolta Holdings Inc., Osaka, Japan) using the CIE L*a*b* colour measuring system [40,43,282] with adaptations for dry ingredients made. Briefly, fifteen grams of each ingredient was placed in a high precision glass tube cell (40.5 x 60 mm; Hellma analytics, Müllheim, Germany) and a smooth, flat surface was created. Measurements were taken at 5 different points. The differential colour index (ΔE) was calculated to determine the difference in ingredient colour with sterilisation and fermentation processes applied compared to BR-UnF using the following equation 12:

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where $\Delta L^* = L^*control - L^*sample$, $\Delta a^* = a^*control - a^*sample$, $\Delta b^* = b^*control - b^*sample$ (12)

5.3.7 Statistical analysis

All analyses were completed in triplicate unless stated otherwise. A one-way ANOVA with post-hoc Tukey test (p value $\leq$ 0.05) was performed using SPSS statistical software (version 28.0.1.0, IBM SPSS, Chicago, IL, USA) to identify significant differences among groups. In the cases where equal variances were not assumed, a correction using the welch test and a Games Howell (p value $\leq$ 0.05) post hoc test was applied to the data. When data was not normally distributed, a non-parametric Kruskal Wallis test ($p < 0.05$) was used.

5.4 Results

5.4.1. Microbial growth and acidification

The microbial kinetics of *L. citreum* TR116, *L. plantarum* FST 1.7, *W. cibaria* MG1, *L. amylovorus* FST2.11 and *L. reuteri* R29 fermentations was analysed to determine their suitability to the BR substrate. Figure 13 displays the pH, TA and cell counts determined during rootlet fermentation. Acidification kinetics were characterised by changes in pH and TA during fermentation (Figure 13 A). Similar initial pH values (5.34 – 5.42) were determined for all strains, with a steep decline observed during the first 24 h. The pH values remained relatively stable for the remainder of the fermentation period. *L. amylovorus* FST2.11 had the lowest final pH (3.11 ± 0.05), with significantly higher final pH values observed for *L. plantarum* FST1.7 (3.35 ± 0.03), *L. reuteri* R29 (3.66 ± 0.01), *W. cibaria* MG1 (4.09 ± 0.02) and *L. citreum* TR116 (4.04 ± 0.01). Corresponding to the decrease in pH, a significant increase in TA was noted in all fermentations. After 24 h the greatest increase in TA was observed with *L. reuteri* R29 (26.10 ± 0.1 mL of 0.1M NaOH), after which the TA remained steady until the end of the fermentation. The lowest increase in TA was observed with *W. cibaria* MG1, reaching a final value of just 14.82 ± 0.10 mL of 0.1M NaOH after 96 h fermentation. For the *L. citreum* TR116 fermentation, the TA value rapidly increased at the beginning of the fermentation, reaching a maximum value of 21.47 ± 0.06 mL of 0.1M NaOH at T48 and slightly declining after this point. *L. plantarum* FST1.7 and *L. amylovorus* FST2.11 displayed similar acidification patterns, with a continuous increase in TA observed during the first 72 h of fermentation, reaching final values of 29.0 ± 1.41 mL of 0.1M NaOH and 29.97 ± 0.21 mL of 0.1M NaOH at T96, respectively.

Initial cell counts for all strains were between 6.8 - 7.4 log CFU/mL (Figure 13 B). Results showed that all strains reached a maximum cell count within the first 24 h (*W. cibaria* MG1, *L. citreum* TR116 and *L. reuteri* R29) and 48 h (*L. plantarum* FST1.7 and *L. amylovorus* FST2.11) of fermentation, with cell counts of 8.97 – 9.68 CFU/ml. *L. plantarum* FST1.7 had the highest cell count and displayed the highest growth curve while *W. cibaria* MG1 showed the weakest growth, entering the decline phase after T24, with a final cell count 8.04 ± 0.05 CFU/mL at T96. Similarly, *L. citreum* TR116 also entered the death phase after T24 with a final cell count of 8.49 ± 0.15 CFU/mL determined. *L. reuteri* R29 showed strong growth until T48 and rapidly declined thereafter and displayed the lowest cell count at T96 (7.57 ± 0.16 CFU/ mL). Both *L. amylovorus* FST2.11 and *L. plantarum* FST1.7, displayed rapid growth

during the first 48 h of fermentation and remained in the stationary phase thereafter, with significantly higher cell numbers (9.16 ± 0.07 CFU/mL and 9.21 ± 0.13, respectively) observed after 96 h of fermentation compared to the other strains.

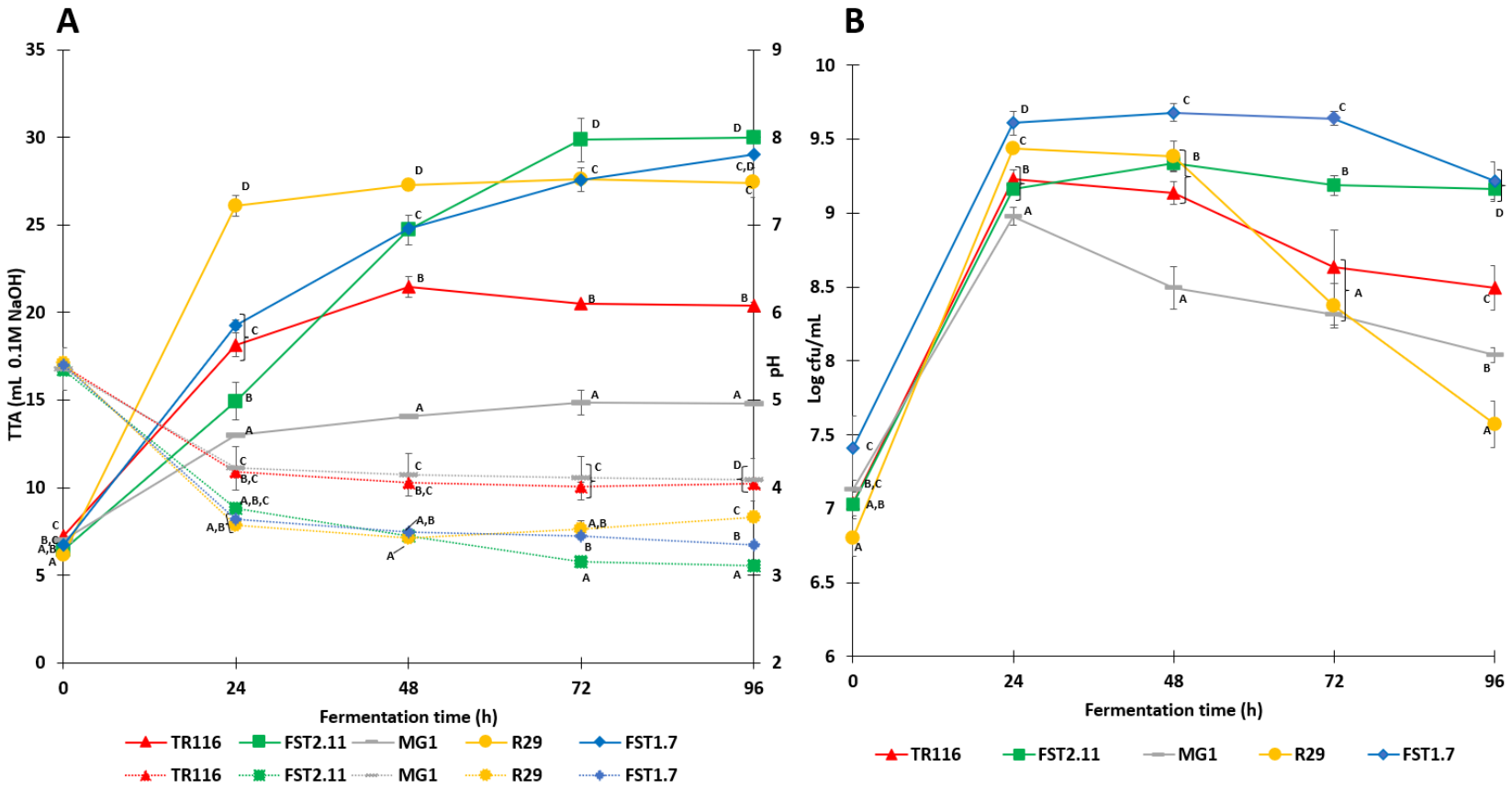

Figure 13. Acidification and microbial growth of *Leuconostoc citreum* TR116 (TR116)( ), *Lactobacillus amylovorus* FST2.11 (FST2.11) ( ), *Weissella cibaria* MG1 (MG1)( ), *Limosilactobacillus reuteri* R29 (R29)( ) and *Lactiplantibacillus plantarum* FST1.7 (FST1.7) ( ). **(A)** Development of Total titratable acids (TTA)(solid line) and pH (dotted line); **(B)** cell count (CFU/mL) during fermentation. Points which share the same letter do not differ significantly**.**

5.4.2 Composition of ingredients

5.4.2.1. Compositional analysis results of BR

Compositional analysis of the raw material (BR-UnF) was completed in order to gain a greater understanding of the basic nutrients present in BR. A protein value of 35.80 ± 1.5 % was determined in BR. In addition, a high amount of fibre (36.64 ± 8.51 %) was present. Of the total fibre, 1.24 ± 0.30 % was soluble fibre whereas 35.40 ± 8.50 % was high molecular weight dietary fibre (combination of insoluble dietary fibre and precipitated soluble dietary fibre). Minimal amounts of fat (1.77 ± 0.11 %) and relatively low levels of ash (5.98 ± 0.30 %) were also determined in BR-UnF.

5.4.2.2. Mono-, di-, trisaccharide profile

Analysis of the mono-, di- and trisaccharides in BR ingredients was determined to provide insight into the effects processing (sterilisation and fermentation) has on their profile. Table 13 illustrates the mono, di- and trisaccharide profile of the BR ingredients after 96 hr fermentation.

Glucose levels were the lowest in BR-UnF (0.261 ± 0.021 g/100g d.m.). A significant increase in glucose levels was noted in BR-Ster (0.521 ± 0.009 g/100g d.m.) while glucose values for BR-TR116 were below the limit of detection. Among the fermented ingredients, the lowest amount of glucose was determined in BR-FST2.11 (2.176 ± 0.029 g/100g d.m) while BR-MG1 had almost four-fold higher amounts of glucose (8.094 ± 0.147 g/100g d.m), significantly higher than that of BR-FST1.7 (4.581 ± 0.050 g/100g d.m.) and BR-R29 (3.190 ± 0.040 g/100g d.m. No significant differences were observed in fructose levels in BR-UnF (1.149 ± 0.076 g/100g d.m) and BR-Ster (1.249 ± 0.023 g/100g d.m). In comparison, a significantly lower amount of fructose was determined in BR-TR116 (0.347 ± 0.006 g/100g d.m) and BR-R29 (0.495 ± 0.004 g/100g d.m). In relation to BR-FST1.7, BR-FT2.11 and BR-MG1 ingredients, significantly higher amounts of fructose were measured with values ranging between 9.991 – 13.234 g/100g d.m. Low levels of sucrose were determined in BR-UnF (0.063 ± 0.002 g/100g d.m.) and BR-Ster (0.016 ± 0.000 g/100g d.m.), with only a minor increase in sucrose contents observed in BR-MG1 (0.586 ± 0.033 g/100g d.m.) and BR-R29 (0.042 ± 0.00 g/100g d.m.). The sucrose content of BR-TR116 was below the limit of detection. Sucrose was present in significant higher amounts in BR-FST1.7 (2.060 ± 0.036 g/100g d.m.) and BR- FST2.11 (2.690 ± 0.069 g/100g d.m.) ingredients, with BR-FST2.11 displaying the highest amount of sucrose. Low levels of maltose and maltotriose were detected in all ingredients ($\leq$ 0.046 g/100g d.m.),

with the exception of the BR-MG1 ingredient which had a significantly higher amount of maltose present (0.137 ± 0.001 g/100g d.m.).

Table 13. Quantities of residual carbohydrates and acids in rootlet ingredients. BR-UnF and BR-Ster represent unfermented barley rootlet and sterilised barley rootlets, respectively. BR-TR116, BR-MG1, BR-FST2.11, BR-29 and FST1.7 represent results for barley rootlets after 96h-fermentation and were fermented with five different lactic acid bacteria: *Leuconostoc citreum* TR116, *Weissella cibaria* MG1, *Lactobacillus amylovorus* FST2.11, *Limosilactobacillus reuteri* R29 and *Lactiplantibacillus plantarum* FST 1.7, respectively. Values reported represent the mean ± standard deviation in g/100g d.m. Values in the same row which share the same uppercase letter do not differ significantly.

	BR-UnF	**BR-Ster**	**BR-TR116**	**BR-MG1**	**BR-FST2.11**	**BR-R29**	**BR-FST1.7**
Sugars [a]							
Glucose	0.261 ± 0.021 ^{A}	0.521 ± 0.009 ^{B}	n.d.	8.094 ± 0.147 ^{F}	2.176 ± 0.029 ^{C}	3.190 ± 0.040 ^{D}	4.581 ± 0.050 ^{E}
Fructose	1.149 ± 0.076 ^{C}	1.249 ± 0.023 ^{C}	0.347 ± 0.006 ^{A}	13.234 ± 0.229 ^{F}	11.870 ± 0.167 ^{E}	0.495 ± 0.004 ^{B}	9.991 ± 0.134 ^{D}
Sucrose	0.063 ± 0.002 ^{C}	0.016 ± 0.000 ^{A}	n.d.	0.586 ± 0.033 ^{D}	2.690 ± 0.069 ^{F}	0.042 ± 0.000 ^{B}	2.060 ± 0.036 ^{E}
Maltose	0.006 ± 0.000 ^{A}	0.137 ± 0.001 ^{D}	n.d.	n.d.	0.041 ± 0.001 ^{C}	0.012 ± 0.001 ^{B}	0.007 ± 0.001 ^{A}
Maltotriose	n.d.	0.026 ± 0.000 ^{B}	0.010 ± 0.000 ^{A}	0.036 ± 0.001 ^{D}	n.d.	0.046 ± 0.001 ^{E}	0.034 ± 0.001 ^{C}
Acids [a]							
Lactic	n.d.	n.d.	2.822 ± 0.035 ^{A}	3.306 ± 0.050 ^{B}	11.743 ± 0.441 ^{D}	5.929 ± 0.036 ^{C}	12.498 ± 0.029 ^{D}
Acetic[b]	0.573 ± 0.019 ^{B}	3.980 ± 0.012 ^{E}	5.523 ± 0.045 ^{G}	1.681 ± 0.024 ^{C}	0.428 ± 0.020 ^{A}	2.060 ± 0.008 ^{D}	4.272 ± 0.031 ^{F}
FODMAPs [a,c]							
Excess Fructose [d]	0.944 ± 0.035 ^{B}	0.764 ± 0.004 ^{B}	0.400 ± 0.004 ^{A}	6.109 ± 0.112 ^{D}	11.780 ± 0.127 ^{E}	0.000 ± 0.000	5.648 ± 0.035 ^{C}
Sorbitol	0.377 ± 0.017 ^{E,F}	0.340 ± 0.002 ^{F}	0.241 ± 0.001 ^{A}	0.283 ± 0.002 ^{D}	0.278 ± 0.002 ^{C}	0.258 ± 0.002 ^{B}	0.292 ± 0.002 ^{E}
Mannitol	0.025 ± 0.000 ^{A}	0.024 ± 0.000 ^{A}	20.074 ± 0.065 ^{D}	n.d.	n.d.	16.779 ± 0.292 ^{C}	0.149 ± 0.001 ^{B}
Σ Polyols	0.402 ± 0.003 ^{B,C}	0.364 ± 0.000 ^{B}	20.315 ± 0.066 ^{E}	0.283 ± 0.002 ^{A}	0.278 ± 0.002 ^{A}	17.037 ± 0.291 ^{D}	0.441 ± 0.003 ^{C}
Raffinose/Stachyose	0.012 ± 0.000 ^{A}	0.035 ± 0.000 ^{B}	n.d.	0.101 ± 0.001 ^{C}	n.d.	n.d.	n.d.
Verbascose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Σ GOS [e]	0.012 ± 0.000 ^{A}	0.035 ± 0.000 ^{B}	-	0.101 ± 0.001 ^{C}	-	-	-
Fructans	1.711 ± 0.083 ^{C}	1.786 ± 0.042 ^{C,D}	0.895 ± 0.022 ^{A}	1.277 ± 0.123 ^{B}	1.277 ± 0.099 ^{B}	1.925 ± 0.014 ^{D}	1.090 ± 0.041 ^{A,B}

[a] n.d. is defined as not detected (below LOD) or levels below 0.002 g/100g (sugars), 0.030 g/100g (acids) and 0.012 g/100g (FODMAPs).[b] Acetic acid values are overestimated due to an unknown compound coeluting at the same retention time. [c] No lactose was detected in any of the FODMAP extracts and therefore was not included in the table. [d] Excess fructose = fructose – glucose. [e] – no α-galactooligosaccarides present.

5.4.2.3. Organic Acids

An analysis of the organic acids present in BR was completed to determine the native acids present in BR as well as providing insight into the extent of acids produced during each LAB fermentation. A summary of the amounts of lactic and acetic acid present in the final BR ingredients are shown in Table 13.

Lactic acid was not determined in the BR-UnF and BR-Ster ingredients, as values were below the limit of detection. Lactic acid was detected in all BR fermented ingredients, with significant differences found between all groups. The highest amount of lactic acid was measured in BR-FST1.7 (12.498 ± 0.029 g/100g d.m.), followed closely by BR-FST2.11 (11.743 ± 0.441 g/100g d.m.). Almost half this amount of lactic acid was determined in BR-R29 (5.929 ± 0.036 g/100g d.m.), while a further two-fold decrease in the amount of lactic acid present was found in BR-MG1 (3.306 ± 0.050 g/100g d.m.) and BR-TR116 (2.822 ± 0.035 g/100g d.m.). Concerning the acetic acid levels illustrated in Table 13, values are likely to be overestimated as chromatogram peaks for acetic acid are impure, due to an unknown compound coeluting at the same retention time as acetic acid. The coeluting compound had a UV max of 267 nm. Further identification of unknown compounds was outside the scope of this study. Nonetheless, trends may still be examined. Low amounts of acetic acid were found in BR-UnF (0.573 ± 0.019 g/100g d.m.). In comparison, significantly higher levels of acetic acid were determined in BR-Ster (3.980 ± 0.012 g/100g d.m.), and in the fermented rootlet ingredients. BR-FST2.11 contained lower amount of acetic acid (0.428 ± 0.020 g/100g d.m.) compared to BR-UnF. Significantly higher amounts of acetic acid were recorded in BR-MG1 (1.681 ± 0.024 g/100g d.m.), BR-R29 (2.060 ± 0.008 g/100g d.m.) and BR-FST1.7 (4.272 ± 0.031 g/100g d.m.), with the highest amount determined in BR-TR116 (5.523 ± 0.045 g/100g d.m.).

5.4.2.4. FODMAPs

FODMAPs analysis was completed to provide fundamental knowledge on the level of these nutrients present in BR as well as showing the altering effects LAB fermentation can have on the FODMAPs present. FODMAPs detected in BR ingredients are illustrated on Table 13. Lactose was below the limit of detection in all samples.

The amount of excess fructose (EF) present in BR-UnF (0.944 ± 0.035 g/100g d.m.) and BR-Ster (0.764 ± 0.004 g/100g d.m.) were comparable, with no significant differences found. EF was not determined in BR-R29, while low levels were present in BR-TR116 (0.400 ± 0.004 g/100g d.m.). In contrast, almost two-fold more EF was measured in BR-FST1.7 (5.648 ± 0.035 g/100g d.m.) and BR-MG1 (6.109 ± 0.112 g/100g d.m.), with the highest amount of EF

determined in BR-FST2.11 (11.780 ± 0.127 g/100g d.m.). The sorbitol levels of BR-UnF (0.377 ± 0.017 g/100g d.m.) and BR-Ster (0.340 ± 0.002 g/100g d.m.) were comparable, while the sorbitol contents of the fermented ingredients were slightly lower ($\leq$ 0.292 ± 0.002 g/100g d.m.). Both BR-UnF (0.025 ± 0.000 g/100g d.m.) and BR-Ster (0.024 ± 0.000 g/100g d.m.) contained low levels of mannitol. In comparison, the mannitol contents in BR-MG1 and BR-FST2.11 were below the limit of detection, while BR-FST1.7 contained a slightly higher amount (0.149 ± 0.001 g/100g d.m.). Significant levels of mannitol were found in BR-R29 (16.779 ± 0.292 g/100g d.m.), with the highest amount measured in BR-TR116 (20.074 ± 0.065 g/100g d.m.). Trends in the levels of total polyols were relative to that of the mannitol and sorbitol contents of the ingredients. Overall, low levels of polyols were determined in BR-FST2.11, BR-MG1 and BR-FST1.7 along with BR-UnF and BR-Ster, with values ranging between 0.278 – 0.441 g/100g d.m. Over 17 g/100g of polyols were found in BR-R29 (17.037 ± 0.291 g/100g d.m.), while the highest content of polyols was measured in BR-TR116 (20.315 ± 0.066 g/100g d.m.). Raffinose/Stachyose levels were detected at low levels in BR-UnF (0.012 ± 0.000 g/100g d.m.) and BR-Ster (0.035 ± 0.000 g/100g d.m.) The highest amount of raffinose/stachyose was determined in BR-MG1 (0.101 ± 0.001 g/100g d.m.) while raffinose/stachyose amounts in BR-TR116, BR-FST2.11, BR-29 and BR-FST1.7 were below the limit of detection. Verbascose was not detected in any of the BR ingredients. With regards to GOS (α-galactooligosaccharides), residual GOS levels were similar to the trends discussed previously for raffinose/stachyose values. Similar amount of fructans were determined in BR-UnF (1.711 ± 0.083 g/100g d.m.) and BR-Ster (1.786 ± 0.042 g/100g d.m.), with no significant differences found between ingredients. In comparison, a significant reduction in the level of fructans present was noted in all BR fermented ingredients, with the exception of BR-R29 where a significant increase in fructans was determined (1.925 ± 0.014 g/100g d.m.).

5.4.2.5. Alpha and beta amylase activity

Determination of alpha and beta amylase activity in BR ingredients was conducted to enhance knowledge on the enzymatic activity of BR ingredients and the effects that processing can have on their activity. Alpha and beta amylase activity levels of the BR ingredients are shown in Table 14.

Alpha amylase activity of BR-UnF was 5.12 ± 0.302 CU/g d.m. A significant decrease in the alpha amylase activity was found post sterilisation of the BR (0.026 ± 0.000 CU/g d.m.). Similar to BR-Ster, very low alpha-amylase activities were determined in all of the fermented BR ingredients, with values ranging from 0.00 – 0.041 CU/g d.m. Beta amylase activity showed

similar trends, with the highest beta amylase activity found in the BR-UnF (0.772 ± 0.185 CU/g d.m.) and significantly lower activity determined for BR-Ster (0.010 ± 0.014 CU/g d.m.) and fermented BR ingredients.

Table 14. Analysis of the functional properties of rootlet ingredients. BR-UnF and BR-Ster represent unfermented barley rootlet and sterilised barley rootlets, respectively. BR-TR116, BR-MG1, BR-FST2.11, BR-29 and BR-FST1.7 represent barley rootlets fermented with: *Leuconostoc citreum* TR116, *Weissella cibaria* MG1, *Lactobacillus amylovorus* FST2.11, *Limosilactobacillus reuteri* R29 and *Lactiplantibacillus plantarum* FST1.7, respectively. The values in the table represent the mean ± standard deviation. No statistical difference was found with values in the same row which share the same letter.

Properties	BR-UnF	BR- Ster	BR-TR116	BR-MG1	BR-FST2.11	BR-R29	BR-FST1.7
Water-Binding Capacity (g H_2O/100 g sample)	500.29 ±11.02 ^{c}	523.90 ± 25.78 ^{c}	427.33 ± 17.20 ^{a,b}	377.76 ± 18.61 ^{a}	459.25 ± 39.09 ^{b,c}	384.07 ± 23.88 ^{a}	418.09 ± 25.25 ^{a,b}
Oil-Binding Capacity (g oil/100 g sample)	237.20 ± 22.74 ^{a}	367.93 ± 21.24 ^{c}	409.39 ± 12.98 ^{d}	287.87 ± 9.62 ^{b}	305.61 ± 3.50 ^{b}	319.90 ± 5.27 ^{b}	290.23 ± 3.84 ^{b}
pH	5.20 ± 0.01 ^{d}	5.16 ± 0.01 ^{c,d}	4.09 ± 0.02 ^{b,c}	4.09 ± 0.01 ^{b,c,d}	3.37 ± 0.01 ^{a}	3.69 ± 0.01 ^{a,b,c}	3.38 ± 0.01 ^{a,b}
Total Titratable Acidity (mL 0.1 M NaOH/g)	4.5 ± 0.13 ^{a}	6.08 ± 0.06 ^{b}	10.55 ± 0.21 ^{d}	8.42 ± 0.0.11 ^{c}	16.82 ± 0.38 ^{f}	17.01 ± 0.25 ^{f}	14.31 ± 0.06 ^{e}
Colour (ΔE - BR-UnF)	-	8.15 ± 0.77 ^{b}	9.13 ± 0.57 ^{b}	10.14 ± 1.15 ^{b}	3.65 ± 0.47 ^{a}	8.32 ± 0.85 ^{b}	9.02 ± 0.25 ^{b}
Alpha amylase (cu/g d.m)	5.120 ± 0.302 ^{b}	0.026 ± 0.000 ^{a}	0.000 ± 0.000 ^{a}	0.025 ± 0.005 ^{a}	0.030 ± 0.000 ^{a}	0.025 ± 0.015 ^{a}	0.041 ± 0.016 ^{a}
Beta amylase (cu/g d.m)	0.772 ± 0.185 ^{b}	0.010 ± 0.014 ^{a}	0.000 ± 0.000 ^{a}	0.006 ± 0.000 ^{a}	0.124 ± 0.000 ^{a}	0.045 ± 0.016 ^{a}	0.035 ± 0.017 ^{a}

5.4.2.6. Metabolite Analysis

Analysis of the metabolomic profile of BR ingredients provides detail on the major pathways occurring during LAB fermentation while also gaining better insight on the level of these metabolites present at the end of fermentation. Figure 14 demonstrates the main metabolites of the tricarboxylic cycle present in the BR ingredients. In relation to the compounds fumaric acid, 2-oxoglutaric acid, cis-aconitic acid and iso-citric acid, negligible amounts (0.022 – 0.298 g/L, results not shown) were measured in all BR ingredients, with little difference observed between samples and were therefore not included on Figure 14.

Pyruvic acid was detected at negligible levels in BR-UnF and BR-Ster (<0.298 ± 0.041). Similarly, low levels of pyruvic acid were found in BR-TR116 (0.376 ± 0.063) and BR-MG1 (0.162 ± 0.029) with a slight increase in pyruvic acid in BR-FST1.7 (0.930 ± 0.006). Pyruvic acid amounts in BR-FST2.11 (2.857 ± 0.022) increased greatly, with the highest amount determined in BR-R29 (5.345 ± 0.915). Significantly higher amounts of citric acid were present in BR-UnF (4.158 ± 0.184 g/L) and BR-Ster (4.444 ± 0.294 g/L), with a reduction observed in BR-FST2.11 (2.546 ± 0.349 g/L), BR-MG1 (2.529 ± 0.031 g/L), and BR-FST1.7 (1.233 ± 0.051 g/L). Citric acid levels in BR-TR116 and BR-R29 were below the limit of detection. Succinic acid was detected in very low amounts in BR-UnF and BR-Ster as well as BR-TR116 and BR-FST1.7, with values in the range of 0.243 – 0.715 g/L. An approximately three-fold increase in succinic acid levels were determined for BR-FST2.11, BR-MG1 and BR-R29 (2.949 – 3.340 g/L). High amounts of malic acid were found in the control ingredients BR-UnF (5.255 ± 0.039 g/L) and BR-Ster (5.510 ± 0.390 g/L), whereas much lower levels were present in in the fermented BR ingredients. Comparing the fermented BR ingredients, BR-FST2.11 contained the highest amount of malic acid (1.208 ± 0.023 g/L) with negligible amounts (0.149 – 0.233 g/L) found in the remaining ingredients. Lactic acid amounts were very low in BR-UnF and BR-Ster ingredients (0.339-0.473 g/L), with little differences observed between samples. High levels of lactic acid were measured in the BR fermented ingredients. The highest amount of lactic acid was determined in BR-FST1.7 (11.252 ± 0.901 g/L) followed sequentially in descending order by BR-FST2.11 (10.781 ± 1.862 g/L), BR-R29 (9.002 ± 0.182 g/L), BR-MG1 (8.755 ± 1.112 g/L) and BR-TR116 (7.051 ± 0.141 g/L).

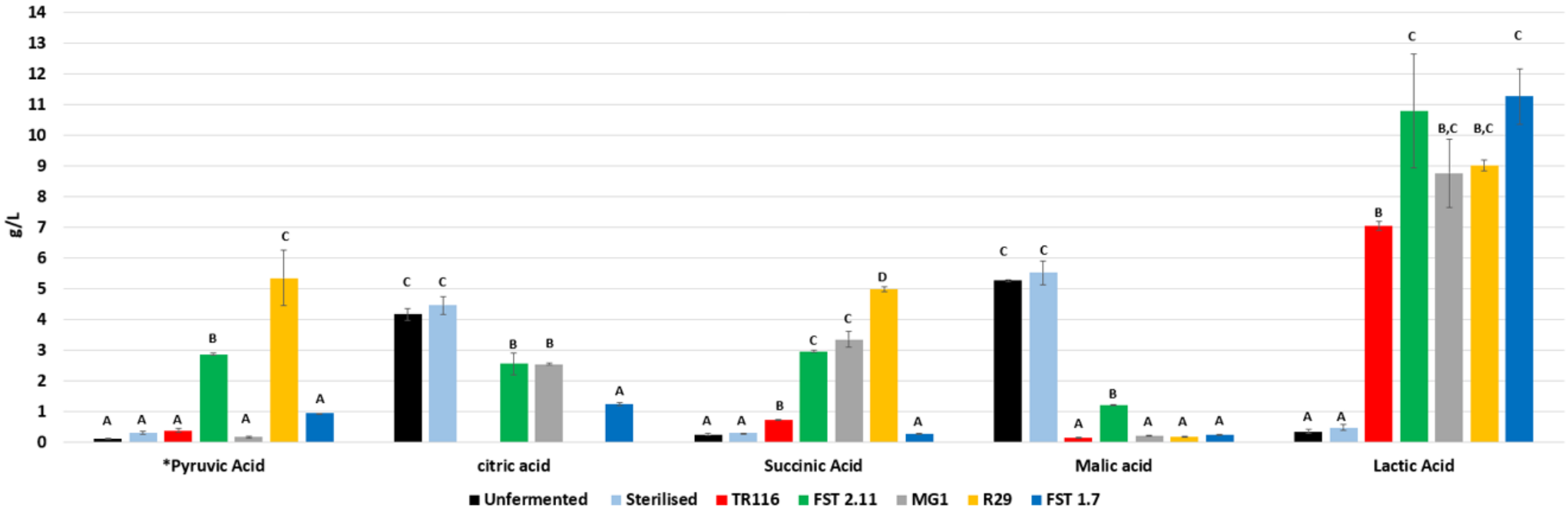

Figure 14. GC-MS Metabolomic analysis of rootlet ingredients. TR116, FST2.11, MG1, R29 and FST 1.7 represent barley rootlets fermented with *Leuconostoc citreum* TR116, *Lactobacillus amylovorus* FST2.11, *Weissella cibaria* MG1, *Limosilactobacillus reuteri* R29 and *Lactiplantibacillus plantarum* FST1.7, respectively. Error bars on the graph indicate the standard deviations found within samples. *Indicates relative peak area values which are normalised based on an internal standard (deuterium labelled alanine). No statistical difference was found with values in the same row which share the same letter.

5.4.3 Scanning Electron Microscopy

Scanning Electron Microscopy analysis was performed on the unfermented, sterilised and LAB fermented ingredients to gain insight into the effects of the processing of BR on the external structure of the ingredient (Figure 15).

As seen in Figure 15 (A) and Figure 15 (B), smooth, fibrous, and intact structures were visible in BR-UnF. The surface of BR-UnF (Fig 15 B) appears to be a continuous matrix with smaller particles dispersed within longer fibrous strands. In contrast, a much more disrupted, broken, and discontinuous structure was evident in BR-Ster (Fig 15 C, Fig 15 D). Particles present in BR-Ster are more fragmented and brittle, indicating some disruption to the fibrous structures present. Figures 15E and 15F characterises BR-R29 and is representative of all fermented ingredients, as little differences were observed between fermented samples. The structure of BR post fermentation more closely resembled BR-Ster rather than BR-UnF, displaying a more severed, uneven surface.

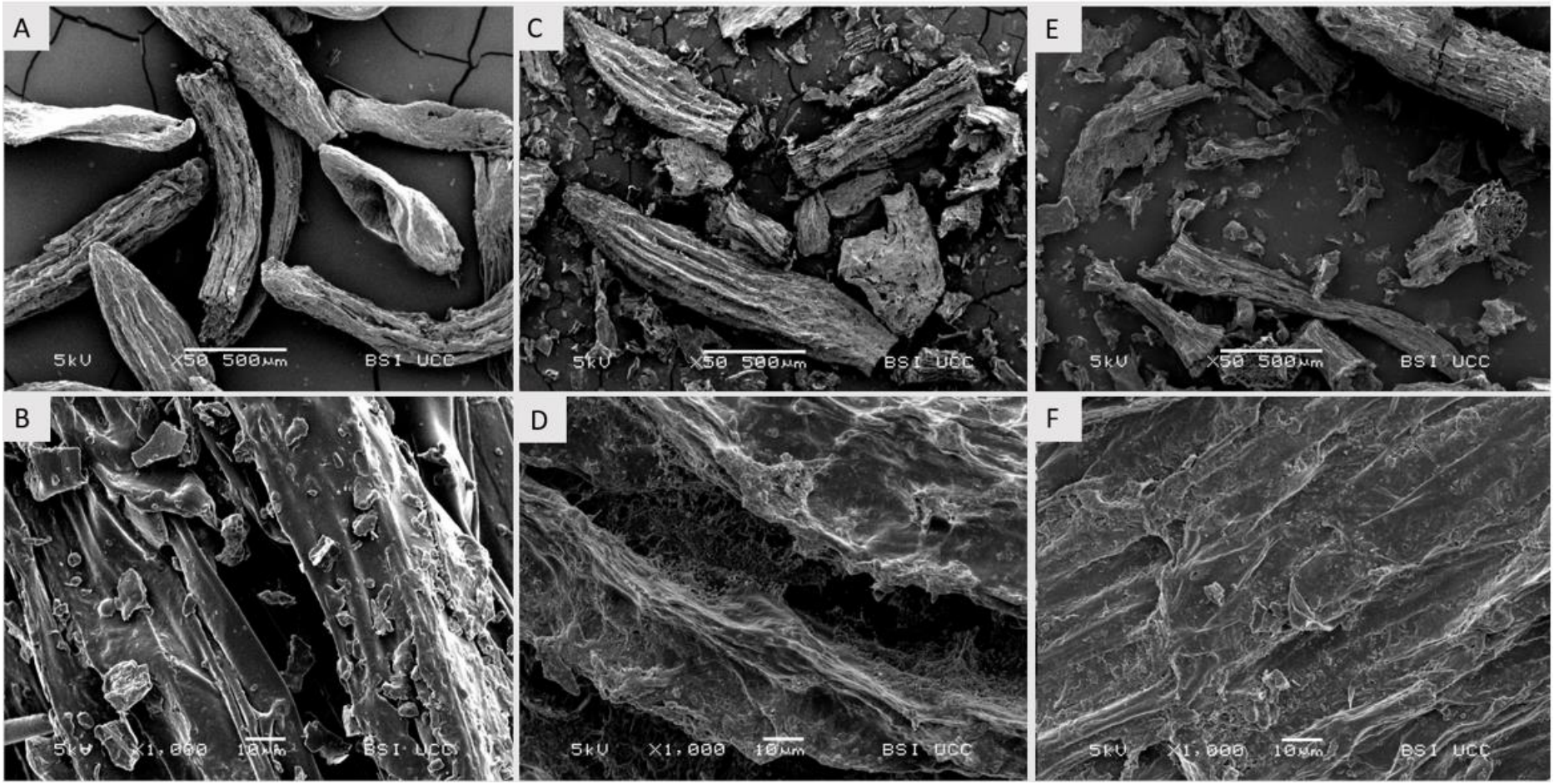

Figure 15. Scanning Electron Micrograph (SEM) of unfermented rootlets (Fig 14A and Fig 14B), sterilised rootlets (Fig 14C and Fig 14D) and rootlets fermented with *Limosilactobacillus reuteri* R29 (Fig 14E and 14F) at magnifications of x50 (A,C,E) and x1000 (B,D,F).

5.4.4 Techno-functional properties of BR ingredients

5.4.4.1. Water and oil binding capacity

The WBC and OBC of the BR ingredients are depicted in Table 14. The highest WBC was determined in BR-Ster (523.90 ± 25.73 g H_2O/ 100g BR) followed by BR-UnF (500.29 ± 11.02 g H_2O/ 100g BR) with no significant differences found between the samples. BR-FST2.11 also had a high WBC (459.25 ± 35.09 g H_2O/ 100g BR) and was comparable to BR-UnF and BR-Ster. Fermentation resulted in a significant reduction in the WBC of the ingredients. A significant reduction in WBC was measured in BR-TR116 (427.33 ± 17.20 g H_2O/ 100g BR), BR-FST1.7 (418.09 ± 25.25 g H_2O/ 100g BR), BR-R29 (384.07 ± 23.88 g H_2O/ 100g BR) and BR-MG1 (377.76 ± 18.61 g H_2O/ 100g BR) compared to BR-UnF and BR-Ster. With respect to the OBCs, BR-UnF had the lowest OBC (237.20 ± 22.74 g oil/100g BR). Heat treatment appeared to increase the OBC significantly, with a value of 367.93 ± 21.24 g oil/100g BR for BR-Ster. Similarly, a significant increase in OBC was also observed after fermentation with the OBC of the fermented BR ingredients ranging from 287.87 – 409.39 g oil/100g BR. The highest OBC was observed in BR-TR116 and the lowest in BR-MG1, with values of 409.39 ± 12.98 g oil/100g BR and 287.87 ± 9.62 g oil/100g BR determined, respectively.

5.4.4.2. pH, TA and colour

The pH, TA and colour of the BR ingredients are shown in Table 14. About the ingredient pH, BR-UnF (5.20 ± 0.01) and BR-Ster (5.16 ± 0.01) had the highest pH values, with no significant differences found between samples. As expected, the fermented BR ingredients had a lower pH. Similar pH values were measured for BR-MG1 (4.09 ± 0.01) and BR-TR116 (4.09 ± 0.02), while BR-R29 had a pH of 3.69 ± 0.01. The lowest pH values were observed for BR-FST1.7 and BR-FST2.11, with values of. 3.38 ± 0.01 and 3.37 ± 0.01 determined, respectively. BR-UnF had the lowest TA value (4.5 ± 0.13 0.1M NaOH/ g) with a significantly higher TA value obtained for BR-Ster (6.08 ± 0.06 0.1M NaOH/ g). The TA values for fermented BR ingredients were significantly higher, ranging from 8.42 ± 0.11 – 17.01 ± 0.25 0.1M NaOH/ g, depending on the LAB strain used for fermentation. Regarding the ΔE values recorded for the BR ingredients, a colour change was noted in the fermented ingredients compared to BR-UnF. No significant differences were found between samples, except for in the case of BR-FST2.11 (3.65 ± 0.47), which had a significantly lower ΔE value compared to all other BR ingredients.

5.5 Discussion

The implementation of by-products into the food chain has been a challenge and often requires further processing to improve their viability. Research relating to the rejuvenation of BR is limited and an in-depth analysis of their potential as food ingredients using fermentation as a processing aid has not yet been explored. The LAB strains *W. cibaria* MG1, *L. amylovorus* FST2.11, *L. plantarum* FST1.7, *L. reuteri* R29 and *L. citreum* TR116 have been used across a variety of studies using various substrates [160,162,295,297,298,174,284,286,287,290,291,293,294] however, their behaviour in a BR system has not yet been investigated.

Firstly, the variation in the growth of the LAB suggests that BR are a more favourable substrate for growth for some LAB than others. The rapid entry of *L. citreum* TR116, *W. cibaria* MG1 and *L. reuteri* R29 into the decline phase and the ability for *L. plantarum* FST1.7 and *L. amylovorus* FST2.11 to maintain high cell counts suggests that BR may serve as a more suitable substrate for *L. plantarum* FST1.7 and *L. amylovorus* FST2.11 over longer fermentation times. However, other factors such as strain origin and acid tolerance are likely to be contributing factors to the growth observed. *L. plantarum* FST1.7 and *L. amylovorus* FST2.11 have been described as strains which are tolerant to acidic environments [286,287,296] which may explain its ability to maintain high cell counts until the end of these fermentations (96 h). In addition, both *L. plantarum* FST1.7 and *L. amylovorus* FST2.11 originate from a brewing environment, indicating they may have had higher adaptability to the BR matrix. In the case of the *W. cibaria* MG1, comparable maximum cell counts were observed after 24 h in a previous study using a quinoa-based fermented yoghurt alternative [293]. Although *W. cibaria* MG1 originates from a cereal environment, its low tolerance to acid may have inhibited growth after T24. Peyer et al. [296] observed similar growth kinetics and pH values with *W. cibaria* PS2 and suggested that *W. cibaria* strains may have lower intrinsic acid tolerance. Growth curves for *L. reuteri* R29 and *L. citreum* TR116 were comparable to those previously described in other studies [162,296], reaching a maximum cell count between 40 – 48 h.

The final pH and TA values obtained during the fermentation coincide and may be explained by the lactic and acetic acid levels determined. Relatively high TA values and greater reductions in pH were observed with *L. amylovorus* FST2.11, *L. reuteri* R29 and *L. plantarum* FST1.7 and were complimented by higher amounts of lactic and acetic acid detected in these ingredients. *W. cibaria* MG1 did not reduce the pH and increase TA measurements to the same

extent as the other LAB which may be explained by the lower lactic and acetic acid contents in BR-MG1, corresponding to the poorer growth kinetics previously discussed. Similar pH values were recorded for BR-TR116 and BR-MG1 however, the higher TA value attained for BR-TR116 is likely due to the higher acetic acid content in BR-TR116 compared to BR-MG1. Acetic acid is a weaker acid with a pKa value of 4.75 verses lactic acid which has a pKa value of 3.86, thus, lactic acid has a stronger influence in reducing pH than acetic acid [303,304]. This type of behaviour has also been discussed in alternative yoghurt systems [160]. In addition, the buffering capacities of lactic and acetic acid could play a role in the differences in TA values observed. Acetic acid has an optimal buffering capacity in the pH range of 3.75 – 5.75 compared to lactic acid which lies in the range of 2.86 – 4.86 [174,305]. As the starting pH of the ingredient was approx. 4.09 and the end point required for the TA analysis was 8.5, this pH dimension favoured the acetic acid buffering capacity range. Thus, higher volumes of 0.1M NaOH were needed to reach pH 8.5, resulting in a higher TA value for BR-TR116 [174].

Lactic and acetic acid quantities are largely dependent on the homo- or hetero- fermentative metabolisms of LAB. The Emden-Meyerhoff-Parnas pathway is employed during homofermentative metabolism (*L. amylovorus* FST2.11) where hexoses are converted to pyruvate, which is later converted to lactic acid catalysed by lactate dehydrogenase [306]. For heterofermentative metabolism (*L. citreum* TR116, *L. plantarum* FST1.7, *L. reuteri* R29, *W. cibaria* MG1) hexoses are metabolised using the phosphoketolase pathway. Similar to homofermentative metabolism, this results in the production of lactic acid from pyruvate, however other end products such as CO_2 and ethanol or acetic acid can also be produced [306]. However, alternative routes of pyruvate must also be considered which are both strain and condition sensitive [306]. As expected, *L. amylovorus* FST2.11 produced almost exclusively lactic acid and has previously been used in beer souring for this purpose [286]. *L. plantarum* FST1.7, has been documented as a strong acidifier in sourdough, acidified wort and malt-based substrates [277,287,296], therefore high amounts of lactic and acetic acid were to be expected. Similarly, *L. citreum* TR116 and *L. reuteri* R29 are capable of producing both lactic and acetic acid. The elevated levels of acetic acid in the BR-TR116 is a reflection of the capability of *L. citreum* TR116 to produce acetic acid using pyruvate dehydrogenase complex as well as the tendency for the strain to produce acetic acid from acetyl phosphate when fructose is present [283]. Although *W. cibaria* MG1 is a heterofermentative strain [288], the lower amounts of lactic and acetic acid in the BR-MG1 might be a reflection of the poorer growth of *W. cibaria* MG1 in BR, as demonstrated in Figure 13B.

The analysis of mono-,di- and trisaccharides in the BR ingredients give a further insight into variations in LAB growth and metabolism. Firstly, the mono-, di and tri-saccharide levels were low in BR-UnF, suggesting few are readily available in BR. Values obtained in this study were lower than previously reported [11], likely due to variances in extraction protocols, diversities in germination processes and also differences in sieved BR fractions. The slight increase observed in the short chain carbohydrates in the BR-Ster ingredient might be explained by enzymes naturally present in BR. BR contain exceptionally high levels of alpha amylase and reasonable levels of beta amylase, with literature also suggesting the presence of invertase [73]. Thus, the gradual increase in temperature during the sterilisation process is likely to activate these enzymes at different time points, resulting in a very slight increase in mono-, di- and tri-saccharide profiles. A comparison of these profiles of the fermented ingredients, primarily glucose, fructose and sucrose, reveals differences in LAB metabolism. The reduction in supplemented fructose and complete depletion of naturally occurring glucose to below detectable levels observed in BR-TR116 can be explained by the phosphoketolase metabolic pathway (glucose) and the presence of mannitol-2-dehydrogenase (fructose) expressed by *L. citreum* TR116 [283]. The *L. reuteri* R29 fermentation was supplemented with sucrose, which was hydrolysed to glucose and fructose, hence its depletion. Results from this study suggest that *L. reuteri* R29 preferred fructose, as levels of this sugar were reduced compared to glucose. Previous studies using *L. reuteri* R29 revealed maltose as the preferred substrate [294,296]. However, as maltose was present in minor amounts in the BR substrate *L. reuteri* R29 likely used fructose as its primary carbon source, activating mannitol-2-dehydrogenase, an enzyme which has been found in some *L. reuteri* strains [307–309]. *W. cibaria* MG1 can utilise sucrose, maltose, fructose, ribose, xylose, gluconate and galactose (using Leloir pathway) as carbon sources [288]. High amounts of glucose and fructose were present in BR-MG1, likely as a result of the poorer growth of *W. cibaria* MG1 and subsequent lower metabolic activity leading to the presence of residual monosaccharides. In the case of the BR-FST2.11 and the BR-FST1.7 ingredients, the residual sucrose, fructose, and glucose in the ingredient again might be related to the growth pattern of the *L. plantarum* FST1.7 and *L. amylovorus* FST2.11 strains. During fermentation, these strains remained in the stationary phase of growth suggesting sufficient sugar substrate was available. The excess of fructose rather than glucose also suggests that *L. plantarum* FST1.7 and *L. amylovorus* FST2.11 had preference for glucose as carbon source when fermented in a BR matrix. Peyer et al. [286,296] also reported a similar trend in sugar metabolism with *L. plantarum* FST1.7 and *L. amylovorus* FST2.11, with both strains showing a preference for glucose during the fermentation of malt-based substrates and in sour beer

production. As sugar substrate was not limiting at the end of fermentation for *L. plantarum* FST1.7 and *L. amylovorus* FST2.11, the restriction to growth might be linked to the low pH and possible limitation of other LAB metabolic growth factors.

Much like the mono- and disaccharide profile, diversities in the FODMAP profile of the fermented BR ingredients were also observed. The term FODMAP refers to a class of short-chain carbohydrates which are poorly digested in the small intestine and migrate to the large intestine where they can be utilised as a carbon source for the gut microflora [310]. This study shows that BR are naturally high in FODMAPs and may contain higher levels than those found in the barley grain itself, depending on the variety of barley used [310]. Thus, for a person who does not suffer from digestive issues such as irritable bowel syndrome, BR can be implemented in the diet as a natural source of prebiotics [311].

The main FODMAPs found in the BR ingredients were polyols, EF and fructans, as all other FODMAPs measured were not found in significant amounts. Among the polyols detected, mannitol is of greater interest since sorbitol levels did not vary greatly during sterilisation and fermentation. The high levels of mannitol in the BR-TR116 and BR-R29 ingredients provide evidence for the expression of mannitol-2-dehydrogenase during fermentation which reduces fructose to mannitol [283,312]. This also gives plausible reason for the lowest amounts of EF found in these ingredients. The mannitol-2-dehydrogenase gene is generally absent in most *W. cibaria* strains which explains the lack of mannitol in the BR-MG1 ingredient [313]. *L. amylovorus* FST2.11 and *L. plantarum* FST1.7, do not appear to synthesise mannitol-2-dehydrogenase when BR is used as the primary substrate, hence the undetected or negligible levels of mannitol. As these strains likely lacked the mannitol-2-dehydrogenase enzyme, subsequently fructose was not converted to mannitol which may justify the high EF levels in the BR-MG1, BR-FST2.11 and BR-FST1.7 ingredients. The hyper production of mannitol in BR-TR116 and BR-R29 may be a favourable characteristic imparted by *L. citreum* TR116 and *L. reuteri* R29 strains, as mannitol may be used as a sugar replacer in food products [174,248,284,298].

Fructans, which are a non-digestible storage carbohydrate in a variety of plants, are comprised of β (2-1) and/or β (2-6) connected fructose chains (both linear and branched) and a single glucose residue [310,314], and were measured in considerable amounts in BR. Previous studies by Ispiryan et al. [314] investigated the effect of malting on the FODMAP profile of a variety of wholegrains (cereals, pseudo cereals, pulses) and revealed that the level of fructans in barley,

wheat and oat increased during the malting process, particularly during the germination phase. Thus, the high levels of fructans in BR is likely related to their synthesis during germination. Different trends were noted in the fermented BR ingredients with some increases (BR-R29) and decreases (BR-TR116, BR-MG1, BR-FST2.11, BR-FST1.7) in fructans observed. The increase in fructans in the BR-R29 ingredient might be explained by the metabolism of *L. reuteri*. *L. reuteri* 121 has previously been shown to synthesise fructans of the levan type and high molecular weight inulin using levansucrase and inulosucrase enzymes [315–318]. Thus, it is plausible that *L. reuteri* R29 may also have the same potential. This may be of benefit when formulating for high fibre products which aim to prevent fructan degradation, particularly in the bread system [319]. The reduction in fructan content in all other BR ingredients indicates that *L. citreum* TR116, *W. cibaria* MG1, *L. amylovorus* FST2.11 and *L. plantarum* FST1.7 can degrade fructans in BR, which may be characteristic of interest for these strains as a bio-technological aid when formulating low FODMAP foods/ingredients [320,321].

Metabolomic analysis provided some insights into LAB metabolism during fermentation of BR. As expected, the most abundant compound of the tricarboxylic acid (TCA) cycle was lactic acid with amounts present following trends of lactic acid values previously discussed. In the case of BR-FST2.11 and BR-FST1.7, the residual levels of pyruvate might be a result of the ongoing pathways previously mentioned, as the growth patterns of both of these strains at the end of fermentation was stable. The residual levels of pyruvic acid in BR-R29 may indicate inhibition of the lactate dehydrogenase enzyme over the course of the fermentation process due to entry into the death phase after T48. Furthermore, pyruvate may also be produced from citric acid, a compound which appears to be naturally present in BR. Citric acid can be used as an alternative carbon source which is converted to oxaloacetate, followed by pyruvate, using the citrate lyase complex and oxaloacetate decarboxylase, respectively [313,322]. In addition, citrate and sugar co-metabolism can result in the excess production of pyruvate which might also give reason for excess pyruvate accumulation in these BR ingredients [322]. Citric acid was fully depleted in BR-R29 and BR-TR116 ingredients and only partially used in the other fermented BR ingredients, highlighting the ability of *L. reuteri* R29 and *L. citreum* TR116 to utilise citric acid as an alternative carbon source. Citric acid metabolism in *L. reuteri* has also been demonstrated in other studies [323–325] while expression of a citrate-lyase gene has been reported for *L. citreum* TR116 [283] as well as other *Leuconostoc* spp. [322]. Citric acid metabolism has also been shown to occur in *L. plantarum* [326,327] which supports the decreased level of citric acid in BR-FST1.7. Citric acid metabolism can also result in the

formation of succinic acid through a series of enzyme catalysed reactions [313,328]. Thus, the higher amounts of succinic acid in BR-R29, BR-MG1 and BR-FST2.11 indicates this pathway might have been favoured during fermentation, particularly during *L. reuteri* R29 fermentation [323,329]. Much like citric acid, malic acid also appears to be a native organic acid present in BR. Malic acid is decarboxylated to lactic acid and CO_2, a reaction which is mediated by malolactic enzyme, with this pathway being strain dependent [330]. The reduction in malic acid levels across all fermented ingredients putatively shows malic acid metabolism may occur in the selected LAB strains when using BR as a substrate. Malic acid metabolism has been found to occur in a variety of LAB [331], including *L. plantarum* [330,332] and *Leuconostoc* spp. [333], however the use of malic acid as a carbon source is more relevant in modern wine production.

Evaluation of the techno-functional properties of the BR rootlet ingredients provides information on how BR may behave in a food system, while also showing how different processing techniques may alter them. BR-UnF and BR-Ster naturally have a very high WHC, which was in agreement with previous studies by Salama et al. [18]. The high WBC of BR is likely due to the significant proportion of fibre in their composition, as fibre in general has a high WBC [210,334]. Fermentation significantly reduced the WHC of BR, which might be linked to a change in protein structure post fermentation. The fermentation process likely unfolds the natural configuration of the proteins present in BR and exposes more hydrophobic regions, resulting in a reduction in the WHC. This theory is also complimented by the increase in the OBC capacity of the fermented BR [165]. In addition, previous literature suggests the freeze-drying process may also have a contributory effect to the reduction in WHC and increase in OBC [165,335]. As expected, trends in the pH and TA values of the BR ingredients followed a similar pattern to the final fermentation values. The significant reduction in alpha and beta enzymatic activity post sterilisation and fermentation is a favourable characteristic primarily imparted by the sterilisation process. In particular, the reduction in the high α-amylase activity is very beneficial, especially for the implementation of BR in a cereal-based application. Excessive amounts of alpha-amylase can lead to processing defects such as sticky doughs, poor crumb structures and darker crusts [336]. The slightly higher amylase activity in BR-FST2.11 is likely linked with the *L. amylovorus* FST2.11 ability to produce extracellular amylases [286]. From an ingredient colour perspective, BR have a naturally dark brown hue, likely due to the Maillard reaction products created during the malting process. However, the sterilisation and fermentation processes slightly altered the colour of the ingredients, reflected in the ΔE values.

A slightly lighter colour is perceived post processing which might be linked with changes in colour pigments during the freeze-drying process. However, overall changes are relatively minimal as freeze-drying tends to preserve colour pigments rather well [337,338].

Aside from the techno-functional characteristics, ingredient structure was also affected post sterilisation and fermentation. The broken, fragmented and enhanced porous structure of the sterilised ingredients might be linked with the freeze-drying process. BR are suspended in high volumes of water for sterilisation and before freeze-drying to a powder, implying BR are fully hydrated. Voda et al. [339] describes how water embedded in the food matrix expands during freezing and applies pressure to the cell walls of the food which ultimately distorts the original cell wall structure and might constitute reason for the degraded structure. The changes observed in the sterilised and fermented ingredients is likely a combination of the aforementioned freeze-drying process as well as some potential enzymatic degradation of the protein and fibre matrix of BR post fermentation. Some degradation of proteins was observed in an SDS-PAGE analysis (results not shown) of the ingredients. Proteases play a vital role in the germination stage of malting [6,340], which is the point at which rootlets are produced likely implying a certain proportion of proteases are present in the rootlets. LAB may also secrete carbohydrase's and proteinases [328] which could also physically degrade polysaccharide and protein structures.

5.6 Conclusion

Revitalisation of by-products for implementation into the food ingredient sector is an important area of research to help address the sustainability goals of the future. The valorisation of BR for use as a food ingredient would aid in improving the carbon footprint of both the malting and brewing industries. However, further processing of BR is an essential part of their future as a food ingredient. This work showcases fundamental knowledge on BR which has been rather limited to date, while also exhibiting the use of LAB fermentation as a valorisation technique for BR. LAB fermentation was found to alter the BR matrix, transforming the compositional and techno-functional profile of the ingredients, with various modifications being strain dependent. *L. amylovorus* FST2.11 and *L. plantarum* FST1.7 displayed optimal growth in BR for longer fermentation times. From a compositional perspective, changes in the residual carbohydrates and acids profile were altered depending on the type of LAB used. FODMAP analysis demonstrated that *L. citreum* TR116 and *L. reuteri* R29 were capable of converting fructose to mannitol, highlighting the potential use of BR-TR116 and BR-R29 ingredients in sugar-reduced products. Fermentation with *L. reuteri* R29 also resulted in an enhanced fructan content suggesting potential for future fibre product development. In addition, metabolomic analysis provided an insight into LAB metabolism utilising BR as a substrate. Finally, from a functionality perspective, LAB fermentation altered the techno-functional characteristics of BR, particularly, the WHC and OBC, where significant changes were observed. In addition, the sterilisation step aided in the reduction of the high alpha-amylase activity of the BR ingredient, which enhances its potential for use as a food ingredient. Further work on the utilisation of the fermented BR ingredients in a food application such as in bakery and/or cereal- based applications where BR could be used as partial flour replacers would be of great interest, to analyse if characteristics imparted by LAB fermentation have further benefits in these applications.

5.7 Acknowledgements

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Chapter 6

FROM WASTE TO TASTE: APPLICATION OF FERMENTED SPENT ROOTLET INGREDIENTS IN A BREAD SYSTEM

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6.1 Abstract

Upcycling of food by-products and their incorporation into food systems as functional ingredients has become a central focus of research. Barley rootlets (BR) are a by-product of malting and brewing industries which can be valorised using lactic acid bacteria fermentation. This research investigates the effects of the inclusion of unfermented (BR-UnF), heat sterilised (BR-Ster), and five fermented BR ingredients (using *Weissella cibaria* MG1 (BR-MG1), *Leuconostoc citreum* TR116 (BR-TR116), *Lactiplantibacillus plantarum* FST1.7 (BR-FST1.7), *Lactobacillus amylovorus* FST2.11 (BR-FST2.11) and *Limosilactobacillus reuteri* R29 (BR-R29)) in bread. The antifungal compounds in BR ingredients and the impact of BR on dough rheology, gluten development and dough mixing properties were analysed. Additionally, their effect on techno-functional characteristics, *in vitro* starch digestibility and sensory quality of bread was determined. BR-UnF showed dough viscoelastic properties and bread quality comparable to the baker's flour (BF). BR-MG1 inclusion ameliorated bread specific volume and reduced crumb hardness. Breads containing BR-TR116 had comparable bread quality to BF, while inclusion of BR-R29 substantially slowed microbial spoilage. Formulations containing BR-FST2.11 and BR-FST1.7 significantly reduced amounts of sugar released from breads during a simulated digestion and resulted in a sourdough-like flavour profile. This study highlights how BR fermentation can be tailored to achieve desired bread characteristics.

6.2 Introduction

In recent years, by-product rejuvenation has become a pivotal point of food research in order to become aligned with the sustainability goals of the future. BR are the main side-stream produced during the malting process and can be generated in volumes which equate up to 5% of the total malt weight [9,10]. Due to the fundamental role of the malting process within the brewing industry, BR are also considered a brewing by-product. To date, the use of BR has been rather limited, with its primary output confined to the animal feed industry. However, BR are of excellent nutritional value, containing a high amount of fibre (9.70 - 43%) [11] and substantial amounts of protein (20 - 38.7%) [278], highlighting their potential as food ingredients for inclusion in the human diet.

Although limited studies exist in the literature regarding the application of BR in food matrices, the implementation of BR into the food chain has promising potential from a nutritional perspective, which can increase the protein and fibre values of foods [68]. However, the inclusion of BR in food products poses difficulties, with losses in food quality and adverse sensory perceptions observed. Chiș et al. [62] found inclusion of BR up to 15% was successful, as undesirable sensory defects were noted beyond this point. Salama et al. [68] reported maximum inclusion levels of BR in biscuits (10%), bread (5%) and sausages (10%), with addition levels beyond these resulting in products being deemed unacceptable by sensory panellists. Similarly, Waters et al. [11] found that 5% addition of BR was the most satisfactory from a sensory perspective, as anything above this level exhibited bitter off-flavours. From a bread quality perspective, the introduction of BR has resulted in lower bread volumes, harder crumb textures and darker crust colours [11].

Lactic acid bacteria (LAB) fermentation has become a process of interest in the enhancement of side-stream products, with numerous studies showcasing their successes. The application of LAB fermentation technology on brewers spent grain and apple by-products showed improvements in a variety of nutritional characteristics; enhanced bread and pasta techno-functional properties; extended shelf life properties; and also produced acceptable sensory attributes [145,154,155,166,168,169,245,282]. LAB fermentation of surplus bread streams and their incorporation into a bread matrix showed improvements in bread specific volume, reduced crumb hardness and also improved the hygienic safety of the recycling process [158]. The application of LAB fermentation technology on side streams from maize and wheat milling industries and their incorporation into a wheat and pasta prototypes exhibited improvements in

product quality, nutritional properties and sensory attributes compared to their unfermented control [157,159,163]. In reference to BR, application of LAB sourdough fermented BR at 10% in a bread matrix displayed enhanced bread texture and flavour quality compared to a wholemeal bread [11].

Neylon et al. [341] performed an in-depth analysis of the effect of heat treatment and LAB fermentation (using *Weissella cibaria* MG1, *Lactiplantibacillus plantarum* FST1.7, *Limosilactobacillus reuteri* R29, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST2.11) on BR. Following on from this, the aim of the current study was to investigate the effects of the inclusion of fermented BR ingredients in a bread matrix. Fermented BR ingredients were incorporated into bread recipes at a 5% replacement level based on bakers' wheat flour. The effect of BR addition on dough quality, techno-functional characteristics, microbial shelf life, sensory experience, and nutritional characteristics (release of reducing sugars during starch digestion) of bread were monitored. Formulations containing bakers' wheat flour (BF), unfermented BR (BR-UnF) and heat sterilised BR (BR-Ster) were used as controls.

6.3 Materials and methods

6.3.1 Raw materials

Ingredients incorporated into bread recipes included: bakers' flour (BF) (Odlums group, Dublin, Ireland); spent barley rootlets (UnF-BR) supplied by Anheuser-Busch InBev (Leuven, Belgium); a sterilised, freeze-dried BR ingredient (BR-Ster) and 5 fermented, freeze-dried BR ingredients [341]. The fermented, freeze-dried BR ingredients were produced using LAB fermentation with the strains and special characteristics listed in Table 15 [341]. Other ingredients used in bread recipes include instant active dried baker's yeast *Saccharomyces cerevisiae* (Puratos, Groot-Bijgaarden, Belgium), sugar (Siúcra, Dublin, Ireland), salt (Glacia British Salt Limited, Cheshire, UK), sunflower oil (Musgraves, Cork, Ireland) and tap water. The compositional analysis of the BF and BR-UnF are illustrated in Table 16, with the composition of BR-UnF previously reported by Neylon et al. [341]. The compositional analysis was determined by an accredited laboratory (Chelab S.r.l, Merieux NutriSciences Corporation, Resana TV, Italy). The protein content was determined by the Dumas method, utilising a modified version of AOAC 992.23 1992 [342]. Fat content was measured using Soxhlet method and was carried out according to the ISTISAN report [299]. Ash was determined using AOAC 923.03 [343]. Moisture was analysed using ISO 712:2009 [344]. Total carbohydrates were calculated by difference based on the AOAC 986.25 method [345]. Fibre was measured by the AOAC 2017.16 method [346]. All chemicals used in experimental analysis were purchased from Sigma-Aldrich (Sigma-Aldrich, Missouri, USA) unless stated otherwise

Table 15. Characteristics of lactic acid bacteria utilised.

Species	***Leuconostoc citreum***	***Lactobacillus amylovorus***	***Weissella cibaria***	***Limosilactobacillus reuteri***	***Lactiplantibacillus plantarum***
Strain	TR116	FST 2.11	MG1	R29	FST 1.7
Metabolism	Heterofermentative	Homofermentative	Heterofermentative	Heterofermentative	Heterofermentative
Fermentation substrate	Fructose	Sucrose	Sucrose	Sucrose	Sucrose
Source	Yellow pea sourdough	Brewing environment	Sourdough	Human intestine	Malted barley
Special traits	Mannitol producer, antifungal producer	Antimicrobial producer, high acid producer	Dextran exopolysaccharide producer	Mannitol producer, Antifungal producer	Antifungal producer, high acid producer
Reference	[160,162,174,283,284]	[285–287]	[277,288–293]	[294–296]	[244,277,287,296,297]

Table 16. Compositional analysis of bakers' flour (BF) and unfermented barley rootlets (UnF-BR) measured in g/100 g. Results are represented as mean values ± standard deviations.

Analyte	BF	BR-UnF
Protein	12.96 ± 0.79	35.80 ± 1.50
Fat	1.20 ± 0.08	1.77 ± 0.11
Ash	0.55 ± 0.05	5.98 ± 0.30
Moisture	12.95 ± 0.30	12.74 ± 0.30
Total Carbohydrate	65.31 ± 1.53	$0 \leq 7.02 \leq 15.72$
Total fibre	7.03 ± 1.27	36.64 ± 8.51
Soluble fibre	2.63 ± 0.63	1.24 ± 0.30
High molecular weight dietary fibre	4.41 ± 1.1	35.40 ± 8.50

6.3.2 Dough analysis

6.3.2.1 Water content adjustment

The Farinograph-TS® (Brabender GmbH and Co KG, Duisburg, Germany) was utilised to determine the amount of water required for each recipe, using an automatic water dosing system (Aqua-Inject). Recipes of a total volume of 300 g were adjusted to a target dough consistency of 500 ± 20 FU (farinograph units) with the mixing chamber temperature set to 30 °C. A 5% addition (based on flour) of the respective BR ingredient was included in bread recipes by replacing BF. The bread recipes used for analysis are included on Table 17.

Table 17. Recipes for bread preparation expressed as % based on flour, which equates to the sum of the baker's flour (BF) and barley rootlet (BR) ingredient. BR-UnF and BR-Ster represent unfermented, and heat sterilised barley rootlets, respectively. BR-TR116, BR-MG1, BR-FST2.11, BR-R29 and BR-FST1.7 express barley rootlets fermented with their respective LAB strain, namely *Leuconostoc citreum* TR116, *Weisella cibaria* MG1, *Lactobacillus amylovorus* FST2.11, *Limosilactobacillus reuteri* R29 and *Lactiplantibacillus plantarum* FST1.7, respectively. FWA % represents results obtained from farinograph water absorption with letters sharing the same subscript numbers not differing significantly ($p < 0.05$).

Ingredient	BF	BR-UnF	BR-Ster	BR-TR116	BR-MG1	BR-FST2.11	BR-R29	BR-FST1.7
BF	100	95	95	95	95	95	95	95
BR ingredient	-	5	5	5	5	5	5	5
Salt	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Sugar	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Sunflower oil	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
Dry Yeast	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Water (FWA %)	64.3^{a}	$68.2^{c,d}$	68.9^{d}	66.90^{b}	67.90^{c}	$67.50^{b,c}$	66.70^{b}	$67.30^{b,c}$

- represents not applicable to recipe.

6.3.2.2 Dough preparation

Bread doughs were prepared by a straight dough process. Firstly, the dry ingredients were mixed using a Kenwood chef classic mixer (Kenwood Manufacturing Co. Ltd., Havant, UK) for 30 s at speed 1. Following this the yeast solution and sunflower oil were added to the dry ingredients. The yeast solution was prepared by adding the dry yeast to the amount of water required for the recipe (water tempered to 25 °C) and allowing to stand for 10 min to activate the yeast. All ingredients were mixed at speed 1 for 1 min, followed by a second mixing stage at speed 2 for 7 min.

6.3.2.3 Dough rheology

The viscoelastic properties of the doughs were evaluated using a Rheometer Physica MCR 301 (Anton PAAR GmBH, Ostfildern, Germany). Bread doughs were prepared as outlined above with the yeast excluded. A serrated plate method was employed for the analysis, with the plates set up in parallel geometry. The lower plate was held at 35 °C and was assembled with an upper plated of 50 mm in diameter. The linear viscoelastic region was evaluated using an amplitude sweep [247] and frequency sweeps were carried out as described by Neylon et al. [282]. The damping factor ($tan\delta \frac{G''}{G'}$) was determined to analyse the changes in dough viscoelastic properties caused by BR ingredient addition.

6.3.2.4 Bread fermentation quality

The bread dough quality was analysed using a Rheofermentometer (Chopin, Villeneuve-la-Garenne CEDEX, France). Briefly, 300 g of bread dough was prepared, placed into the fermentation chamber and assembled with a 1500 g cylindrical weight on top of the dough. The fermentation chamber was sealed, and the BR doughs were left to ferment for 3 h at 30 °C. The maximum dough height (Hm) in mm, CO_2 retention coefficient (%) and the volume of CO_2 produced during fermentation (mL) were analysed.

6.3.2.5 Dough development and starch pasting properties

The mixing and pasting behaviour of the doughs were analysed using Mixolab (Chopin, Villeneuve-la-Garenne CEDEX, France) according to the method detailed by Rosell et al. [347]. Briefly, flour blends (Table 17) were mixed with the required water content according to Farinograph-TS® values (Table 17) to reach a dough weight of 75 g. Samples were mixed at a constant rate of 75 rpm. A heating profile was applied to the dough starting at 30 °C until

maximum dough development, heated to 90 °C over 15 min at a rate of 4 °C/min and held at 90 °C for a total of 7 min. A cooling profile was then applied over 10 min to 50 °C at a rate of 4 °C/min and held at 50 °C for a total of 5 min. The following parameters were analysed: dough development time (DDT), C2, C3, C4 and C5. As described by Rosell et al. [348], DDT is determined as the time taken (min) for the maximum torque to be reached during the first stage of mixing at a temperature of 30 °C. C2 is the minimum value of torque when the dough is exposed to mechanical and temperature stress and provides information on the protein destabilisation or protein weakening. C3 is the maximum torque reached during the heating and mixing stage which provides information on the starch swelling and hydration properties. C4 is deemed as the minimum torque reached and provides an insight into the physical breakdown of the starch granules in the dough. Finally, C5 is the final, maximum torque reached during the cooling phase, providing information on the extent of starch retrogradation.

6.3.2.6 Gluten network development

GlutoPeak (Brabender GmbH and Co KG, Duisburg, Germany) was used to determine gluten network development. Flour blends, (Table 17), were prepared and mixed to ensure homogeneity. For analysis, 9 g of sample (based on 14% moisture) was added to deionised water (36 °C) to reach a final volume of 18 g. Measurement parameters were set to shear speed of 2750 rpm at a chamber temperature of 36 °C. Torque was monitored over time (s) with the following parameters measured: torque maximum (TM) in Brabender units (BU) and peak maximum time (PMT) in seconds (s).

6.3.3 Bread production process

Dough quantities of 550 g were prepared for each recipe outlined in Table 17. The bread dough was divided into eight 65 ± 1 g dough pieces, moulded and placed into greased tins. The dough pieces were proofed (30 °C, 85% relative humidity (RH)) in a proofing chamber (KOMA SunRiser, Roermond, The Netherlands) for 75 min. After proofing, the doughs were transferred to a deck oven (MIWE Condo, Arnstein, Germany) and baked at 210 °C top/bottom heat for 14 min. To optimise conditions during baking, 700 mL of steam was injected into the oven before loading. After baking, the bread loaves were allowed to cool for 1 hour before analysis.

6.3.4 Bread analysis

6.3.4.1 Bake loss

Bake loss was calculated to account for the amount of water lost during the baking process. The bake loss of 4 breads per batch were analysed and calculated according to the below formulas:

$$Weight\ of\ the\ dough\ (g) - weight\ of\ baked\ bread\ (g) = moisture\ lost\ during\ bake\ (g) \quad (13)$$

$$\frac{Moisture\ lost\ during\ bake\ (g)}{Weight\ of\ dough\ (g)}\ x\ 100\ = Bake\ loss\ (\%) \quad (14)$$

6.3.4.2 Specific volume

The specific volume of each bread loaf was determined using the Volscan Profiler (Stable Micro Systems, Surrey, UK) and expressed as mL/g. Four bread loaves were analysed per batch.

6.3.4.3 Bread crumb structure

The C-Cell Imaging System (Calibre Control International Ltd, Warrington, UK) was used to analyse the cell diameter of the breads. Four bread loaves per batch were cut into slices of 20 mm thickness (3 slices per loaf) with crust slices omitted from the analysis.

6.3.4.4 Bread crumb texture

Crumb texture of bread slices was measured using the TA-XT2i Texture Analyser (Stable MicroSystems, Surrey, UK). The TA-XT2i Texture Analyser was equipped with a 25 kg load cell and a two-compression test with a strain of 40%, test speed of 5 mm/s, a trigger force of 0.05 N. A waiting time of 5 s between the two compressions was used. A 20 mm cylindrical probe was attached for the analysis. Bread slices of 20 mm thickness were analysed for their hardness and resilience on the day of baking. Crumb hardness was calculated as the maximum force of the first compression. Bread crumb resilience was calculated by dividing the energy required during the upstroke action of compression one by the energy required during the downstroke action of compression one.

6.3.5 Extraction and quantification of antifungal compounds from BR ingredients

BR ingredients were screened for the presence of 15 phenolic compounds, namely: catechol, 4-hydroxyphenyllactic acid, 4-hydroxybenzoic acid, hydrocaffeic acid, vanillic acid, caffeic acid, phloretic acid, 3-phenyllactic acid, hydroferulic acid, p-coumaric acid, ferulic acid, benzoic acid, salicylic acid, hydrocinnamic acid, methylcinnamic acid. Extraction of the antifungal compounds was carried out based on the QuEChERS (quick, easy, cheap, effective, rugged and safe) procedure first described by Brosnan et al [349] and further modified by Hoehnel et al. [162]. Briefly, 2 g of ingredient was suspended in 10 mL of ultrapure water, followed by addition of 10 mL ethyl acetate containing 0.1% (v/v) formic acid. The samples were mixed thoroughly using a vortex shaker. Following this, 4 g of $MgSO_4$ and 1g of NaCl were added and the samples were shaken by hand for exactly 1 min. The samples were centrifuged at 4800 x *g* for 10 min and the supernatant was retained and transferred to solid-phase extraction (SPE) tubes (Bond Elut QuEChERS Dispersive kit; Agilent Technologies Inc., Santa Clara, CA, USA). The tube contents were thoroughly mixed using a vortex shaker, centrifuged at 2300 x *g* for 10 min and 5 mL of the supernatant was added to 100 µL dimethlsulfoxide (DMSO). Samples were concentrated using a vacuum centrifuge (Scanvac, Scanspeed; 2 h, 500 rpm, 45 °C) before reconstitution with 400 µL of ultrapurewater/ acetonitrile (90:10, v/v). Samples were filtered through 0.2 µm syringe driven filters. The separation and quantification of antifungal compounds present in BR ingredients was performed according to chromatographic test parameters detailed by Hoehnel et al. [162]. Antifungal quantities were calculated based on dry matter (D.M.).

6.3.6 Shelf-life evaluation

The microbial shelf-life of the breads was measured using the mould environmental challenge method [244,249,294] with some minor modifications. Briefly, 8 centre slices of 20 mm thickness per batch were left to rest on a sterile metal rack. Bread crumbs of both sides of the bread slice were exposed to the bakery environment for 5 min. The bread slices were packed singly in sterile bags, heat-sealed and a filter pipette was placed in each bag to allow for airflow to persist (aerobic conditions). The samples were stored at 20 ± 1 °C and 50% RH in a pre-sterilised and temperature-controlled chamber (KOMA SunRiser, Roermond, Netherlands). Samples were analysed daily for 14 days. The mould growth of each bread slice was visually

assessed and rated as “mould free”, “mould growth <10%”, “10–24% mould growth”, “25–49% mould growth” and “mould growth >50%”.

6.3.7 Release of reducing sugars

The release of reducing sugars was investigated by means of an *in vitro* digestion assay using an enzymatic degradation of starch. The method was carried out as previously described by Brennan & Tudorica [181] which was designed for fibre-enriched products. Briefly, 4 g of ground bread crumb was treated with a pepsin solution (115 U/ mL) for 30 min at 37 °C. Following this, samples were placed in dialysis tubing (1-inch diameter, molecular weight cut off 14000 Daltons) and suspended in a sodium potassium phosphate buffer (pH 6.9) and incubated with pancreatic alpha-amylase solution (110 U/ mL). During the incubation period, the dialysis tubing was inverted three times to simulate the peristalsis effect. Samples were collected from the buffer solution at 30 min intervals. A 100 µL aliquot of sample was diluted with 100 µL sample of 3,5-dinitrosalicyclic acid and heated (100 °C, 15 min). Following this, the heated samples were left to cool for 5 min on ice and were subsequently diluted with 1 mL of deionised H_2O. The absorbance of the samples at 546 nm was measured and the reducing sugar release % (RSR) over time was determined using the below formula [181].

$$RSR\ \% = \frac{(\mathrm{A}\,sample)\ x\ 500\ ml\ x\ 0.95}{(A\ maltose)\ x\ available\ carbohydrate(mg)}\ x\ 100 \qquad (15)$$

Where (Asample) is the absorbance of the active sample at 546 nm; 500 mL is the total volume of the solution which was analysed; 0.95 is the conversion factor for maltose to starch; (Amaltose) is the absorbance of 1 mg pure maltose/ mL buffer and available carbohydrates is the amount of readily digestible carbohydrates present in the 4 g sample. The available carbohydrate was determined using the digestible carbohydrate values obtained from the K-RAPRS kit (Megazyme, Bray, Ireland).

6.3.8 Sensory evaluation

The sensory properties of the bread crumb were investigated by performing a descriptive analysis test using an experienced sensory panel (n=9, age range 23-33) recruited from the Food Science department at University College Cork. Panellists evaluated the intensity of the odour attributes ‘overall intensity, ‘citrus’, ‘vegetable; ‘cereal/grains’, the flavour attributes ‘overall intensity’, ‘muddy/earthy’, ‘fruity’, ‘vegetable’, ‘aftertaste’, the taste attribute ‘sour’ and the texture attributes ‘hardness’, ‘chewiness’ on a scale from 0, ‘not present’ to 10,

‘extremely’. Panellists were also asked to rank the overall acceptability of the breads. Sensory analysis of all breads was performed in duplicate and bread crumb samples were analysed on the same day they were baked.

6.3.9 Statistical analysis

Experimental analysis was carried out in triplicate unless stated otherwise. In the case of normally distributed data, a one-way ANOVA with post-hoc Tukey test (p value < 0.05) was performed using SPSS statistical software in order to determine significant differences between sample groups. Where equal variances were not assumed, a correction using the welch test and a Games Howell (p value < 0.05) post hoc test was applied. When data was not normally distributed, a non-parametric Kruskal Wallis test ($p < 0.05$) was used to identify significant differences among samples.

6.4 Results

6.4.1 Compositional analysis

The composition of BF and BR-UnF are presented in Table 16. The compositional analysis of BR-UnF has been reported in a previous publication [341]. The protein content in BR-UnF (35.80 ± 1.50 g/100g) was much higher than the protein content quantified in BF (12.96 ± 0.79 g/ 100g). BF and BR-UnF had relatively similar quantities of fat (1.20 ± 0.08 g/100 g and 1.77 ± 0.11 g/100 g, respectively), however, higher amounts of ash were quantified in BR-UnF (5.98 ± 0.30 g/100 g) compared to BF (0.55 ± 0.05 g/100 g). The moisture contents measured in BF (12.95 ± 0.30 g/100g) and BR-UnF (12.74 ± 0.30 g/100 g) were comparable. BF contained 65.31 ± 1.53 g/100g of total carbohydrate, which was much lower than that measured in BR-UnF ($0 \leq 7.02 \leq 15.72$ g/ 100 g). The total fibre in BF equated to 7.03 ± 1.27 g/100g, of which 2.63 ± 0.63 g/100g was deemed as soluble fibre and 4.41 ± 1.1 g/100g was determined as high molecular wight dietary fibre. Higher quantities of total fibre (36.64 ± 8.51 g/100 g) and high molecular weight dietary fibre (35.40 ± 8.50 g/100 g) were quantified in BR-UnF, with a slightly lower amount of soluble fibre quantified in BR-UnF (1.24 ± 0.30 g/ 100 g) than BF.

6.4.2 Dough analysis

6.4.2.1 Water absorption

The optimal water content for each recipe was determined to ensure optimal dough consistency for favourable dough and gluten network development. The results from farinograph analysis are displayed in Table 17. The lowest water absorption capacity (WAC) was found in the BF recipe (64.3%). The inclusion of the BR ingredients significantly increased the WAC of the recipes. The highest WAC was determined in the BR-Ster recipe (68.9%) followed closely by the BR-UnF recipe (68.2%), with no significant differences found between these samples. The WAC of the BR-UnF recipe and the recipes containing BR-MG1 (67.90%), BR-FST2.11 (67.50%) and BR-FST1.7 (67.30%) were similar. A WAC of 66.90% and 66.70% was measured in BR-TR116 and BR-R29 recipes, respectively, which was significantly lower than BR control recipes, BR-UnF and BR-Ster.

6.4.2.2 Gluten network development

Attributes of the gluten network development were determined to investigate any differences in gluten aggregation kinetics. The torque maximum (TM) and the time taken for gluten development (PMT) are shown in Table 18, while the torque (BU) versus time (s) of the gluten network development is displayed in Figure 16. A curve typical for wheat flour was obtained

for BF with a TM of 78.33 ± 1.53 BU and a PMT of 39.7 ± 1.53 s. The inclusion of BR-UnF and BR-Ster significantly reduced the gluten network strength with TM values of 64.0 ± 1.0 BU and 61.66 ± 2.52 BU determined, respectively. No significant differences were noted in the times required for gluten development with BF (39.7 ± 1.53 s) or BR-UnF (44.0 ± 1.0 s) and BR-Ster (40.00 ± 2.00 s). The inclusion of fermented BR ingredients resulted in a decreased gluten network strength compared to the BF control formulation but was similar to BR-UnF and BR-Ster results. The weakest gluten aggregation strength was noted in the cBR-R29 (58.67 ± 1.53 BU) recipe, followed closely by recipes with BR-FST1.7 (60.00 ± 1.00 BU) and BR-TR116 (60.33 ± 4.04 BU) inclusion. An increase in gluten strength was found for BR-MG1 formulations (62.33 ± 1.53 BU) with the strongest gluten network amongst the fermented formulations observed with BR-FST2.11 (65.33 ± 1.13 BU) inclusion. In relation to the gluten network development time, all fermented ingredients enhanced the speed of gluten aggregation in the dough, resulting in a decrease in PMT compared to BF, BR-UnF and BR-Ster. The fastest gluten network development was observed in formulations supplemented with BR-FST2.11 (25.70 ± 1.15 s) and BR-FST1.7 (26.70 ± 1.15 s), with no significant differences found. The PMT observed for BR-TR116 (33.70 ± 3.21 s), BR-MG1 (32.70 ± 2.08 s) and BR-R29 (32.30 ± 0.58 s) recipes were significantly longer than that of fermented samples BR-FST1.7 (26.667 ± 1.155 s) and BR-FST2.11 (25.667 ± 1.155 s). However, they were still significantly faster than the PMT values obtained for BF, BR-UnF and BR-Ster controls (39.67 ± 44.00 s).

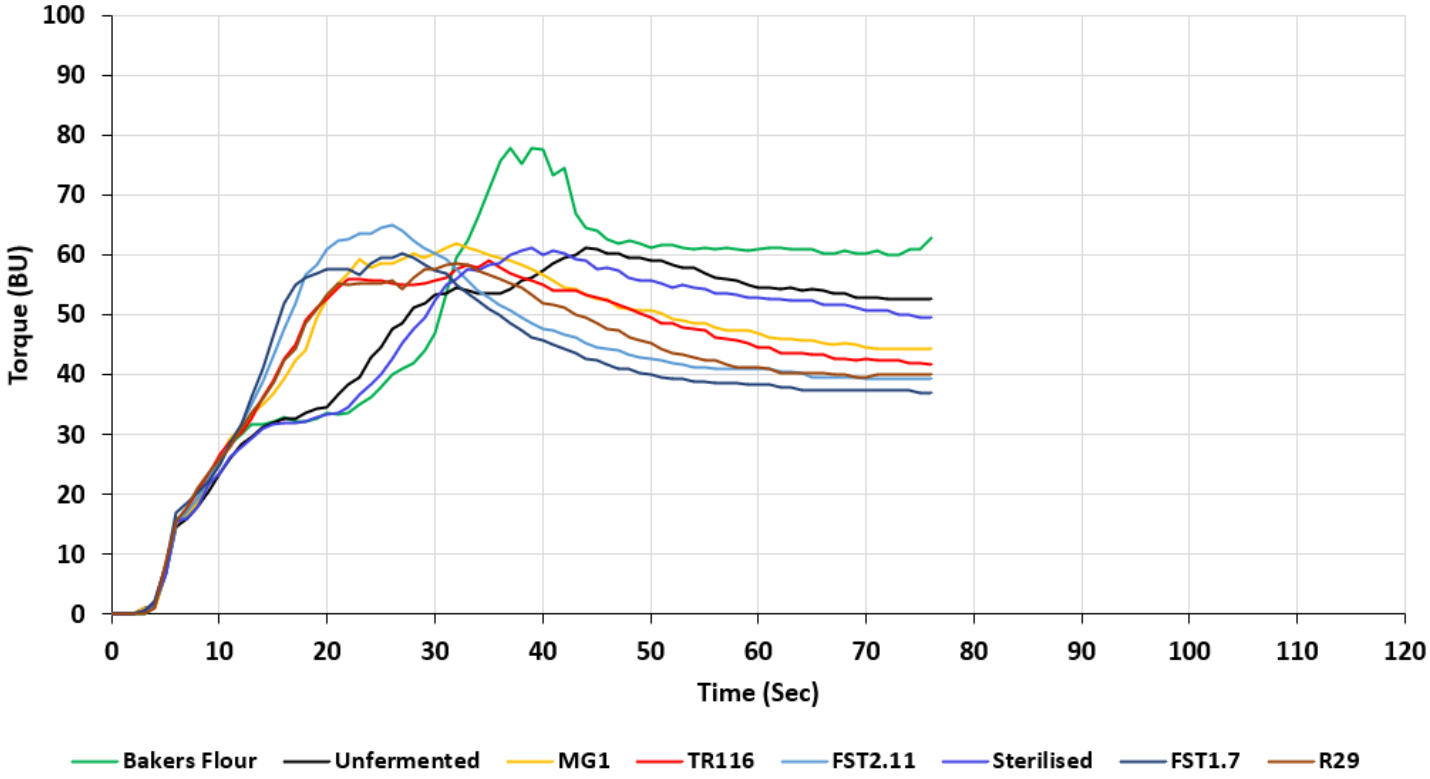

Figure 16. Effect of unfermented and fermented rootlets on gluten network development of bakers' flour as dough torque (BU) over mixing time (s). TR116, FST2.11, MG1, FST1.7 and R29 denote wheat flour blends with 5% inclusion of the fermented rootlets, fermented with *Leuconostoc citreum* TR116, *Lactobacillus amylovorus* FST2.11, *Weissella cibaria* MG1, *Lactiplantibacillus plantarum* FST1.7 and *Limosilactobacillus reuteri* R29, respectively. Bakers' flour, 5% supplementation of unfermented rootlets and 5% supplementation of sterilised rootlet flour blends are also represented on the illustration.

Table 18. Dough characteristics with BR inclusion. BF, BR-UnF and BR-Ster represent control recipes using bakers' flour, 5% unfermented barley rootlets inclusion and 5% sterilised, freeze-dried barley rootlet inclusion, respectively. BR-TR116, BR-MG1, BR-FST2.11, BR-R29 and BR-FST1.7 denote recipes including fermented rootlets at 5% addition level and fermented using *L. citreum* TR116, *W. cibaria* MG1, *L. amylovorus* FST2.11, *L. reuteri* R29 and *L. plantarum* FST1.7, respectively. Results are illustrated as mean values ± standard deviations. Samples in the same row which share the same subscript letter had no significant statistical differences ($p < 0.05$).

	BF	**BR-UnF**	**BR-Ster**	**BR-TR116**	**BR-MG1**	**BR-FST2.11**	**BR-R29**	**BR-FST1.7**
Torque Max (BU)	78.333 ± 1.538^{c}	$64.000 \pm 1.000^{a,b}$	$61.667 \pm 2.527^{a,b}$	$60.333 \pm 4.041^{a,b}$	$62.333 \pm 1.528^{a,b}$	65.333 ± 1.155^{b}	58.667 ± 1.538^{a}	60.000 ± 1.000^{a}
Peak Max Time (s)	39.667 ± 1.528^{c}	44.000 ± 1.000^{c}	40.000 ± 2.000^{c}	33.667 ± 3.215^{b}	32.667 ± 2.082^{b}	25.667 ± 1.155^{a}	32.333 ± 0.577^{b}	26.667 ± 1.155^{a}
DDT (min)	1.320 ± 0.269^{a}	7.037 ± 0.225^{d}	7.493 ± 0.318^{d}	5.610 ± 0.066^{c}	$4.950 \pm 0.996^{a,b,c,d}$	4.213 ± 0.029^{b}	3.660 ± 0.147^{b}	3.870 ± 0.101^{b}
C2 (Nm)	0.427 ± 0.008^{g}	$0.328 \pm 0.004^{b,c}$	0.379 ± 0.003^{f}	$0.354 \pm 0.012^{d,e}$	0.361 ± 0.002^{e}	$0.311 \pm 0.005^{a,b}$	$0.338 \pm 0.003^{c,d}$	0.303 ± 0.005^{a}
C3 (Nm)	1.631 ± 0.013^{d}	1.235 ± 0.019^{a}	1.519 ± 0.007^{b}	$1.559 \pm 0.015^{b,c}$	1.533 ± 0.004^{b}	$1.625 \pm 0.003^{c,d}$	$1.598 \pm 0.012^{c,d}$	$1.621 \pm 0.007^{c,d}$
C4 (Nm)	$1.496 \pm 0.010^{e,f}$	0.706 ± 0.025^{a}	1.375 ± 0.007^{b}	$1.442 \pm 0.014^{c,d}$	$1.411 \pm 0.011^{b,c}$	1.506 ± 0.006^{f}	$1.465 \pm 0.010^{d,e}$	$1.499 \pm 0.008^{e,f}$
C5 (Nm)	2.549 ± 0.029^{d}	1.119 ± 0.030^{a}	2.231 ± 0.027^{b}	2.339 ± 0.022^{c}	$2.279 \pm 0.012^{b,c}$	2.647 ± 0.028^{e}	$2.594 \pm 0.048^{d,e}$	$2.613 \pm 0.016^{d,e}$
Hm (mm)	50.933 ± 2.479^{c}	$46.400 \pm 1.308^{b,c}$	$40.433 \pm 0.569^{a,b}$	43.167 ± 3.256^{b}	$44.867 \pm 1.518^{b,c}$	37.000 ± 0.300^{a}	36.567 ± 2.532^{a}	34.967 ± 3.099^{a}
Vol of CO_2 (mL)	$2045 \pm 72.808^{a,b,c}$	2451 ± 30.139^{d}	2399 ± 99.571^{d}	$2029 \pm 69.241^{a,b}$	2547 ± 67.656^{d}	$2318 \pm 130.750^{c,d}$	1919 ± 58.506^{a}	$2302 \pm 173.283^{b,c,d}$
CO_2 retention coefficient (%)	97.200 ± 3.329^{a}	99.633 ± 0.115^{a}	99.700 ± 0.000^{a}	99.567 ± 0.153^{a}	99.733 ± 0.115^{a}	99.667 ± 0.058^{a}	99.600 ± 0.173^{a}	99.733 ± 0.058^{a}
Damping Factor	0.390 ± 0.012^{d}	0.383 ± 0.009^{d}	0.357 ± 0.010^{c}	$0.346 \pm 0.012^{a,b,c}$	$0.352 \pm 0.011^{b,c}$	0.328 ± 0.006^{a}	$0.344 \pm 0.011^{a,b,c}$	$0.333 \pm 0.014^{a,b}$

6.4.2.3 Dough development and starch pasting properties

Mixolab analysis was conducted to investigate the effects observed on dough mixing characteristics and dough pasting properties under mechanical and thermal stresses (Table 18).

The shortest DDT was observed for the BF formulation (1.320 ± 0.269 min), with significantly higher DDT observed in BR-UnF (7.037 ± 0.225 min) and BR-Ster (7.493 ± 0.318 min) formulations. Significant reductions in DDT (3.660 – 5.610 min) were determined with the inclusion of most fermented BR ingredients. The BR-MG1 formulation was the exception to this trend, with no significant difference in DDT noted when compared to the BF, BR-UnF and BR-Ster bread formulations.

The BF control recipe resulted in the highest C2 value (0.427 ± 0.008 Nm), with a significant reduction in C2 values recorded with inclusion of both BR-UnF (0.328 ± 0.004 Nm) and BR-Ster (0.379 ± 0.03 Nm). Comparing fermented formulations, C2 values for doughs containing BR-MG1 (0.361 ± 0.002 Nm) and BR-TR116 (0.354 ± 0.012 Nm) were the highest. C2 values for BR-MG1 and BR-TR116 recipes were significantly lower than BF and BR-Ster, however, were significantly higher than the BR-UnF formulation. Inclusion of BR-R29 reduced the C2 value to 0.338 ± 0.003 Nm, with an even greater reduction observed with doughs containing BR-FST2.11 (3.11 ± 0.005 Nm) and BR-FST1.7 (0.303 ± 0.005 Nm).

BF had the highest C3 value recorded (1.631 ± 0.013 Nm), which was significantly higher than the BR-Ster value (1.519 ± 0.007 Nm). In contrast, the BR-UnF formulation had the lowest C3 value (1.235 ± 0.019 Nm). C3 values for fermented formulations were in the range of 1.533 – 1.625 Nm.

Comparing C4 values, BR-UnF had the lowest value recorded (0.706 ± 0.025 Nm). A significant increase in C4 was observed for the BR-Ster formulation (1.375 ± 0.007 Nm) with an even great increase reported for BF (1.496 ± 0.010 Nm). C4 values for BR-MG1 (1.411 ± 0.011 Nm) was comparable to the BR-Ster formulation. No significant difference was found between BR-MG1 and BR-TR116 (1.442 ± 0.014 Nm). The BR-R29 (1.465 ± 0.010 Nm), BR-FST1.7 (1.499 ± 0.008 Nm) and BR-FST2.11 (1.506 ± 0.006 Nm) C4 values were comparable to the BF recipe.

Trends for C5 values mirrored those reported for C4 values. BR-UnF doughs had the lowest C5 value recorded (1.119 ± 0.030 Nm), while significant increases were observed in C5 values for doughs containing BR-Ster (2.231 ± 0.027 Nm) and BF (2.549 ± 0.029 Nm). C5 values for fermented formulations were in the range of 2.279 – 2.647 Nm.

6.4.2.4 Dough fermentation capacity

The fermentation capacity of each dough was analysed to provide information on the yeast fermentation process (Table 18).

Of the formulations tested, BF resulted in the highest Hm (50.933 ± 2.479 mm). Inclusion of BR-UnF maintained the dough height (46.400 ± 1.308 mm), with no significant difference observed compared to the BF control. A greater reduction in Hm was observed when BR-Ster was included (40.433 ± 0.569 mm). Comparing fermented BR formulations, the highest Hm was recorded with BR-MG1 inclusion (44.867 ± 1.518 mm) and was comparable to all control recipes. Inclusion of BR-TR116 resulted in a slight decrease of Hm (43.167 ± 3.256 mm) but was not significantly different to BR-MG1 Hm results. Compared to BF and BR-UnF, the addition of BR-FST2.11, BR-R29 and BR-FST1.7 reduced Hm significantly with values of 37.000 ± 0.300 mm, 36.567 ± 2.532 mm and 34.967 ± 3.099 mm determined, respectively. However, these values were not found to be statistically different from the Hm of the BR-Ster recipe. The volume of CO_2 recorded varied between recipes. Firstly, comparing controls, the lowest amount of CO_2 produced was recorded in the BF recipe (2045 ± 73 mL), while significant increases were observed in volume of CO_2 produced with BR-UnF (2451 ± 30 mL) and BR-Ster (2399 ± 100 mL) inclusion. The incorporation of BR-MG1 produced the highest amount of CO_2 during the analysis (2547 ± 68 mL) and was comparable to BR-UnF and BR-Ster controls. Similarly, the BR-FST2.11 and BR-FST1.7 formulation maintained high amounts of CO_2 production resulting in values of 2318 ± 131 mL and 2302 ± 172 mL, respectively. A slight drop in the level of CO_2 produced was observed in the BR-TR116 recipe (2029 ± 69 mL) which was more comparable to BF and BR-FST1.7 formulations. The lowest amount of CO_2 produced was noted in the BR-R29 recipe (1919 ± 59 mL), which was similar to the volume of CO_2 produced in the BF fermentation process. Finally, the CO_2 retention coefficient for all recipes was comparable with no significant difference found between recipes.

6.4.2.5 Dough rheology

To investigate dough elastic (solid) and viscous (liquid) parts, oscillation measurements were conducted, and the damping factor (DF) was determined. A material is considered an ideal elastic if the damping factor is 0, implying that the material contains no viscous elements. An increase in the DF signifies an increase in viscous parts of the dough system.

As shown in Table 18, BF had the highest DF (0.390 ± 0.012) indicating the highest viscous parts in this dough. The BR-UnF formulation showed similar dough rheological properties (0.383 ± 0.009) to BF. The DF determined in the BR-Ster formulation was significantly lower

(0.357 ± 0.010) than the BF and BR-UnF formulations, indicating a dough with more elastic properties. The inclusion of fermented BR ingredients resulted in a significant reduction in the DF compared to BF and BR-UnF formulations. However, no significant difference was noted in BR-TR116 (0.346 ± 0.012), BR-MG1 (0.352 ± 0.011) and BR-R29 (0.344 ± 0.011) formulations compared to the BR-Ster control. A significant reduction in DF was found in BR-FST1.7 (0.333 ± 0.014) and BR-FST2.11 (0.328 ± 0.011) formulations compared to control recipes, with the lowest DF noted in the BR-FST2.11 formulation.

6.4.3 Baked bread analysis

A visual representation of the final baked bread products is illustrated in Figure 17.

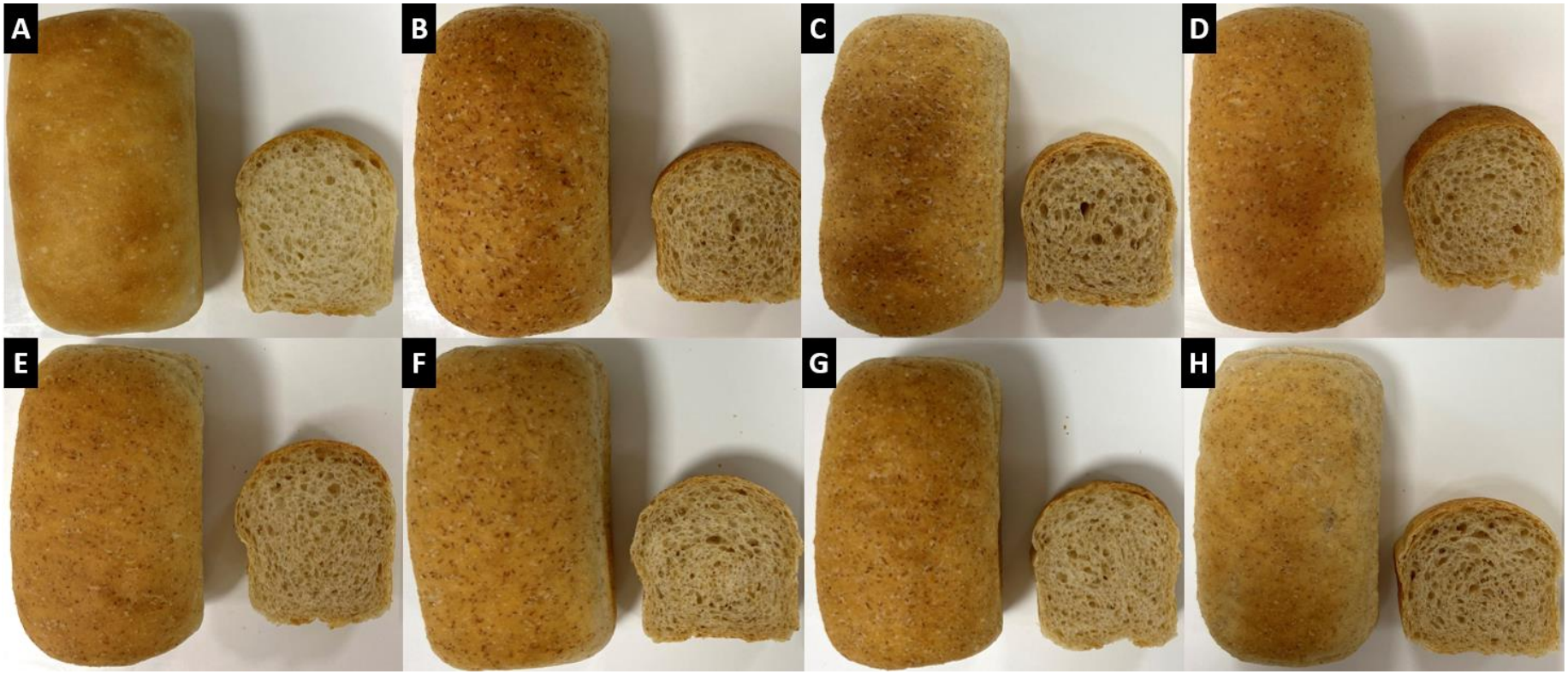

Figure 17. Images of rootlet breads produced during baking trials. Image A, B and C represent control recipe breads made with bakers' flour, unfermented barley rootlets and sterilised barley rootlets, respectively. Images D, E, F, G and H illustrate breads made with fermented barley rootlets with the strains *Leuconostoc citreum* TR116, *Weissella cibaria* MG1, *Lactobacillus amylovorus* FST2.11, *Limosilactobacillus reuteri* R29 and *Lactiplantibacillus plantarum* FST1.7, respectively.

6.4.3.1 Baking loss

Bake loss (BL) was determined to analyse the extent of water loss during the bake. Results from this analysis are depicted on Table 19. BF bread had a BL of 14.24 ± 0.91 % which was not significantly different to any of the formulations tested. A similar BL was observed in BR-UnF (13.54 ± 0.58 %) and BR-Ster (13.56 ± 0.53 %) formulations, with minor differences noted between samples. Among fermented BR ingredient formulations, the highest BL was observed in the BR-TR116 formulation (14.33 ± 0.47 %), followed closely by BR-MG1 (14.19 ± 0.38 %) and BR-FST2.11 (13.85 ± 0.31 %). A minor reduction in BL was observed in BR-R29 (13.36 ± 0.58 %) and BR-FST1.7 (13.54 %) formulations, however all were comparable to the BF control.

Table 19. Bread quality characteristics with inclusion of barley rootlets and novel fermented barley rootlet ingredients (5% inclusion, based on flour). BF represents control recipe using bakers' flour only. BR-UnF and BR-Ster denote control recipes using unfermented barley rootlets and sterilised, freeze-dried barley rootlets, respectively. BR-TR116, BR-MG1, BR-FST2.11, BR-R29 and BR-FST1.7 illustrates fermented rootlet recipes, with ingredients fermented using *L. citreum* TR116, *W. cibaria* MG1, *L. amylovorus* FST2.11, *L. reuteri* R29 and *L. plantarum* FST1.7, respectively. Results are represented as means ± standard deviation. No significant differences were found between samples which share the same subscript letter in the same row ($p < 0.05$).

	BF	**BR-UnF**	**BR-Ster**	**BR-TR116**	**BR-MG1**	**BR-FST2.11**	**BR-R29**	**BR-FST1.7**
Predicted fibre content (g/100 g)	4.74	5.57	5.55	5.66	5.63	5.61	5.61	5.60
Digestible starch content (g/100 g)	40.61 ± 1.46^{c}	38.68 ± 0.80^{a}	$35.64 \pm 0.56^{a,b}$	$36.80 \pm 1.14^{a,b}$	37.17 ± 0.66^{b}	$36.11 \pm 1.02^{a,b}$	$36.32 \pm 0.76^{a,b}$	$36.85 \pm 0.73^{a,b}$
Bake loss (%)	$14.24 \pm 0.91^{a,b,c,d,e}$	$13.54 \pm 0.58^{b,c,e}$	$13.56 \pm 0.53^{b,c}$	$14.33 \pm 0.47^{d,e}$	$14.19 \pm 0.38^{a,d,e}$	$13.85 \pm 0.31^{a,b,c,d,e}$	$13.36 \pm 0.58^{a,b,c}$	$13.54 \pm 0.93^{a,b,c,d,e}$
Specific Volume (mL/g)	$3.74 \pm 0.20^{d,e}$	$3.55 \pm 0.14^{c,d}$	3.09 ± 0.18^{b}	$3.68 \pm 0.22^{c,d,e}$	3.80 ± 0.13^{e}	2.80 ± 0.15^{a}	$3.35 \pm 0.29^{b,c}$	2.63 ± 0.19^{a}
Cell diameter (mm)	2.93 ± 0.15^{e}	2.16 ± 0.15^{d}	$1.94 \pm 0.18^{b,c}$	$1.95 \pm 0.17^{b,c}$	$2.05 \pm 0.16^{c,d}$	1.83 ± 0.22^{b}	1.83 ± 0.15^{b}	1.63 ± 0.16^{a}
Bread crumb hardness (N)	2.33 ± 0.32^{c}	$1.77 \pm 0.26^{a,b}$	3.14 ± 0.42^{d}	1.96 ± 0.30^{b}	1.61 ± 0.22^{a}	3.59 ± 0.53^{d}	2.39 ± 0.35^{c}	4.11 ± 0.55^{d}
Bread crumb resilience (N)	$0.56 \pm 0.01^{c,d}$	0.53 ± 0.02^{b}	$0.55 \pm 0.01^{b,c}$	$0.55 \pm 0.02^{b,c}$	0.56 ± 0.01^{d}	0.52 ± 0.02^{a}	0.54 ± 0.01^{b}	0.53 ± 0.01^{a}

6.4.3.2 Specific Volume

The specific volume of each bread was analysed as an important parameter to investigate the rise and expansion of the breads. The results from the specific volume analysis are presented in Table 19. Comparing control breads, BF and BR-UnF formulations had similar specific volumes, with values of 3.74 ± 0.20 mL/g and 3.55 ± 0.14 mL/g measured, respectively. A significant reduction in the specific volume was noted with the inclusion of BR-Ster (3.09 ± 0.18 mL/g).

In relation to the fermented ingredients, the formulation including BR-MG1 resulted in the highest specific volume recorded (3.80 ± 0.13 mL/g), which was comparable to BF and significantly higher than BR-UnF and BR-Ster controls. BR-TR116 had a specific volume of 3.68 ± 0.22 mL/g which was comparable to BF and BR-UnF. A significant reduction in specific volume was observed in BR-R29 (3.35 ± 0.29 mL/g). The lowest specific volumes recorded were in BR-FST2.11 (2.80 ± 0.15 mL/g) and BR-FST1.7 (2.63 ± mL/g) formulations, which were significantly lower than BF, BR-UnF and BR-Ster control recipes.

6.4.3.3 Crumb structure - Cell diameter

The cell diameter was determined to provide information of the cell structure of the crumb. Results are depicted on Table 19.

The largest cell diameter was determined in the BF control bread (2.93 ± 0.15 mm). A reduction in cell diameter was observed in BR-UnF (2.16 ± 0.15 mm) and BR-Ster (1.94 ± 0.18 mm) breads compared to the BF.

Amongst the fermented formulations, BR-MG1 had the highest cell diameter (2.05 ± 0.16 mm), which was not significantly different to BR-UnF and BR-Ster controls. The BR-TR116 formulation had a cell diameter of 1.95 ± 0.17 mm, followed by BR-FST2.11 (1.83 ± 0.22 mm), BR-R29 (1.83 ± 0.15 mm) and BR-FST1.7 (1.63 ± 0.16 mm).

6.4.3.4 Bread crumb texture

Bread crumb texture was determined to provide information the bread crumb quality. Results of crumb texture parameters hardness and resilience are represented on Table 19.

6.4.3.4.1 Hardness

Among control recipes, the softest crumb was noted in the BR-UnF formulation (1.77 ± 0.26 N). A significant increase in bread crumb hardness was observed in BF (2.33 ± 0.32 N), with an even larger increase recorded in the BR-Ster (3.14 ± 0.42 N).

The lowest bread crumb hardness was found in BR-MG1 (1.61 ± 0.22 N) followed by BR-TR116 (1.96 ± 0.30 N), with both recipes having comparable crumb hardness to the BR-UnF formulation. An increase in crumb hardness was found in BR-R29 formulation (2.39 ± 0.35 N), which was more comparable with the BF control recipe. In relation to the BR-FST2.11 and BR-FST1.7 recipes, a significant increase in crumb hardness was observed with hardness values of 3.59 ± 0.53 N and 4.11 ± 0.55 N recorded, which were aligned with the BR-Ster hardness value.

6.4.3.4.2 Resilience

The highest bread crumb resilience was noted in the BF control (0.56 ± 0.01 N), with some reductions in bread crumb strength found in BR-UnF (0.53 ± 0.02 N) and BR-Ster (0.55 ± 0.01 N). Comparing fermented ingredients, inclusion of BR-MG1 resulted in the highest bread crumb resilience (0.56 ± 0.01 N). A reduction in bread crumb strength was observed with BR-TR116 (0.55 ± 0.02 N) and BR-R29 inclusion (0.54 ± 0.01 N), aligning with BR-Ster and BR-UnF values. BR-FST2.11 (0.52 ± 0.02 N) and BR-FST1.7 (0.53 ± 0.01 N) inclusion had the greatest reduction in crumb strength which was significantly different to BF, BR-UnF and BR-Ster formulations.

6.4.3.5 Microbial shelf-life properties

6.4.3.5.1 Antifungal compounds in BR ingredients

The BR ingredients used in the study were screened for the presence of 15 phenolic-type antifungal compounds to investigate naturally present antifungal compounds, as well as the extent of those produced during LAB fermentation. Results from the analysis are presented on Table 20.

Table 20. Analysis of the antifungal compounds present in BR ingredients in g/100 g dry matter. BR-UnF and BR-Ster represent control ingredients unfermented barley rootlets and sterilised, freeze-dried barley rootlets, respectively. BR-TR116, BR-MG1, BR-FST2.11, BR-R29 and BR-FST1.7 illustrates fermented rootlet ingredients which were fermented using *L. citreum* TR116, *W. cibaria* MG1, *L. amylovorus* FST2.11, *L. reuteri* R29 and *L. plantarum* FST1.7, respectively. Results are represented as means ± standard deviation. No significant differences were found between samples which share the same subscript letter in the same row ($p < 0.05$).

Antifungal compound	BR-UnF	BR-Ster	BR-TR116	BR-MG1	BR-FST2.11	BR-R29	BR-FST1.7
Hydroxyphenyllactic acid	n.d.	n.d.	0.637 ± 0.091^{a}	n.d.	2.483 ± 0.247^{b}	2.239 ± 0.087^{b}	9.136 ± 0.104^{c}
4-Hydroxybenzoic acid	0.928 ± 0.006^{d}	$1.215 \pm 0.109^{c,d}$	1.177 ± 0.036^{c}	$0.881 \pm 0.101^{a,c,d}$	$0.864 \pm 0.078^{a,b,d}$	0.653 ± 0.034^{a}	$0.861 \pm 0.004^{b,c}$
Vanillic acid	1.316 ± 0.018^{b}	1.551 ± 0.171^{b}	1.270 ± 0.038^{b}	1.367 ± 0.150^{b}	$1.144 \pm 0.157^{a,b}$	1.199 ± 0.057^{b}	0.553 ± 0.006^{a}
Phenyllactic acid	n.d.	n.d.	5.254 ± 0.179^{b}	0.562 ± 0.078^{a}	5.223 ± 0.740^{b}	15.645 ± 0.589^{c}	13.387 ± 0.074^{c}
Hydroferulic acid	n.d.	n.d.	n.d.	0.924 ± 0.050^{a}	0.973 ± 0.132^{a}	n.d.	0.815 ± 0.019^{a}
Coumaric acid	n.d.	0.537 ± 0.090^{a}	0.842 ± 0.057^{b}	n.d.	n.d.	n.d.	n.d.
Ferulic acid	1.008 ± 0.095^{a}	1.656 ± 0.197^{b}	2.220 ± 0.079^{c}	n.d.	n.d.	4.068 ± 0.164^{d}	n.d.

n.d defined as not detected.

Hydroxyphenyllactic acid was not detected in quantifiable amounts in BR-UnF, BR-Ster or BR-MG1. The highest amount of hydroxyphenyllactic acid was determined in BR-FST1.7 (9.136 ± 0.104 g/100 g D.M). In comparison to BR-FST1.7, a significant reduction in hydroxyphenyllactic was observed in BR-FST2.11 (2.483 ± 0.247 g/100 g D.M) and BR-R29 (2.239 ± 0.087 g/100 g D.M), with the lowest amount recorded in BR-TR116 (0.637 ± 0.091 g/100 g D.M). In relation to 4-hydroxybenzoic acid, the highest amount detected was in BR-Ster (1.215 ± 0.190 g/100 g D.M). No significant differences were found in levels of 4-hydroxybenzoic acid between BR-Ster and BR-UnF (0.928 ± 0.006 g/100 g D.M). Among the fermented ingredients, relatively similar amounts of 4-hydroxybenzoic acid were determined. BR-TR116 (1.177 ± 0.036 g/100 g D.M) contained the highest amount of 4-hydroxybenzoic acid, followed sequentially by BR-MG1 (0.881 ± 0.101 g/100 g D.M), BR-FST2.11 (0.861 ± 0.078 g/100 g D.M) and BR-FST1.7 (0.861 ± 0.004 g/100 g D.M) with amounts observed comparable to BR-UnF and BR-Ster. The lowest amount of 4-hydroxybenzoic acid was recorded in BR-29 (0.653 ± 0.034 g/100 g D.M). With regards to vanillic acid levels, no significant differences were found between samples as contents ranged between 1.144 -1.551 g/100 g D.M, except in the case of BR-FST1.7, which had a lower amount (0.553 ± 0.006 g/100 g D.M). Phenyllactic acid was not detected in BR-UnF and BR-Ster. Among fermented ingredients, BR-R29 and BR-FST1.7 contained the highest amounts of phenyllactic acid, with values of 15.645 ± 0.589 g/100 g D.M and 13.387 ± 0.074 g/100 g D.M quantified, respectively. A significantly lower amount of phenyllactic acid was determined in BR-TR116 (5.254 ± 0.090 g/100 g D.M) and BR-FST2.11 (5.223 ± 0.740 g/100 g D.M) with the lowest amount of phenyllactic acid quantified in BR-MG1 (0.562 ± 0.078 g/100g D.M). Hydroferulic acid was detected only in BR-MG1 (0.924 ± 0.050 g/100 g D.M), BR-FST2.11 (0.973 ± 0.132 g/100g D.M) and BR-FST1.7 (0.815 ± 0.019 g/100 g D.M) with no significant differences found between samples. Coumaric acid was quantified in BR-Ster (0.537 ± 0.090 g/100 g D.M) and BR-TR116 (0.842 ± 0.057 g/100 g D.M), with the amount quantified BR-TR116 significantly higher than the amount determined in BR-Ster. BR-UnF contained 1.008 ± 0.095 g/100 g D.M of ferulic acid and was the lowest amount detected among ingredients. A significantly higher amount of ferulic acid was noted in BR-Ster (1.656 ± 0.197 g/100 g D.M). A significant increase in ferulic acid content was measured in BR-TR116 (2.220 ± 0.079 g/100 g D.M), with the highest amount recorded in BR-R29 (4.068 ± g/100 g D.M). Ferulic acid was not quantified in BR-MG1, BR-FST2.11 and BR-FST1.7.

6.4.3.5.2 Bread microbial shelf-life

The extent of mould growth on the bread crumb of the breads was analysed to investigate the shelf life of the bread. The results of the microbial shelf-life analysis are illustrated in Figure 18.

The first mould growth on the BF was noted after two days which was similar to the BR-UnF formulation. Relatively similar kinetics were observed in the BF and BR-UnF recipes. Incorporation of BR-Ster prolonged the microbial shelf life of the bread by one day, with kinetics of the mould growth also slightly slower compared to control recipes. Like the BR-Ster recipe, formulations containing BR-MG1 and BR-FST1.7 remained mould free until day 3, however the extent of mould growth over time in BR-MG1 and BR-FST1.7 recipes was much slower. A further increase in the number of mould-free days (+ 1 day) was observed with BR-TR116 and BR-FST2.11 addition, with a considerable delay in mould growth over time compared to BR-Ster, BR-UnF and BF controls. Inclusion of BR-R29 had the most substantial effect in microbial shelf-life extension with no mould growth observed until day 4 and little to no increase in mould growth for up to 8 days.

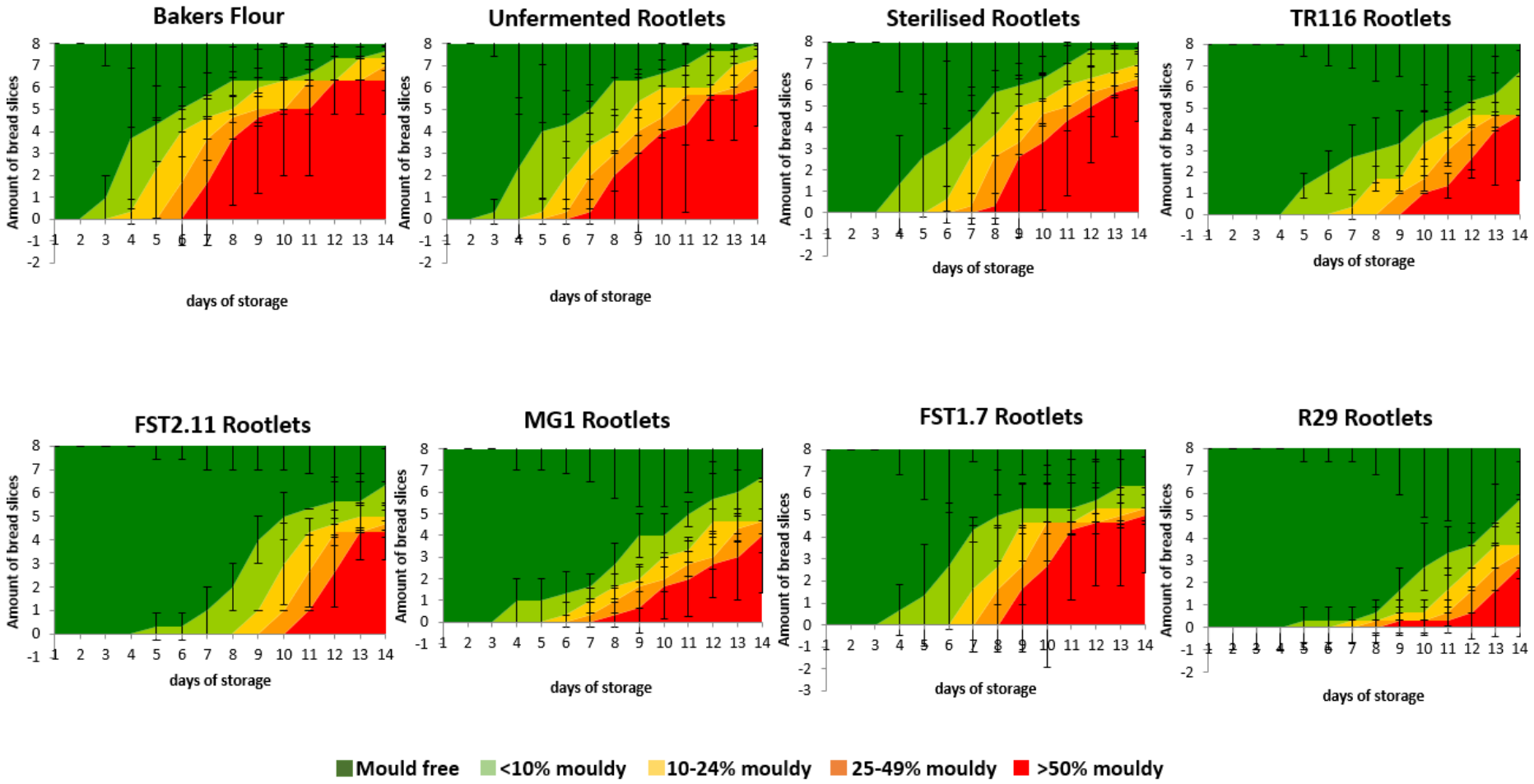

Figure 18. Microbial shelf-life evaluation of bread containing unfermented and fermented BR. TR116, FST2.11, MG1, FST1.7 and R29 denote BR recipes fermented with the strains *Leuconostoc citreum* TR116, *Lactobacillus amylovorus* FST2.11, *Weissella cibaria* MG1, *Lactiplantibacillus plantarum* FST1.7 and *Limosilactobacillus reuteri* R29, respectively. The graph represents the means of three independent batches with error bars representing the standard deviation between batches.

6.4.3.6 In vitro starch hydrolysis

Starch digestibility was examined to provide an insight into the nutritional characteristics of the bread. The results of the release of reducing sugars (RSR) over time are illustrated on Figure 19.

Breads with supplementation of BR-UnF resulted in the highest RSR curve. A slight reduction in the RSR value was noted in BF and BR-TR116 bread formulations compared to BR-UnF. A further reduction in RSR release was also observed in BR-Ster, BR-MG1 and BR-R29 compared to BR-UnF but was not statistically significant from BF and BR-TR116 formulations. Bread formulations with inclusion of BR-FST2.11 and BR-FST1.7 slowed the release of reducing sugars to the greatest extent, particularly in the case of BR-FST1.7 where the lowest RSR was recorded, which was significantly lower than control formulations BF, BR-UnF and BR-Ster.

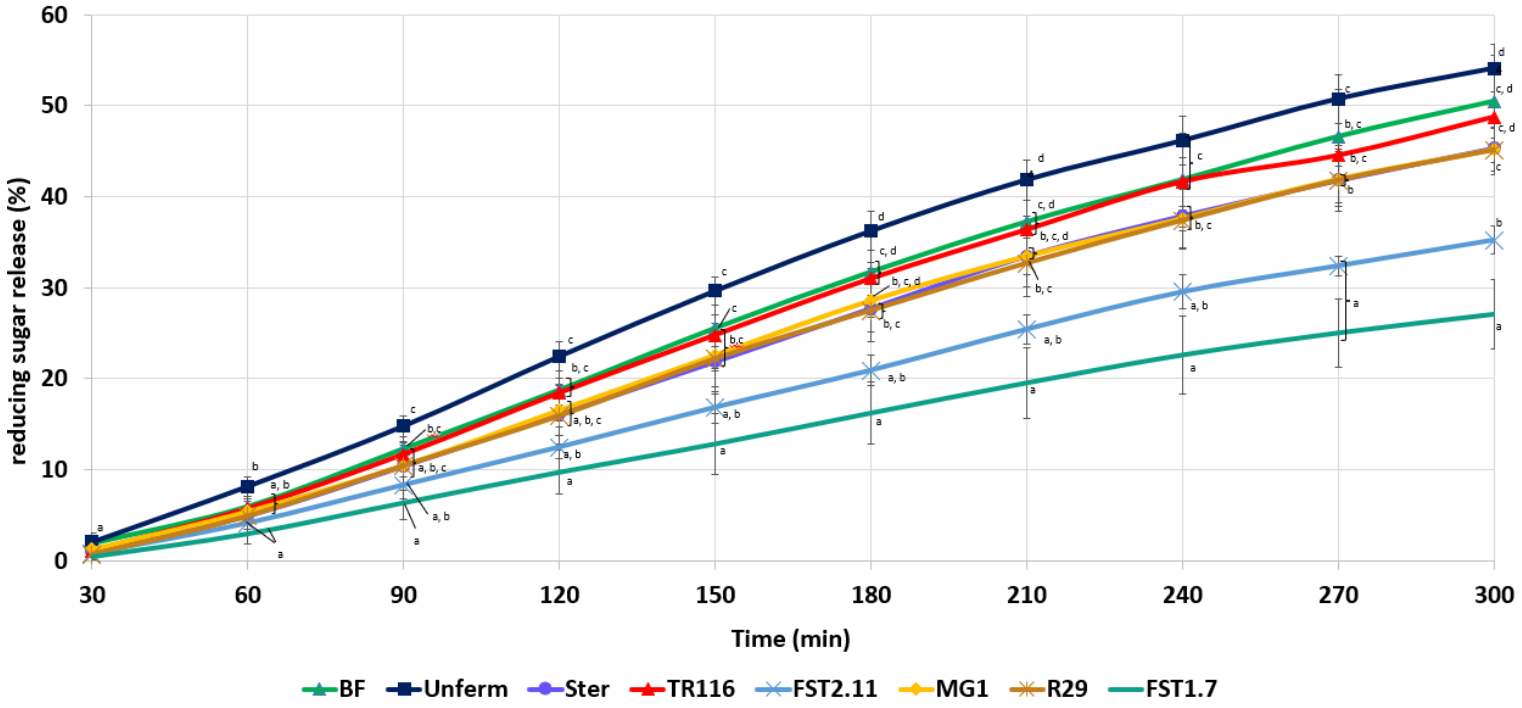

Figure 19. Release of reducing sugars during in-vitro starch hydrolysis of BR breads. BF, UnFerm and Ster represent control recipes made using bakers' flour, 5 % supplementation with unfermented rootlets and sterilised rootlets, respectively. TR116, FST2.11, MG1, FST1.7 and R29 represent the recipes with 5% replacement of fermented rootlets made using *Leuconostoc citreum* TR116, *Lactobacillus amylovorus* FST2.11, *Weissella cibaria* MG1, *Lactiplantibacillus plantarum* FST1.7 and *Limosilactobacillus reuteri* R29, respectively. Graph illustrates the mean values of triplicate samples with standard deviations represented by error bars. Graph points which share the same letter at each time point do not differ significantly.

6.4.3.7 Sensory analysis of the BR breads

A sensory analysis of the BR breads was conducted to determine the effect of BR inclusion on the overall sensory experience of the bread and to determine their palatability. Results are represented in Table 21.

Overall, the odour perception of all BR breads was acceptable and comparable to the BF control. No significant differences were found between bread samples with reference to any of the odour attributes. In relation to taste parameters analysed, some significant differences were found between BR breads. The lowest sourness was perceived in the BR-Ster (0.72 ± 0.83) and BF (0.78 ± 1.26) controls. An increase in sourness was noted in the BR-UnF breads (1.61 ± 1.42), however, this was not significantly different to the BR-Ster and BF formulations. Inclusion of fermented BR resulted in an increase in sourness. The BR-TR116, BR-MG1 and BR-R29 breads had sourness values of 2.17 ± 1.86, 2.22 ± 1.66 and 2.89 ± 2.14, respectively, which was comparable to the BR-UnF formulation but was significantly higher than the BF and BR-Ster formulations. An even greater increase in sourness was recorded in BR-FST2.11 (4.22 ± 2.34) and BR-FST1.7 (4.33 ± 2.83) which was significantly higher than all control recipes as well as fermented samples BR-TR116 and BR-MG1. No significant difference was noted in flavour intensity, aftertaste and in the muddy/earthy flavour parameters, however, significant differences were detected between samples for fruity and vegetable flavour compounds in BR breads. Fruity flavours were lowest in BF (0.44 ± 0.51) and BR-Ster (0.61 ± 0.70). An increase in fruity flavours was noted in the BR-UnF (1.56 ± 1.76), BR-TR116 (1.39 ± 1.38) and BR-MG1 (1.56 ± 1.50) compared to BF and BR-Ster. The values for fruity flavours found in BR-R29 (2.39 ± 1.42) and BR-FST1.7 (2.89 ± 2.49) were slightly higher than the those observed in the other fermented samples; however, this was not found to be significant. The highest perception of fruity flavours was detected in the BR-FST2.11 formulations, which was comparable to BR-R29 and BR-FST1.7 formulations but was significantly higher than all control samples. In regard to the vegetable notes in the rootlet breads, BF had the lowest value (1.06 ± 0.94), whereas inclusion of BR-Ster resulted in the highest perception of vegetable flavours (3.06 ± 1.55). BR-TR116 addition also resulted in an elevated vegetable flavour which was similar the BR-Ster sample. Among the remaining samples (BR-UnF, BR-MG1, BR-FST2.11, BR-R29 and BR-FST1.7), no difference in vegetable flavour was detected between samples, with values ranging from 1.89 - 2.50. Hardness and chewiness values ranged between 3.33 - 4.00 and 4.50 – 5.22, respectively, with no significant differences observed between

samples. In addition, all breads analysed were ranked highly in overall acceptability (<7), with no significant differences found among the breads tested

Table 21. Descriptive sensory analysis of BR breads. BF represents results obtained from bakers' flour wheat flour breads. BR-UnF and BR-Ster denote control recipes made with 5% inclusion of unfermented rootlets and sterilised rootlets, respectively. BR-TR116, BR-MG1, BR-FST2.11, BR-R29 and BR-FST1.7 illustrate sensory results from bread recipes made with 5% addition of fermented rootlets produced using *L. citreum* TR116, *W. cibaria* MG1, *L. amylovorus* FST2.11, *L. reuteri* R29 and *L. plantarum* FST1.7, respectively. Results are represented as mean values ± standard deviations. Values in the same row which share the same subscript letter are not significantly different ($p < 0.05$).

	BF	**BR-UnF**	**BR-Ster**	**BR-TR116**	**BR-MG1**	**BR-FST2.11**	**BR-R29**	**BR-FST1.7**
Odour								
Intensity	5.78 ± 2.21^{a}	6.39 ± 1.85^{a}	6.67 ± 2.28^{a}	6.33 ± 2.11^{a}	6.78 ± 1.83^{a}	6.67 ± 2.11^{a}	6.22 ± 1.86^{a}	6.22 ± 2.07^{a}
Citrus	1.67 ± 1.46^{a}	2.06 ± 1.26^{a}	1.50 ± 1.86^{a}	1.72 ± 1.36^{a}	1.83 ± 0.99^{a}	2.56 ± 1.65^{a}	2.17 ± 1.34^{a}	2.78 ± 1.80^{a}
Vegetable	1.28 ± 1.27^{a}	2.28 ± 1.49^{a}	2.44 ± 1.50^{a}	1.83 ± 0.99^{a}	1.78 ± 1.44^{a}	2.44 ± 1.38^{a}	2.06 ± 1.70^{a}	1.67 ± 1.14^{a}
Cereals/grain	6.06 ± 2.36^{a}	7.11 ± 1.32^{a}	7.28 ± 1.36	7.00 ± 1.41^{a}	6.83 ± 1.34^{a}	6.06 ± 1.30^{a}	6.06 ± 1.43^{a}	6.56 ± 1.54^{a}
Taste								
Sour	0.78 ± 1.26^{a}	$1.61 \pm 1.42^{a,b}$	0.72 ± 0.83^{a}	2.17 ± 1.86^{b}	2.22 ± 1.66^{b}	4.22 ± 2.34^{c}	$2.89 \pm 2.14^{b,c}$	4.33 ± 2.83^{c}
Flavour								
Intensity	4.39 ± 1.61^{a}	5.72 ± 1.64^{a}	5.67 ± 2.11^{a}	5.44 ± 1.85^{a}	5.11 ± 2.05^{a}	6.22 ± 1.77^{a}	5.94 ± 1.95^{a}	6.17 ± 1.95^{a}
Muddy/earthy	0.89 ± 1.02^{a}	2.22 ± 2.37^{a}	2.89 ± 2.37^{a}	2.50 ± 2.38^{a}	1.67 ± 1.81^{a}	2.17 ± 2.33^{a}	1.83 ± 2.20^{a}	1.44 ± 1.25^{a}
Fruity	0.44 ± 0.51^{a}	$1.56 \pm 1.76^{b,c}$	$0.61 \pm 0.70^{a,b}$	$1.39 \pm 1.38^{b,c}$	$1.56 \pm 1.50^{b,c}$	3.56 ± 2.45^{d}	$2.39 \pm 1.42^{c,d}$	$2.89 \pm 2.49^{c,d}$
Vegetable	1.06 ± 0.94^{a}	$2.39 \pm 1.46^{a,b}$	3.06 ± 1.55^{b}	2.61 ± 1.65^{b}	$2.39 \pm 1.58^{a,b}$	$2.50 \pm 1.47^{a,b}$	$2.50 \pm 1.86^{a,b}$	$1.89 \pm 1.41^{a,b}$
Aftertaste	1.28 ± 1.74^{a}	2.72 ± 2.44^{a}	2.94 ± 2.21^{a}	2.22 ± 1.96^{a}	2.11 ± 1.64^{a}	3.39 ± 2.66^{a}	3.06 ± 2.41^{a}	3.17 ± 2.36^{a}
Texture								
Hardness	4.00 ± 2.28^{a}	3.44 ± 2.28^{a}	3.83 ± 1.95^{a}	3.44 ± 1.62^{a}	3.50 ± 1.76^{a}	3.89 ± 1.45^{a}	3.33 ± 1.71^{a}	3.61 ± 1.65^{a}
Chewiness	4.50 ± 2.23^{a}	4.67 ± 2.11^{a}	4.50 ± 2.01^{a}	4.78 ± 1.77^{a}	5.22 ± 2.07^{a}	4.94 ± 1.63^{a}	4.56 ± 1.98^{a}	4.94 ± 1.83^{a}
Overall acceptability	8.17 ± 1.72^{a}	7.56 ± 1.82^{a}	7.33 ± 2.11^{a}	7.67 ± 1.64^{a}	8.11 ± 1.41^{a}	7.28 ± 1.41^{a}	7.50 ± 1.98^{a}	7.44 ± 1.46^{a}

6.5 Discussion

The introduction of food by-products into the food chain has become an important part of research to address various aspects of the sustainability goals of the future. Fortification of staple foods with by-products poses difficulties, with deterioration in food prototype quality observed, particularly at higher levels of inclusion [11,68,145,146,350]. Hence, further valorisation and additional processing is required. Neylon et al. [341] demonstrated the significant potential of LAB fermentation to improve BR quality at a fundamental level while this study illustrates translation of various benefits imparted by individual LAB fermentations when incorporation into bread protypes.

The addition of BR, regardless of the processing applied, significantly reduced the strength of the gluten network suggesting that BR interfere with the gluten network formation. The reductions in gluten network strength are likely a contribution of the changes in minerals, and protein and fibre interaction with BR addition. BR contain a variety of minerals [11] which could introduce a charge effect and negatively impact the fundamental bonds required for gluten network development [351]. Inclusion of BR also introduces a certain amount of protein to the system, containing a variety of charged amino acids [11] which can also interfere with the intramolecular gluten network bonds. Finally, fibre constitutes a large proportion of the BR composition which has been seen to alter gluten development and techno-functional characteristics which may contribute to the negative defects observed [199,254]. Fibres have been shown to create a physical hindrance in gluten development; negatively interfere with the hydration of the gluten network; and facilitate gluten-fibre interactions which results in weakened gluten techno-functional characteristics [199,254]. Inclusion of fermented BR reduced the amount of time taken for the gluten network to develop which might be due to enhanced hydrophobic interactions. The inclusion of a fermented ingredient putatively creates a more acidic environment which may change the configuration of proteins, further exposing the hydrophobic regions of proteins [162,292]. This might amplify hydrophobic interactions during gluten development which is believed to stabilise gluten development [352], thus reducing the amount of time required for the network to form. In addition to this, fermentation of BR increases mineral bioavailability [353] resulting in a possible charge screening effect which may further help expose apolar amino acid chains and encourage hydrophobic interactions to a greater extent.

The time taken for gluten network development also correlated positively ($r = 0.899$, $p < 0.05$) to the damping factor from rheological measurements, indicating that the time take for gluten network development was influential in determining viscoelastic properties. Inclusion of BR-UnF maintained the same viscoelastic properties as BF which could be linked with the alpha amylase activity of BR-UnF. Alpha amylases degrade starch molecules to small chain dextrins which reduce dough viscosity [354] and thus likely increases the viscous proportion of the dough. Moreover, proteases play a vital role in the germination stage of malting [6,340], which is where BR are sourced from. Thus, BR-UnF likely contain natural proteases present which may also induce a dough softening effect and enhance viscous parts of the dough [355,356]. In contrast, BR-Ster increased elastic (solid) proportions of the dough. This was likely due to the elimination of the enzymatic effects associated with BR-UnF with heat treatment applied and the overall reduction in starch and gluten within the dough matrix with flour replacement resulting in a dough with enhanced resistance to deformation. For fermented formulations, the enhanced hydrophobic interactions discussed previously may putatively stabilise the gluten network development in the dough matrix, resulting in a dough with greater resistance to deformation. The greater increase in elastic parts in BR-FST2.11 and BR-FST1.7 compared to the other fermented ingredients could be due to the greater acid contents present in these ingredients [341]. This may enhance the amount of positive charges present, facilitating hydrophobic interactions during gluten development [206] and producing a stiffer dough.

The DF also had an influence on the maximum dough rise (Hm) achieved during the yeast fermentation ($r= 0.885$, $p < 0.05$), indicating that viscoelastic properties facilitate dough expansion during the fermentation. Interestingly, the BR-MG1 formulation achieved a high Hm which may be explained by exopolysaccharides present in the BR-MG1 ingredient post fermentation. *W. cibaria* MG1 is capable of producing exopolysaccharides of the dextran type and gluco-oligosaccharide type from sucrose [289,290] which can be used to enhance bread structure and mimic behaviour of hydrocolloids [357]. A previous study investigating the use of *W. cibaria* MG1 in sourdough attributed the enhanced loaf volume and reduced crumb hardness to the dextran exopolysaccharides [358]. Thus, higher Hm values during proofing could be achieved through a re-enforced dough structure with the presence of exopolysaccharides in BR-MG1. Galle et al. [358] also observed increased production of CO_2 with the inclusion of *W. cibaria* MG1 in sourdough, suggesting the increased production was as a result of enhanced level of sugars present in the sourdough favouring yeast fermentation. Results from Vtotal CO_2 values in this study compliment these findings. The BR-MG1 (as well

as BR-FST2.11 and BR-FST1.7) ingredient contains higher levels of residual glucose and fructose [341] which could enhance yeast metabolism and increase the volume of CO_2 produced during proofing. However, it is important to note that inclusion of BR regardless of the processing applied enhanced CO_2 production, indicating BR did not interfere negatively with yeast metabolism, but rather improved it. This suggests BR are likely a suitable substrate for inclusion in yeast leavened products. Thus, the adverse effects in dough rise observed are likely more connected to the observations made in DF values previously discussed. However, in contrast to this trend was the BR-R29 formulation which behaved differently during the yeast fermentation with a reduced amount of CO_2 produced. This could be linked to the levels of antifungals present (discussed in later sections) as well as the probable reuterin present [295] in the ingredient which inhibited yeast metabolism and thus dough expansion to a certain extent. However, volumes of CO_2 produced were relatively similar to amounts produced in the BF control indicating that the negative impacts observed was not of major significance.

Hm values correlated positively to bread specific volume ($r = 0.90$, $p < 0.05$) indicating the height reached during yeast fermentation had an effect on bread volumes. BR-MG1 breads had an improved specific volume, reaching volumes higher than that observed for the BF wheat control, suggesting fermentation of BR with *W. cibaria* MG1 had a positive influence on bread quality. The enhancement observed can be attributed to the more stabilised dough structure with the presence of exopolysaccharides. Exopolysaccharides have been described to perform similar to hydrocolloids in a bread system [359] and increasing bread specific volumes [334]. The inclusion of BR-TR116 also maintained bread specific volume which was comparable to BF. Although *L. citreum* TR116 can also produce exopolysaccharides [283], the presence of exopolysaccharides was unlikely, as the fermentation was supplemented with fructose which facilitates the production of mannitol and acetate [341]. Higher volumes of acetate have been reported in literature to negatively influence dough extensibility and volume [360], however this did not appear to negatively impact the BR-TR116 bread quality. This might be linked with the addition level of the ingredient which may not have reached the threshold for this hypothesis to take effect. Incorporation of BR-UnF also achieved specific volumes comparable to the BF likely due to the similarities observed in viscoelastic properties as well as the enhanced yeast metabolism. The lower specific volumes with inclusion of BR-Ster, BR-FST2.11, BR-R29 and BR-FST1.7 could be associated with the higher elastic properties of these doughs restricting dough rise. The higher acidification effect in the FST1.7 and FST2.11 formulations may have had an extra contributory effect and may constitute reason for the

lowest volumes observed in these formulations. In the case of BR-R29, the reductions observed in bread specific volume coincide and can be explained by the inhibited yeast fermentation. The variations observed in crumb hardness may be attributed to the differences noted in bread specific volume, as crumb hardness and bread specific volume had a strong negative correlation ($r = -0.957$, $p < 0.01$). Addition of BR-UnF produced a soft crumb likely due to alpha amylase activity present in BR-UnF [341] which has a positive influence on crumb texture [361]. Inclusion of BR ingredients fermented with *W. cibaria* MG1 and *L. citreum* TR116 also positively influenced crumb hardness as even lower crumb hardness (compared to BF) was observed highlighting further enhancements to bread quality using BR-MG1 and BR-TR116 inclusion. Although variations in crumb hardness were observed, overall, relatively similar trends in crumb resilience suggest inclusion of BR does not negatively impact the crumb integrity. Dough characteristics such as Hm and DF also had an impact on crumb cell structure with significant positive correlations observed with cell diameter values ($r= 0.97$, < 0.05 and $r= 0.91$, $p < 0.05$, respectively). Thus, variations in cell diameters observed with BR inclusion are a result of the restrictions imposed on viscoelastic properties constricting the cell diameter size.

Aside from gluten network development and dough rheological analysis, in depth analysis of the effects on gluten and starch pasting during mixing and heating provided by Mixolab analysis gives further insight into bread quality. Inclusion of BR increased dough development times, likely due to the higher amount of fibre inclusion in bread formulations which has previously shown to extend dough development times [210,348,362]. This may be due to the increase in competition for water with fibre inclusion [208,334] as BR-UnF and BR-Ster have an exceptionally high water binding capacity [341]. The addition of fermented BR ingredients reduced the amount of time required for the dough to form. This may be linked with the reduced water binding capacity of the fermented ingredients [341] which would likely reduce the competition for hydration. In addition, the reductions in dough development times with inclusion of fermented ingredients aligns with the shorter gluten network development times seen previously, indicating optimal dough development occurs at an earlier stage with fermented ingredient inclusion. Thus, as fully developed doughs reflect optimal dough quality results from this study suggest alterations in dough mixing times during the baking process may further enhance BR bread dough quality. The reductions in C2 values suggest a protein weakening effect [347] with BR inclusion and compliment the results observed previously with decreased gluten network strength. The greatest reductions in C2 observed with BR-UnF, BR-

FST2.11 and BR-FST1.7 suggest that the protein destabilisation effect was process dependent. The protein weakening effect with BR-UnF could be a result of active proteases in BR-UnF which can weaken the integrity of the gluten network strength [292]. The enhanced protein weakening effect in the BR-FST2.11 and BR-FST1.7 might be linked with the higher acid content of these BR ingredients [341]. The higher acid content, which as previously stated may enhance the formation of the gluten network, could also activate some proteolytic activity in the wheat flour [363], resulting in weakening the integrity and the functionality of the gluten network overtime. Reductions in C3 values indicate lower levels of starch swelling. The reduction in starch swelling with BR inclusion was to be expected with the inclusion of a fibre/protein rich material such as BR which limits the amount of starch available to contribute to this reaction [208,245,282]. The more pronounced reduction in starch swelling in BR-UnF reflects the high alpha amylase activity of the unfermented BR ingredient [341] which adversely effects starch granule integrity [356], hence, negatively impacting its hydration properties. The higher enzymatic activity hypothesis can also be applied to rupturing of starch granules (C4 values) and starch retrogradation properties (C5 values) of the BR-UnF dough. Higher alpha amylase activity accumulates low molecular weight dextrin and thus reducing viscosity, which explains the lower C4 values [364]. Furthermore, higher alpha amylase activities restrict realignment during the retrogradation process with increasing low molecular weight dextrins [365,366]. The minor differences observed with BR ingredient inclusion across all other formulations indicates no major differences were detected in the starch pasting properties, indicating little strain dependency effects.

Observations from microbial shelf-life kinetics show inclusion of BR-UnF resulted in a microbial shelf life similar to BF, indicating BR-UnF did not influence the microbial shelf life. The one day increase in microbial shelf life with BR-Ster addition illustrates the sterilisation process mitigates some natural microflora present in BR which may help to extend the microbial shelf life. The further extension in microbial shelf life (+1 day) with fermented BR addition can be linked with the antifungal metabolites imparted on the BR ingredients during fermentation. Sourdough technology with numerous LAB strains has been previously shown to provide a natural antimicrobial effect due to the synergistic effect of the variety of antifungal metabolites and organic acids produced during fermentation [267,294]. BR-MG1 had the lowest amount and variety of antifungal compounds present suggesting *W. cibaria* MG1 does not give an enhanced antifungal effect and provides explanation to the similar shelf-life kinetics observed with BR-Ster inclusion. Among the fermented formulations, BR-R29 provided the

most significant reduction in microbial growth kinetics due to the highest amount/variety of antifungal compounds and their synergistic effect with lactic and acetic acid creating an even greater hurdle effect [294]. Previous studies using *L. reuteri* R29 found success in microbial shelf life extension in bread and attributed this primarily to the exceptionally high levels of phenyllactic acid produced during fermentation [294,295], complimenting the results found in this study. *L. amylovorus* FST2.11, *L. plantarum* FST1.7 and *L. citreum* TR116 have also been documented as antifungal producers across a variety of substrates [160,162,244,285,367], however in this study, the low BR addition level may have limited their potency as a natural shelf life extender. Thus, fermentation of BR with *L. reuteri* R29 and their addition into bread at 5% inclusion may aid in the formulation of clean label bread products and reduce the level of chemical preservatives such as propionate or sorbate in formulations [268,368].

Due to the high fibre content of the BR ingredient, incorporation into a bread formulation will enhance the fibre content of the bread [68], thus enhancing nutritional benefits of the bread product [67]. However, analysis of changes in the extent of sugar release over time from bread delves further into the potential nutritional benefits of BR inclusion. BR-UnF did not slow sugar release over time but in fact increased the amount of sugar released from the bread matrix. This might be due to the enhanced enzymatic activity of BR-UnF, which favours the hydrolysis of starch during proofing and increases the amount of readily available sugars capable of being released from bread matrix. This also explains the slightly lower sugar release observed with BR-Ster inclusion as enzymatic activity influence was eliminated post sterilisation. The reductions observed with BR-Ster, BR-TR116, BR-R29 and BR-MG1 are likely due to the overall reduction in starch available for hydrolysis with the replacement of a fibre ingredient. Interestingly, BR-FST2.11 and BR-FST1.7 showed a different trend and a notably lower sugar release curve over time was observed. Inclusion of fermented by-products has previously been shown to inhibit starch hydrolysis in bread [157,159,166,282], which in this study, appears to be a strain dependent characteristic. The reduction in the level of starch hydrolysis may be attributed to the much higher amounts of lactic acid in these ingredients [341] which negatively interferes with starch hydrolysis. Studies from Östman et al. [233] show the presence of lactic acid during heat treatment encourages interactions between starch and gluten which limits the starch available for hydrolysis. Thus, fermentation of BR with *L. plantarum* FST1.7 and *L. amylovorus* FST 2.11 might be more beneficial for the engineering of products for consumers with diabetes. The higher levels of acid in BR-FST1.7 and BR-FST2.11 could also have led to changes observed in the sensory perception of these bread prototypes with enhancements noted

in sour taste and fruity flavours. Increases in fruity and sour tastes are typically found in wheat sourdough breads [369] indicating a sourdough-like bread flavour may be achieved through fermentation of BR by *L. plantarum* FST1.7 and *L. amylovorus* FST 2.11. The high comparability of BF bread and BR breads across all sensory attributes indicate that 5% BR inclusion level in a bread system is highly acceptable and compliments bread sensory results previously seen [11,68].

6.6 Conclusion

To date, the inclusion of BR and investigation of their effects in a bread application has been limited however, this study shows a promising future for BR in the baking industry, particularly in yeast leavened products. Furthermore, upcycling processes such as LAB fermentation prove to be a viable processing tool for BR with unique fermentation characteristics being translated to the bread matrix. The addition of BR into the bread system at a 5% addition level was found to decrease the strength of the gluten network, however, the introduction of the fermented ingredients amplified the speed required for gluten development. In relation to the unfermented BR formulation, positive impacts were also observed in the viscoelastic properties of the dough which was mainly attributed to the enzymatic activity of the unprocessed ingredient. The addition of the BR-MG1 and BR-TR116 ingredients showcased their potential to improve bread quality with high specific volumes and softer crumbs observed. Moreover, inclusion of BR-R29 illustrates its power to reduce the microbial growth rate to a significant extent through natural production of high amounts of antifungal compounds. The incorporation of BR-FST1.7 and BR-FST2.11 shows promising potential to further improve the nutritional characteristics of the BR with a slower release of sugars overtime during starch digestion. Furthermore, inclusion of BR-FST1.7 and BR-FST2.11 altered the sensory experience of wheat bread, creating a bread with flavours which might compare more similarly to that of a conventional sourdough. Thus, this study showcases how LAB fermentation of BR can be tailored, based on desired requirements in the bread application at a later stage.

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Chapter 7

GENERAL DISCUSSION

7.1 Discussion, conclusions, and future work.

In recent years, interest in food by-product upcycling and incorporation of the valorised by-products into new applications has been on the rise. The sustainable development goals [1] have highlighted the impending urgency behind the demand to change our way of living at a global level, with food industrial processing playing a key role in this change. As conclusively highlighted in the literature of this thesis, the brewing and malting industries generate large quantities of by-products annually [3,10] including BSG and BR; which are of great nutritional benefit [3,278]. Hence, utilising BSG and BR in applications destined for human consumption is of interest, especially in cereal-based applications. However, the inclusion of both BSG and BR into cereal matrixes such as bread and pasta has been challenging [11,68,145,147,153,154] highlighting the need to further process these ingredients. LAB fermentation represents a processing tool that can be applied in BSG and BR processing, with the use of this technology imparting a variety of enhanced features such as antifungal properties; increased nutritional factors; improved techno-functional capacity; and improved sensory experiences [11,145,154,155,162,166,168,169,174]

The literature review discussed in **Chapter 2** focusses on the fundamentals in relation to BR and brought attention to the biological production of BR within the germination step of the malting process [9,10]. Investigation into the processing techniques for BR removal from the parent malt identified deculming machines (pneumatic device) or deculming screws are implemented; with BR often collected simultaneously with various other malting by-products (barley dust, corns, husks, acrospires) and pelletised for their use in animal feed [9,10,36]. Analysis of the limited basic compositional data available on BR revealed protein (20.34 – 38.70 %) and fibre (9.7 – 43.0 %) constituted the main components in BR, with reasonable levels of minerals (2.8 - 8.7%) and minimal amounts of fat (<5%). The exploration of the basic composition of BR revealed their potential to be supplemented into food matrixes, particularly in cereal-based applications. In addition to their potential in food applications, **chapter 2** also explored the literature available regarding the use of BR as enzyme sources (primarily 5'-phosphodiesterase); a nutrient source during LAB fermentation; a source of antioxidants (vitamin C and vitamin E); and also, their potential in biochar production. Investigation into the current status of BR highlighted the present output for BR (animal feed/landfill) underutilises their maximum potential and also the need for a quality control system to be considered in BR processing to ensure optimal BR quality. **Chapter 2** also includes an excerpt

regarding some of the current knowledge on BSG, an area much more extensively explored compared to BR. The general consensus from literature details inclusion of BSG improves fibre and protein contents of products, however; a major challenge faced is the deterioration in food quality with BSG inclusion, with higher amounts resulting in greater defects. Despite this, LAB fermentation technology has been highlighted as a useful valorisation technique for BSG. The studies applying LAB fermentation technology on BSG have documented notable improvements in product quality such as: softer bread crumbs; increased crumb springiness, increased bread specific volumes; altered product sensory profiles; increased antioxidant potential; enhanced protein digestibility (*in vitro*); reduced glycaemic effect (*in vitro*); and an increased positive effect on the modulation of the gut microbiome (*in vitro*) [145,154,155,168–170]. Hence, suggesting a future for LAB fermentation technology as a by-product valorisation technique

In **chapter 3**, BSG and FBSG were applied in a pasta matrix and the effect of LAB fermentation on BSG in a pasta matrix was investigated. The fortification of BSG and FBSG altered gluten network properties, amplifying the kinetics of the gluten network, with higher levels of inclusion resulting in more significant effects. This was related to the protein, minerals, and fibre present in BSG/FBSG. BSG/FBSG contain a significant amount of proteins (Table 6) which amplified the development of a protein network mainly by their charged amino acids [145]. The higher levels of minerals in BSG/FBSG induced a charge screening effect exposing apolar protein residue and encouraged stronger hydrophobic interaction with increased protein aggregation [202]. BSG/FBSG addition may also shift the balance of glutenin and gliadins present in the flour, enhancing the glutenin proportion and enhancing gluten strength. Higher amounts of FBSG (FBSG HF) resulted in two peaks (secondary protein network other than gluten) during gluten analysis revealing additional protein modifications to FBSG. This concluded that the fermentation modified the proteins present in BSG via proteolysis (figure 6) and changed the tertiary structure due to the drop in pH post lactic acid production. In acidic conditions, gluten development is hindered and alterations in charges facilitates the formation of new secondary bonds [206]. Furthermore, the modified protein/peptides may vary in solubility compared to gluten, which also affects the protein aggregation [40] and contributes to the formation of two peaks during the measurement [40]. The modifications to the protein aggregation were found to reduce pasta tensile strength/pasta firmness and increase cooking times when compared to a semolina control. Pasta stickiness was also enhanced in FBSG formulations more than BSG formulations, which was attributed to the differences in starch

pasting properties. The increased α-amylase activity in FBSG increased production of starch degradation products [226] and thus, enhanced pasta stickiness. Furthermore, the protein modifications in FBSG likely negatively influence pasta structure, allowing for a greater amount of amylose to leach onto the pasta surface. Differences in pasta structure revealed a layer in association with starch granules (primarily at higher levels of inclusion) showing evidence for fibre-protein-starch interactions. This was primarily associated with the gel-like effect of the arabinoxylan addition [48,220] which are naturally occurring in BSG [3] and the interaction effect within the gluten network via a chemically mediated effect (ferulic acid) and physical effect (increased viscosity depleting protein interaction) [201]. From a nutritional perspective, addition of BSG/FBSG was found to reduce the overall predicted glycaemic index of the pasta. Interestingly, FBSG HF formulations reduced the predicted *in vitro* glycaemic index to a greater extent compared to BSG HF and highlighted the greater nutritional benefit of including a fermented ingredient. This was associated with the restricted access to starch granules during amylase hydrolysis due to enhanced interactions between starch-gluten-heat in the presence of lactic acid [233].

Chapter 4 explored the fortification of BSG and FBSG in a bread system. A breads quality is largely dependent on gluten network properties; dough rheological properties; and also the yeast fermentation capacity. The gluten aggregation profiles revealed inclusion of BSG and FBSG weakened the gluten technical capacity [250–252] with fibres [145,146,155,199,201,250,254], proteins [205] and minerals [202,253] in BSG negatively interfering with gluten development and gluten techno-functional characteristics. However, this weakened gluten functionality was observed to a lesser extent with FBSG formulations. As previously highlighted in **chapter 3,** the two peaks formed during gluten kinetics with FSBG HF formulations as well as the gluten-fibre-starch interactions in bread structure also translated to the bread system. Dough viscoelastic properties showed enhanced elastic proportions in BSG/FBSG formulations restricting dough expansion, but this effect occurred to a lesser extent in FBSG formulations. The acidic effect of the FBSG ingredient weakened the gluten network development [256] and putatively enhanced the proteolytic activity of the flour, reducing dough elasticity to a greater extent [255,256] and facilitating expansion. Deterioration in bread techno-functional characteristics with BSG/FSBSG was observed compared to BF and was associated with the negative effects observed in gluten network capacity and dough rheological properties. However, FBSG formulations performed more favourably than BSG formulations, showcasing the potential of LAB fermentation in BSG

processing. FBSG formulations (particularly at HF) showed higher bread volumes; softer crumbs; slowed the microbial kinetics during shelf life analysis due to antifungal characteristics imparted after LAB fermentation [267,268]; and slowed the release of reducing sugars overtime, further enhancing the nutritional characteristics of the FBSG breads.

The successes observed in applying LAB fermentation to BSG as a processing aid in **chapter 3** and **4** sparked the interest in the application of LAB fermentation technology in BR processing, an area much less extensively explored. **Chapter 5** showcased the changes observed with LAB technology at a fundamental level and also observed the changes to the BR ingredient depending on the strain used for fermentation. In general, BR were a viable substrate for all LAB. *Lactiplantibacillus plantarum* FST 1.7 and *Lactobacillus amylovorus* FST2.11 particularly thrived using BR as a substrate, reflecting their high adaptability to BR coming from the brewing environment and high acidic tolerance [286,287,296]. The changes in the BR composition (sugar, FODMAPs, acid) and metabolomic profiles of the ingredients showed variances between strains, highlighting the unique characteristics that can be imparted by each LAB and further explored their metabolism. A comparison of the glucose, fructose and sucrose profiles of the BR ingredients reflected the differences in LAB growth and metabolism, with preferences in sugar substrate highlighted. FODMAP profiles demonstrated *Leuconostoc citreum* TR116 and *Limosilactobacillus reuteri* R29 as high mannitol producers (Table 13) indicating potential for BR-TR116 and BR-R29 in sugar reduced products. Additionally, while most LAB reduced the level of fructans present, *Limosilactobacillus reuteri* R29 produced fructans, likely of levan type and high-molecular-weight inulin type [315–318], showing potential as a fibre fortification ingredient particularly when limited fructan degradation is desired [319]. In relation to acid composition in BR, *Lactiplantibacillus plantarum* FST 1.7 and *Lactobacillus amylovorus* FST2.11 produced large amounts of lactic acid while lower amounts were found in BR ingredients fermented with *Weissella cibaria* MG1, *Leuconostoc citreum* TR116 and *Limosilactobacillus reuteri* R29 (Table 13). The metabolomic profiles of the BR ingredients delved further into the metabolism of the LAB using BR as a substrate showcasing the changes observed in the TCA compounds. Evaluation of the techno-functional properties of BR ingredients provided information on how BR may behave in a food system, while also showing how different processing techniques may alter them. Following fermentation technology, changes in ingredient structure (SEM); a decrease in WHC; increase in OBC and a reduction in α-amylase activity was observed. SEM micrographs also showed a physical change in BR structure post processing, highlighting a more broken, fragmented and

enhanced porous structure, likely as a combined effect of the freeze-drying [339], anticipated natural proteolytic activity in BR [6,340] and enzymes secreted during LAB fermentation [328].The reductions in WHC were associated with changes in protein structure post fermentation, unfolding the natural configuration of the proteins present in BR and exposing more hydrophobic regions, resulting in a reduction in the WHC. This theory was also complimented by the increase in the OBC capacity of the fermented BR [165]. The reductions noted in α-amylase activity also showed greater prospects for BR in cereal-based applications, as excessive amounts of α-amylase can lead to processing defects such as sticky doughs, poor crumb structures and darker crusts [336].

The LAB used in **chapter 5** displayed great potential in altering the functionality of BR as a food ingredient while also showcasing LAB fermentation as a viable processing aid for BR valorisation. The developed BR ingredients showed suitable characteristics for cereal-based applications, particularly bread. **Chapter 6** applied the developed BR ingredients as partial flour replacers (5% supplementation based on flour) to analyse whether characteristics imparted by LAB fermentation translated to the bread application. The addition of BR into the bread system at this level was found to decrease the strength of the gluten network, however, the introduction of the fermented ingredients amplified the speed required for gluten development. The reductions in gluten network strength are likely a contribution of the changes in minerals, protein and fibre balance with BR inclusion [11] and their influence in the fundamental bonds required for gluten development [199,254,351]. The fermented ingredients reduced the amount of time needed for gluten development, likely as a result of amplified hydrophobic interactions via protein structure change [221,292] and increased mineral bioavailability [353] after fermentation. This also gave reason for the higher elastic properties in fermented doughs. The unfermented BR formulation positively impacted the viscoelastic properties of the dough which was mainly attributed to the enzymatic activity of the unprocessed ingredient. However, changes in bread quality were observed depending on the type of BR ingredient applied. The addition of the BR-MG1 showcased its potential to improve bread specific volume and reduce crumb hardness. This was associated with the exopolysaccharides produced by *Weissella cibaria* MG1 [289,290] which have been described to perform similar to hydrocolloids in a bread system [359] stabilising dough structure and increasing bread specific volumes [334]. Similar to BR-MG1, inclusion of BR-TR116 also maintained bread crumb and bread volume quality. The addition of BR-R29 illustrated its power to reduce the microbial growth rate to a significant extent through natural production of

high amounts of antifungal compounds. The BR-R29 ingredient had the highest amount/variety of antifungal compounds and their synergistic effect with lactic and acetic acid created an even greater hurdle effect [294]. The incorporation of BR-FST1.7 and BR-FST2.11 showed promising potential to further improve the nutritional characteristics of the BR breads and slowed the release of sugars overtime during starch digestion. Like in **chapter 3 and 4**, this was associated with the acid contents of the BR ingredients (Table 13) inhibiting starch hydrolysis [233]. Furthermore, inclusion of BR-FST1.7 and BR-FST2.11 altered the sensory experience of the wheat bread, increasing the sourness taste and fruity flavours, creating a bread with flavours which might compare more similarly to that of a conventional sourdough [369]. The study showcased how LAB fermentation of BR can be tailored and engineered, depending on the quality requirements of the bread at a later stage.

In conclusion, the implementation of BSG and BR into cereal-based matrixes such as bread and pasta is a challenging task, as deterioration in product quality was often observed. However, as epitomised in this thesis for a variety of reasons, LAB fermentation proves to be a viable processing aid in the valorisation of BSG and BR, and aids in maintaining and/or improving product quality. This thesis also exemplifies how LAB fermentation can be engineered for specific requirements, especially in bread applications, synergising the food and biotechnology industries even further. In relation to future work in this area of research, numerous avenues are left to explore. For BSG, investigation into the refining and purification of BSG to high protein/high fibre ingredients could be of interest as this could lower levels of replacement required to reach food claims and help maintain product quality. In relation to BR, with literature much more limited in this area, there is even greater capacity to uncover their untapped potential. As a starting suggestion, research into unprocessed BR as dough improvers could be of interest in the development of clean label products for the cereal applications industry; delving further into the nutritional quality and/or digestibility of BR may reveal further benefits of BR; and lastly, further refinement of BR could improve prospects and diversify potential food applications for BR in the future. In regard to LAB fermentation technology, it would be of great interest to research if the unique characteristics from singular LAB fermentations could be combined through co-fermentations. Moreover, the application of LAB fermentation technology is likely not limited to this area and could be translated to a variety of food industry processes. The LAB pre-fermentation step of raw materials could be applied to alternative protein sources (eg. soy, pea, rice, chickpea, faba bean, insect, algae), purified fibres (eg. cellulose, arabinoxylans, resistant starch, beta-glucan), and side-streams of

multiple food industry processes (eg. fruit & vegetable processing, wine making, vegetable oil processing, pressed juices, milling, meat, dairy). The application of LAB fermentation technology could improve various nutritional aspects (reduce GI, solubilisation of fibres, increase bioavailability of amino acids); food safety aspects (reduce presence of biogenic amines and pathogenic micro-organisms); and antinutritional concerns (reduce phytic acid, tannins, saponins) of the aforementioned ingredients [167], ultimately improving their viability in the human food chain. In relation to the implications of this technology for human health and consumers, with the improved nutritional aspects of the ingredients post LAB fermentation, this will aid towards greater nourishment of individuals across a variety of demographics, with future work focussing on human intervention studies to confirm this. In addition, the development of new food formulations from LAB processed ingredients would increase the variety of food choices for the consumer and with the correct knowledge transfer; consumers will also benefit from an informed food choice, helping them to take steps towards more sustainably processed foods.

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Appendix

Publications

Neylon, E.; Arendt, E.K.; Lynch, K.M.; Zannini, E.; Bazzoli, P.; Monin, T.; Sahin, A.W. Rootlets, a Malting By-Product with Great Potential. Fermentation 2020, 6, 117. https://doi.org/10.3390/fermentation6040117

Neylon, E.; Arendt, E.K.; Zannini, E.; Sahin, A.W. Fundamental study of the application of brewers spent grain and fermented brewers spent grain on the quality of pasta. Food structure 2021, 30, https://doi.org/10.1016/j.foostr.2021.100225

Neylon, E.; Arendt, E.K.; Zannini, E.; Sahin, A.W. Fermentation as a Tool to Revitalise Brewers' Spent Grain and Elevate Techno-Functional Properties and Nutritional Value in High Fibre Bread. Foods 2021, 10, 1639. https://doi.org/10.3390/foods10071639

Neylon, E.; Nyhan, L.; Zannini, E.; Monin, T.; Muench, S.; Sahin, A.W.; Arendt, E.K. Food ingredients of the future: in-depth analysis on the effect of lactic acid bacteria fermentation on spent barley rootlets - Fermentation 2023, 9, https://doi.org/10.3390/ fermentation9010078

Neylon, E.; Nyhan, L.; Zannini, E.; Sahin, A.W.; Arendt, E.K. From waste to taste: application of fermented spent rootlets in a bread system. Foods, 2023, 12, 1549, https://doi.org/10.3390/foods12071549

Borowska M.; Ispiryan L.; Neylon E.; Sahin A.W.; Murphy C.P.; Zannini E.; Arendt E.K.; Coffey A. Screening and application of novel homofermentative lactic acid bacteria results in low-FODMAP whole wheat bread. Fermentation, 2023, 9, 336. https://doi.org/10.3390/fermentation9040336

Awards

2nd place prize for **best oral presentation** at the 8th international symposium on sourdough, June 14 - 17 2022, Bolzano, Italy.

1st place prize for **best oral presentation** at the UCC/IFSTI 50th food science and technology conference - Frontiers in AgriFood Science: Addressing the Problems of Today for a Brighter Tomorrow, December 6 & 7 2022, Cork, Ireland.

Oral presentations

Neylon, E.; Arendt, E.K.; Zannini, E.; Nyhan L.; Jaeger, Alice.; Sahin, A.W. (2022). Fermentation as a Tool to Revitalise Brewers' Spent Grain and Elevate Techno-Functional Properties and Nutritional Value in High Fibre Bread. *8th international symposium on sourdough, Bolzano, Italy, June 14 – 17.*

Neylon, E.; Nyhan, L.; Zannini, E.; Sahin, A.W.; Arendt, E.K. (2022). From waste to taste: application of fermented spent rootlets in a bread system. *UCC/IFSTI 50th food science and technology conference - Frontiers in AgriFood Science: Addressing the Problems of Today for a Brighter Tomorrow, Cork, Ireland. December 6 & 7.*

Poster presentations

Neylon, E.; Arendt, E.K.; Zannini, E.; Sahin, A.W. (2021). Fundamental study of the application of brewers spent grain and fermented brewers spent grain on the quality of pasta. *4th food structure and functionality symposium, online live and on-demand, 19-20 Oct 2021.*

Neylon, E.; Arendt, E.K.; Zannini, E.; Nyhan, Laura.; Sahin, A.W. (2021). Fundamental study of the application of brewers spent grain and fermented brewers spent grain on the quality of pasta. *International Symposium on Lactic Acid Bacteria online, 23rd - 25th August 2021.*

Neylon, E.; Arendt, E.K.; Zannini, E.; Nyhan, Laura.; Pop, B.; Sahin, A.W. (2022). Fermentation as a tool to revitalise brewers spent grain and extend the microbial shelf life of high fibre bread. *SafeConsume International Conference, Bucharest, 27-28th June*.